A thesis entitled

# Regulatory studies of the mammalian RNA polymerase III transcriptional apparatus

Presented by

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to

**The University of Glasgow**

for the degree of

**Doctor of Philosophy**

*April 2001*

**Division of Biochemistry and Molecular Biology**
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**Abstract**

Although many studies have shown that pol III transcription is strongly regulated in higher eukaryotes, it is poorly understood how this regulation is achieved. The basal pol III factors TFIIIB and TFIIIC have been implicated as common targets for regulation. I have developed reproducible purification protocols for yielding partially purified active human TFIIIB and human TFIIIC. The purity of hTFIIIB and hTFIIIC obtained are a significant improvement upon that of hTFIIIB and hTFIIIC typically used in our laboratory, allowing regulatory studies to be conducted with a much higher level of confidence than previously.

One established repressor of pol III transcription is the tumour suppressor RB. Recently, the related proteins p107 and p130 have also been shown to inhibit pol III transcription. Here, I show that endogenous p107 and p130 cofractionate and coimmunoprecipitate with endogenous TFIIIB, suggesting that, like RB, p107 and p130 stably associate with TFIIIB under physiological conditions. I have also investigated why the binding of RB to TFIIIB inhibits pol III transcription. For several genes transcribed by pol II, RB represses transcription through the recruitment of the histone deacetylase HDAC1, which is thought to deacetylate histones at the promoter resulting in the formation of a more compact chromatin structure less accessible to transcription factors. However, the repression of pol III transcription in vitro by RB is unaffected by the presence of the histone deacetylase inhibitor trichostatin A. Using an immunoisolated pol III complex that contains pol III, TFIIIC and TFIIIB, I show that recombinant RB can specifically disrupt the interaction between TFIIIB and TFIIIC.

The serine/threonine kinase CKII is identified as a novel activator of mammalian pol III transcription and is shown to stably interact with endogenous hTFIIIB. Significantly, CKII kinase activity appears to promote the binding of TFIIIB to TFIIIC. The receptor tyrosine kinase neu (erbB2) is also implicated in the regulation of pol III transcription. A rodent ovarian epithelial cell line transformed by an activated neu oncogene is found to display elevated pol III activity. TFIIIC2 B-block binding activity is specifically elevated. Using the purified TFIIIB and TFIIIC fractions, I show that TFIIIC is limiting in the untransformed control cell line, indicating that upregulation of TFIIIC2 activity in response to neu transformation can at least partly account for the increase in pol III activity.

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# Abbreviations

| | |
|---|---|
| BRF | TFIIB-related factor |
| CDK | Cyclin-dependent kinase |
| CKII | Casein kinase II |
| cvs | Column volume |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA-PK | DNA-dependent protein kinase |
| DPG | 2,3-diphosphoglycerate |
| DRB | 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole |
| DTT | Dithiothreitol |
| EBV | Epstein Barr virus |
| EDTA | Ethylene diamine tetra-acetic acid |
| EGFR | Epidermal growth factor receptor |
| EMSA | Electrophoretic mobility shift assay |
| FT | Flowthrough |
| GSK-3 | Glycogen synthase kinase-3 |
| GST | Glutathione-S-transferase |
| HAT | Histone acetyltransferase |
| HBV | Hepatitis B virus |
| HDAC | Histone deacetylase |
| Hepes | *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulphonic acid |
| $His_6$ | 6 histidine residues |
| HMG | High mobility group |
| HPV | Human papillomavirus |
| HTLV-1 | Human T-cell leukaemia virus 1 |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| kD | Kilodalton |
| LEF/TCF | Lymphoid-enhancing factor/T-cell factor |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PC | Phosphocellulose fraction |
| PIC | Preinitiation complex |

| | |
|---|---|
| PMA | *p*-hydroxymercuribenzoic acid |
| PMSF | Phenylmethylsulfonyl fluoride |
| Pol III | RNA polymerase III |
| RB | Retinoblastoma protein |
| rt | Room temperature |
| RTK | Receptor tyrosine kinase |
| SDS | Sodium dodecyl sulphate |
| Ser | Serine |
| SP- | Sulphopropyl- |
| SV40 | Simian virus 40 |
| TBS | Tris buffered saline |
| TF | Transcription factor |
| Thr | Threonine |
| Tris | Tris(hydroxymethyl)methylamine |
| ts | Temperature sensitive mutation |
| TSA | Trichostatin A |
| Tween 20 | Polyoxyethylene sorbitan monolaurate |

# Acknowledgements

Firstly, I would like to thank my supervisor, Bob White, for his enormous support and encouragement over the past three years, for his meticulous reading of this manuscript and for providing me with the opportunity to work in the wonderful world of pol III transcription. I would also like to thank the other members of the lab, past and present, for making long hours in the lab more fun and for their endless help and practical advice. I find it difficult to single people out but in particular I would like to thank Jo and Zoë for their humour and a sympathetic ear, Imogen for letting me muscle in on CKII, and Pam for her endless help and encouragement and for making weekends in the lab less lonely. I also feel indebted to members of the Cathcart lab for letting me take over their cold room and for providing me with numerous reagents.

I would like to thank Mum and Dad without whom none of this would ever have been possible, for their unwavering belief in my ability and endless support throughout my years of study.

Finally, thanks to my future wife Eleni for all her support and understanding, for providing me with perspective and for always keeping a smile on my face and in my heart.

# Declaration

I am the sole author of this thesis. All the work presented in this thesis was performed by myself, unless otherwise acknowledged.

Simon J Allison, April 2001

Dedicated with love to Eleni

# Publications

Winter, A. G., G. Sourvinos, **S. J. Allison**, K. Tosh, P. H. Scott, D. A. Spandidos, and R. J. White. 2000. RNA polymerase III transcription factor TFIIIC2 is overexpressed in ovarian tumours. Proc. Natl. Acad. Sci. USA. 97:12619-12624.

Sutcliffe, J. E., T. R. P. Brown, **S. J. Allison**, P. H. Scott, and R. J. White 2000. Retinoblastoma protein disrupts interactions required for RNA polymerase III transcription. Mol. Cell. Biol. 20:9192-9202.

Sutcliffe, J. E., C. A. Cairns, A. McLees, **S. J. Allison**, K. Tosh, and R. J. White 1999. RNA polymerase III transcription factor IIIB is a target for repression by pocket proteins p107 and p130. Mol. Cell. Biol. 19:4255-4261.

# Chapter 1.
# Introduction

## 1.1 Eukaryotic RNA polymerases

In prokaryotes, a single RNA polymerase is responsible for the production of all cellular RNA. This contrasts with the situation in eukaryotes in which there are multiple DNA-dependent RNA polymerases (80, 476). There are three distinct nuclear RNA polymerases and mitochondria and chloroplasts each have their own unique RNA polymerase (80, 476). The mitochondrial and chloroplast RNA polymerases reflect the partial maintenance by these organelles of their own genetic system derived from their endosymbiotic origin. The three nuclear RNA polymerases are thought to have evolved from a common ancestor. An initial fortuitous gene duplication event is likely to have first given rise to two RNA polymerases prior to the evolution of the present day tripartite system found in all eukaryotes (80).

The three nuclear RNA polymerases (pols) are not redundant activities as they are unable to functionally compensate for one another, as demonstrated by the lethality of separate mutations in each one of the pols. There is a precise division of labour; each of the three RNA polymerases is responsible for the transcription of a different set of genes. Thus, RNA polymerase I (pol I) synthesises the 45S ribosomal RNA (rRNA), RNA polymerase II (pol II) synthesises all messenger RNA (mRNA) and most small nuclear RNA (snRNA), and RNA polymerase III (pol III) synthesises 5S rRNA, transfer RNA (tRNA), U6 snRNA and a variety of other small RNAs. The evolution of three distinct nuclear RNA polymerases functionally specialised for the dedicated expression of different sets of genes may have been necessary to achieve the increased, more complex regulatory control upon gene expression required in eukaryotes.

## 1.2 Pol III transcripts

RNA polymerase II, being entirely responsible for the transcription of genes that encode proteins, transcribes by far the largest variety of different genes. In

contrast, pol I is only responsible for the production of a single transcript, 45S rRNA. Nonetheless, pol I transcription constitutes ~70% of total nuclear transcription in an actively growing cell (410). Each 45S rRNA molecule is cleaved to generate one molecule each of 5.8S rRNA, 18S rRNA and 28S rRNA, essential RNA components of the ribosome, the site of cellular protein synthesis. The gene encoding the 45S rRNA is highly re-iterated in the eukaryotic genome, which may be necessary to ensure sufficient levels of 5.8S rRNA, 18S rRNA and 28S rRNA are produced to make enough ribosomes to support the biosynthetic requirements of the cell (410). It has been estimated that ~10 million copies of each of these rRNA molecules must be synthesised each cell generation in an actively growing higher eukaryotic cell.

Approximately 10% of nuclear transcription is carried out by pol III (569). The genes transcribed by pol III, so-called class III genes, encode a variety of small stable RNAs, many of which have critical roles in cellular metabolism (568). The most abundantly expressed class III genes are those encoding 5S rRNA and tRNA molecules, highly conserved pol III transcripts, both of which have essential functions in the complex process of translation (568). 5S rRNA, the smallest of the ribosomal RNAs and the only one transcribed by pol III, is ~120 nt in length and is found in all eukaryotes as part of the large subunit of ribosomes. As for the 45S rRNA gene, the 5S rRNA gene is found in multiple copy number; there are ~300-400 5S rRNA genes in the haploid human genome (495). tRNAs, which are ~70-90 nt long, act as translational adaptors serving to convert the genetic information contained within the nucleotide sequence of a messenger RNA into a particular order of amino acid residues in a protein, as specified by the genetic code. The fidelity of this process is dependent on the appropriate amino acid having been attached to each tRNA molecule in accordance with the anticodon sequence of the tRNA molecule. Base-pairing of the anticodon of a tRNA molecule with the complementary codon in the mRNA ensures that the correct amino acids are covalently linked, as dictated by the nucleotide sequence of the mRNA. The recently published draft of the human genome sequence contains 497 tRNA genes; these are thought to encode approximately 60-90 different tRNA species (199).

Other important class III genes include those encoding U6 snRNA, H1 RNA and MRP RNA, each of which is involved in post-transcriptional processing (568, 583). U6 snRNA functions in pre-mRNA splicing as part of the spliceosome, a large ribonucleoprotein (RNP) complex that catalyses the removal of introns and accurate

splicing of exons (353). U6 snRNA is the most highly conserved of the spliceosomal RNAs and has been shown to be essential in yeast (56, 258). H1 RNA is part of RNaseP, an endoribonuclease involved in the processing of the 5′-termini of pre-tRNA (26). MRP RNA, which is closely related to H1 RNA and can fold into a similar secondary structure, is part of MRP RNase, another endoribonuclease, which has an important role in the endonucleolytic cleavage of the 45S precursor rRNA (383). The influence of pol III transcription on protein synthesis may therefore extend beyond the production of 5S rRNA and tRNAs, indirectly through its effects on post-transcriptional processing.

Another essential class III gene is that encoding 7SL RNA, which is involved in the correct targeting of proteins to their appropriate intracellular location. 7SL, a highly conserved 300 nt transcript, forms the scaffold of the signal recognition particle (SRP), which is required for the cotranslational insertion of nascent polypeptides into the endoplasmic reticulum (547).

The $VA_I$ and $VA_{II}$ genes of adenovirus and the EBER1 and EBER2 genes of Epstein-Barr virus are also transcribed by pol III. The adenoviral VA RNAs, expressed at high levels during late stages of viral infection, are involved in subverting the translational machinery of the host cell towards the more effective production of viral proteins (519). A similar role is likely for the EBER RNAs as multiple copies of these short RNAs can substitute for $VA_I$ during adenovirus infection (35).

Other class III genes encode transcripts that are hitherto of unknown function (568, 583). These include 7SK and the various gene families of short interspersed nuclear elements (SINEs). SINEs are quantitatively the most important class III genes in higher organisms (250). Rodent species contain several SINE families; these include the ID gene family and the B1 and B2 gene families. The B2 family, which is rodent-specific, is represented by ~80,000 copies of the B2 gene in the haploid mouse genome, constituting ~0.7% of the total genomic DNA (29). Approximately 1% of the genome of Xenopus laevis is comprised of a SINE DNA called satellite 1 (309). The major SINE in primates is the Alu gene, of which there are about 500,000 to one million copies in the haploid human genome (251). This constitutes ~5% of the total human genomic DNA. Clearly, a significant proportion of the genome of a variety of eukaryotes consists of SINE DNA.

The extraordinarily high copy numbers of the various SINEs are thought to have arisen by retrotransposition (558). This involves the reverse transcription of the SINE transcripts and integration of the resulting DNA into novel genomic sites. For some genes amplified by retrotransposition, the transcription of a gene copy requires its fortuitous insertion in the DNA adjacent to an active promoter. This dependency on external promoter elements for transcription severely limits the rate of transposition. However, SINEs, like the majority of class III genes, contain internal promoter elements. Therefore, each gene copy produced contains the necessary information for its own transcription and is not reliant on its site of integration in the DNA for expression and the generation of further copies. This may explain the very high rate of transposition of SINEs.

The major SINE families appear to be derived from class III genes of known physiological significance. Genes from the B2 and ID families share significant homology with tRNA genes and their transcripts can form similar secondary structures (110). In contrast, the B1 and Alu families are thought to have evolved from the 7SL gene (531). Such SINEs may therefore simply represent particularly mobile pseudogenes and may be totally devoid of function. Alternatively, perhaps certain of these SINEs have acquired functions during the course of evolution. Although a variety of functions have been proposed for particular SINEs (227), a functional role for a SINE family has yet to be convincingly demonstrated. Even if the individual SINE families have no specific function, the mobility of these short repetitive elements and their integration into novel genomic locations will inevitably have had major effects on the structure and evolution of the genome. The transposition of SINEs creates novel genetic combinations within the DNA. Such a large number of small homologous sequences are also likely to cause an increase in the levels of recombination, a major source of genetic variability.

These genetic changes induced by SINEs make a significant contribution to the fluidity and adaptability of the genome. However, such genetic change can also be highly detrimental; for example, essential genes may be disrupted. Clearly, SINEs have the potential to be potent mutagens and inflict serious genetic damage, which may partly explain why SINEs are only found to be expressed at extremely low levels in cells. Although there may be as many as one million Alu genes in the haploid human genome, a growing HeLa cell only contains about 100-1000 Alu transcripts (335). In contrast, approximately one million 7SL transcripts were

detected per HeLa cell, yet there are only four 7SL genes in the entire genome. Alu genes are derived from 7SL; however, they lack the upstream promoter sequences of 7SL and the internal promoter elements may also have acquired changes further reducing Alu expression (531).

## 1.3 Promoter structure of class III genes

Class III genes show a marked diversity of promoter organisation, consistent with the broad range of different genes that are transcribed by pol III. There are three basic classes of promoter recognised by pol III, type I, type II and type III (157, 411, 583). Genes with a type I or type II promoter, which together constitute the majority of class III genes, are unusual in that they require intragenic sequences, so-called internal control regions (ICRs), for their expression. In contrast, transcription of genes with type III promoters is entirely determined by sequences that lie upstream of the start site (299). Type III promoters resemble those of genes transcribed by pol I or pol II in that they are completely independent of intragenic sequence elements.

### 1.3.1 Type I promoters

The type I promoter is unique to 5S rRNA genes. Extensive mutagenesis of a somatic 5S rRNA gene from Xenopus laevis defined three essential sequence blocks for transcription, each located in the coding region of the gene: the A-block (+50 to +64), the intermediate element (+67 to +72) and the C-block (+80 to +97) (39, 416, 417). The identities of the bases in between these individual sequence elements have no effect on transcription efficiency; however, changes in the spacing of the elements were poorly tolerated (417). Linker scanning mutagenesis of the sequence between the A-block and the transcriptional start site suggests that this sequence can have a major influence on 5S rRNA production under conditions that are suboptimal for transcription (150, 270, 596). The 5′- and 3′- flanking regions can also affect transcription levels under less favourable conditions. These effects are purely

modulatory, however; under optimal conditions the region from +50 to +97, encompassing the A-block, the intermediate element and the C-block, suffices for efficient transcription.

A synthetic 5S gene consisting solely of the coding region of a human 5S rRNA gene is transcribed in a HeLa extract (584). Clearly, the expression of 5S rRNA genes is possible in the absence of extragenic sequence. The 5′ flanking region of the human 5S rRNA gene was capable of stimulating transcription ~10 fold, however (495). Although the flanking sequences can clearly influence transcription, they are poorly conserved and can generally tolerate mutations reasonably well. In contrast, the A- and C- blocks and the intermediate element are strongly conserved between species and mutations in these regions abolish transcription.

### 1.3.2 Type II promoters

Most class III genes, including the tRNA, VA, Alu, B1, and B2 genes, have a type II promoter (568, 583). Transcription of these genes, like 5S rRNA genes, is dependent upon discontinuous intragenic sequence elements. The internal control region of type II promoters consists of two essential sequence elements, an A-block and a B-block, each ~10bp, optimally separated by ~30-60nt of non-essential DNA, although larger distances of up to 365bp can be tolerated (18, 138). The A-blocks of type I and type II promoters are homologous and sometimes interchangeable (94). However, the A-block of type II promoters are much closer to the start site (at ~+10- to +20) than the corresponding region of type I promoters, which tend to be found ~40bp further upstream (158). This difference may reflect the dominant role of the A-block of type II promoters in start site selection (94, 138).

The tRNA genes and adenoviral $VA_I$ gene have been extensively studied as model templates for genes with a type II promoter. The A- and B- blocks of tRNA genes are remarkably well conserved, both between genes encoding different tRNA isoacceptors and between tRNA genes from different species (158). Even certain bacterial and chloroplast tRNA genes contain similar sequences and can therefore be transcribed by pol III (149, 176). Chimeras constructed from the 5′ half of a $tRNA^{Leu}$

gene and the 3′ half of a $tRNA^{Met}$ gene, or vice-versa, were active for transcription, demonstrating the functional compatibility of the A- and B- blocks from different genes (158). The A- and B- blocks of tRNA genes encode the D- and T- loops respectively of tRNA molecules, both of which are absolutely essential for tRNA function. The high level of sequence conservation of the A- and B- blocks of tRNA genes is therefore likely to reflect selection both for tRNA and promoter function. Nevertheless, point mutations in the A- and B- blocks can severely affect transcription efficiency. Consensus sequences of TGGCNNAGTGG for the A-block and GGTTCGANNCC for the B-block have been derived (158). Mutational analysis of the yeast SUP4 $tRNA^{Tyr}$ gene suggests that the consensus sequences closely coincide, although not perfectly, with the sequences that give optimal promoter activity (8). The A- and B- blocks together constitute the minimal promoter requirements for accurate tRNA transcription. A chemically synthesised oligonucleotide corresponding to these two sequence elements separated by a 51 bp spacer was able to direct efficient transcription in a HeLa cell extract (389). As for 5S rRNA genes, flanking regions are able to modulate transcription efficiency but are generally poorly conserved. The 5′ flanking regions of tRNA genes show little or no homology even between different genes that encode the same tRNA isoacceptor (568). There is evidence to suggest that this sequence variation is involved in the differential regulation of tRNA genes, which is necessary for the tRNA population to be adapted to differing codon and amino acid usage in different cell types (568).

All type II promoters contain sequences highly homologous to the A- and B-blocks of tRNA genes. The promoter requirements are very similar for different genes with type II promoters; indeed, B2 promoter sequences have been shown to cross-compete with those of tRNA or $VA_I$ genes (182, 577). As for tRNA genes, the A- and B- blocks of the adenoviral $VA_I$ gene have been shown to be sufficient for its transcription in vitro (430, 444, 553, 599). Linker scanning mutagenesis has identified both positive and negative modulatory sequences upstream of the start site of the $VA_I$ gene, although the overall effect of surrounding sequences is stimulatory (430). The $VA_I$ gene is transcribed more strongly than tRNA genes; however, the sequences responsible for this difference in transcription efficiency have yet to be elucidated. Flanking sequences that are able to influence transcription levels are not conserved between different genes with type II promoters and are poorly

characterised. These differences, as well as how closely the sequences of the A- and B- blocks match those that are optimal for transcription, are likely to be responsible for the differential promoter strength of different class III genes with type II promoters.

### 1.3.3 Type III promoters

A minority of pol III templates, such as the vertebrate U6 and 7SK genes and mammalian MRP genes, have a type III promoter (112, 281, 390, 610). The distinguishing feature of this type of class III promoter is the notable absence of intragenic promoter elements. The type III promoter resides exclusively in extragenic sequence, specifically in the 5′ flanking region of the gene (157, 568, 583). The entire coding sequence of human and mouse U6 genes can be replaced without any effect on transcription, either in vitro or in vivo (112, 301). The same has also been shown to be true of the human 7SK and MRP genes (281, 390, 610).

Whereas the U6 snRNA gene is transcribed by pol III, the other U snRNAs, U1, U2, U4 and U5, are products of pol II transcription (300, 379, 432). Nevertheless, despite being transcribed by different RNA polymerases, the promoters of vertebrate U6 snRNA genes are remarkably alike those of the U snRNA genes transcribed by pol II (302). Upstream sequences required for efficient transcription of human U6 genes are a TATA box between –30 and -25, a proximal sequence element (PSE) between –66 and –47 and a distal sequence element (DSE) between -244 and –214 (19, 72, 112, 302, 338). The human U6 PSE and DSE show substantial homology with elements found at comparable positions in the promoters of the class II U snRNA genes (19, 72, 111, 302, 338). Indeed, the PSEs of the human U2 and U6 promoters are identical at 13 out of 17 positions and are functionally interchangeable (338, 406). The DSEs of the human U2 and U6 promoters are also interchangeable (19, 72, 302). However, a TATA box, a very common feature of class II genes, is absent from the promoters of U snRNA genes transcribed by pol II. Paradoxically, the TATA element is a major determinant of polymerase specificity (338, 339, 362). Inactivation of the TATA box allows the U6 snRNA gene to be transcribed by pol II (362). Conversely, the insertion of a TATA

box into the corresponding position of a U2 promoter can convert the U2 gene into a pol III template (338). However, the U6 TATA box alone is insufficient to confer recognition by pol III upon the U1 gene (329). To switch the polymerase specificity of the U1 gene also requires that the PSE be moved 4 bp further upstream so that the distance between it and the TATA box is the same as in the U6 promoter (329). Pol III transcription of the U6 snRNA gene is severely impaired by changes in the separation of the PSE and TATA box (167, 339). The exact sequence requirements for a class III TATA box also differ slightly from those of a class II TATA box, as revealed by the differential sensitivity of pol II and pol III transcription to particular mutations of the Xenopus U6 TATA box (486). Clearly, the choice of polymerase for transcription of the individual U snRNA genes can be influenced by a number of factors.

The promoter structure of the human 7SK gene is very similar to that of vertebrate U6 genes with TATA, PSE and DSE sequences all located upstream of the coding region in almost identical positions to their U6 counterparts (568). In contrast to this similarity, the promoters of vertebrate and yeast U6 genes are vastly different from one another. The U6 promoter of the yeast Saccharomyces cerevisiae is tripartite, with upstream, intragenic and downstream promoter elements (56, 57, 137). An A-block at +21 to +31 is essential for transcription, both in vitro and in vivo (67, 137). Efficient transcription of yeast U6 genes in vivo is also dependent upon a functional B-block sequence, unusually located 120bp downstream of the termination site in S. cerevisiae (57, 137). This novel positioning of the B-block may reflect an incompatibility of the B-block sequence with the function of the highly conserved U6 transcript. The upstream sequence of the gene contains a consensus TATA box at –30 to –25 . Although this sequence can influence start site selection and stimulates expression in vitro, it has little effect on transcription in vivo (67, 79, 137, 357). Additionally, a sequence around –55 shows partial homology to PSEs of vertebrate U6 promoters but has little or no effect on the level of transcription (67, 137).

Whereas the vertebrate U6 promoter resides entirely upstream of the coding sequence, transcription of the yeast U6 genes is dependent upon downstream A- and B- blocks. The yeast U6 promoters are therefore similar to the type II promoters of tRNA and VA genes (583). The totally extragenic promoter organisation of the U6 genes has therefore evolved relatively recently, since the divergence of the metazoan

and yeast lineages. The most obvious advantage of acquiring an extragenic promoter is that the promoter sequences are no longer constrained by serving a dual function and having to be compatible with transcript function. The promoter can evolve independently of the coding sequence, which may allow the evolution of a more complex promoter capable of more intricate control and fine-tuning of transcriptional output.

In contrast to the diversity of promoter organisation of the U6 snRNA genes, the promoters of most class III genes are quite well conserved between yeast and vertebrates (568).

### 1.3.4 Mixed promoters

A few class III genes have promoters that cannot be easily categorised into any of the aforementioned promoter types; these include the human 7SL and Epstein-Barr virus EBER2 genes (411). These genes rely on both internal and upstream sequences for their efficient expression. For example, the human 7SL gene has intragenic A- and B- blocks that are required for expression. However, these are fairly degenerate compared to the A- and B- blocks that are typical of type II promoters (8). Sequences upstream of +1 are therefore also necessary for significant levels of transcription of the human 7SL gene (51, 532). Indeed, 5′ deletion to –37 reduces transcription more than 20-fold in transfected HeLa cells (51). A binding site for activating-transcription-factor (ATF) occurs at –51 to –44 (51). Point mutation of this binding site mimics the effect of 5′ deletion to –37 (51). Moreover, extracts from cells pretreated with forskolin so as to induce the cAMP signal transduction pathway known to stimulate ATF activity support substantially enhanced levels of transcription of the human 7SL gene (51). In contrast, expression of the human 7SK gene, which has no ATF binding site, was unaffected by forskolin treatment demonstrating the specificity of this effect (51). Sequences downstream of -37 are also required for efficient transcription, which may include a putative TATA box at –28 to –24 (51).

The EBV EBER2 gene similarly requires a combination of internal and external promoter elements for its efficient expression (228). The EBER2 promoter

has intragenic A- and B- blocks that are essential for transcription; these are less degenerate than are those of the human 7SL gene (228). Nevertheless, deletion of sequences upstream of -46 reduces EBER2 expression in transfected cells to ~7% of the wild-type level (228). This is thought to be due to the loss of upstream binding sites for ATF and Sp1 (228). In addition, a TATA box at –28 to –23 can stimulate transcription ~5-fold (228).

The EBV EBER2 and human 7SL promoters are examples of promoters with diverse combinations of sequence elements that can cooperate to direct efficient pol III transcription (583). Thus, A- and B- blocks, homologous to those of type II promoters, can be effectively combined with sequence elements such as ATF- and Sp1- binding sites, which were originally defined as cis-acting regulatory elements of class II promoters (51, 228). The A- and B- blocks can also be combined with sequence elements from type III promoters, as the 5′ flanking sequence of the human 7SL gene can be efficiently substituted by that of the 7SK gene (282). The structure of these genes is analogous to the modality of class II promoters. It is also noteworthy that a few cis-acting elements are common to both class II and class III genes, including the TATA box, ATF- and Sp1- binding sites, and octamer motifs found in DSEs.

## 1.4 RNA polymerase III

The existence of multiple forms of nuclear RNA polymerase was originally demonstrated by DEAE Sephadex chromatography of solubilised nuclei preparations (443). The polymerase activity of extracts from embryonic sea urchin nuclei could be resolved into three distinct peaks by gradient chromatography on DEAE Sephadex (443). Moreover, the chromatographic properties of the individual peaks of activity were maintained during their separate rechromatography, suggesting the resolved polymerase activities are not interconvertible but represent distinct entities (443). The three nuclear RNA polymerases, named according to their order of elution from DEAE Sephadex, have subsequently been shown to display differential chromatographic behaviour on a variety of columns (80, 440, 441, 467). They can also be distinguished by their sensitivity to the toxin α-amanitin, produced by the

poisonous Amanita mushrooms (268). α-amanitin inhibits transcription elongation by interfering with polymerase translocation (116). In mammals, whereas pol I activity is highly resistant to α-amanitin toxicity, pol II is extremely susceptible with 50% inhibition at 25ng/ml α-amanitin and pol III is moderately sensitive with 50% inhibition at 20μg/ml α-amanitin (467). These differential sensitivities to α-amanitin were used to determine which polymerase is responsible for transcribing particular genes.

The eukaryotic nuclear RNA polymerases are large multisubunit protein complexes made up of twelve or more different polypeptides. RNA polymerase III, the largest of the three nuclear pols, has been purified from a variety of organisms including human (247, 553, 554), mouse (490), frog (440, 441), silkworm (489), fruitfly (181), wheat (252, 516) and yeast (83, 184, 535). The polypeptide composition is quite well conserved between the different species. Saccharomyces cerevisiae pol III, which is the most extensively characterised, both biochemically and genetically, consists of seventeen subunits, ranging in size from 10 to 160 kD (83). The genes for sixteen of the seventeen subunits have now been cloned; all of them were found to be unique and essential for yeast cell viability (83). In contrast, several pol II subunits are dispensable for growth (598, 609).

The largest and second largest polypeptides of yeast pol III, C160 and C128, are structurally and immunologically related to the largest and second largest polypeptides of pols I and II (9, 49, 63, 368). Moreover, they display substantial homology to β′ and β respectively of Escherichia coli RNA polymerase (9, 249, 368, 506). The β′ and β subunits together harbour the active site of the polymerase and are involved in basic pol functions, such as interactions with the DNA template and nascent RNA and the binding of nucleoside triphosphate substrates. The largest two subunits of pols I, II and III are thought to be functionally equivalent to β′ and β (122, 368, 526). C160 and C128 of yeast pol III both have zinc finger motifs suggesting they may participate in DNA binding and the C128 subunit is labelled by nucleotide analogues (438, 526). The phenotypic effects of mutations in C160 and C128 suggest that they are involved in similar core pol functions to the prokaryotic β and β′ subunits (122, 436, 478). Two other subunits of yeast pol III, AC40 and AC19, which are shared with pol I, show some sequence homology to the prokaryotic α subunit, which functions in the assembly of the pol (120).

Furthermore, a temperature-sensitive mutation in AC40 causes a defect in the in vivo assembly of pols I and III, suggesting functional equivalency with E.coli α subunits (354). Together, C160, C128, AC40 and AC19 constitute the enzymatic core of yeast pol III.

Of the remaining thirteen subunits, five of these are also found in pols I and II (ABC10α, ABC10β, ABC14.5, ABC23, ABC27) (83). Although highly conserved, with 40-75% amino acid identity between the yeast and human homologues, the absence of homologous subunits in prokaryotic RNA polymerases suggests that they are probably not directly involved in the catalytic steps of transcription (483). At least one of the subunits, ABC23, is thought to be required for the structural and functional integrity of the pols (311). The other shared subunits may perform similar structural roles. They may also provide the opportunity for co-ordinate regulation of transcription by pols I, II and III. Yeast pol III also contains eight unique subunits (83). These pol III-specific subunits are required for specific transcription by pol III. Indeed, mutations in several of these subunits or antibodies raised against particular subunits, have been shown to have no effect on the efficiency of pol III transcription of non-specific templates, but the accurate transcription of pol III templates is substantially reduced (236, 244, 522). However, these pol III-specific subunits are insufficient for the accurate and specific recruitment of pol III to class III genes. This is also true of pols I and II. The purified nuclear pols have no sequence-specificity for DNA and thus are unable to specifically recognise the promoter elements of genes that together define the pol specificity of the gene and specify the site of transcription initiation. The assistance of additional proteins, so-called transcription factors, is required that serve to recruit the pol to the appropriate start sites of the appropriate sets of genes, thus ensuring accurate and specific initiation of transcription (405). Each of the nuclear pols utilises a different set of transcription factors (411, 613).

## 1.5 Transcription factors utilised by pol III

The transcription factor requirements for basal pol III transcription are not the same for every pol III template; this depends on the promoter structure of the gene

(411, 568). Thus, genes with a type I promoter have different transcription factor requirements to genes with type II or type III promoters. For example, 5S rRNA genes are the only genes that require transcription factor IIIA (TFIIIA) (134, 473). TFIIIA is therefore a gene-specific transcription factor. However, although the exact requirements differ between genes of the different promoter types, there is also considerable overlap. Transcription factor IIIB (TFIIIB) and transcription factor IIIC (TFIIIC) are required for the transcription of all class III genes and may therefore be regarded as general pol III transcription factors (473, 568).

### 1.5.1 TATA-binding protein

The TATA-binding protein (TBP) is an essential component of TFIIIB. It was originally thought that its involvement in transcription was confined to TATA box-containing class II genes. The surprising discovery that a minority of class III genes, typified by the vertebrate U6 snRNA gene, also contain a TATA box as an essential component of their promoters raised the possibility that TBP may also be required for the transcription of a restricted set of pol III templates. It was first assumed that a TATA-binding factor distinct from TBP was involved, because the TATA box of class III genes has similar but distinct sequence requirements to the TATA boxes associated with class II genes (362, 391, 486). Moreover, the class III TATA box is a major determinant of the pol III specificity of these genes (338, 362). Subsequently, it was shown that TBP and the TATA-binding factor required for the transcription of TATA box-containing class III genes copurify and that cloned TBP can efficiently substitute for this factor in supporting U6 transcription (339, 356, 485). It is now clear that the class III TATA-binding factor and TBP are the same.

Rather unexpectedly, it was found that the transcription of TATA-less class III genes also requires TBP (573, 575). This was first suggested by the observation by White et al. that transcription of TATA-less pol III templates is dramatically inhibited by the preincubation of cell extracts with short oligonucleotides containing TATA box sequences from class II promoters (575). This was shown for six different TATA-less pol III templates in all, 5S, tRNA, VA, Alu, B1 and B2, as well as the TATA-containing U6 and EBER2 genes (575). In contrast, oligonucleotides with mutated TATA boxes unable to bind TBP failed to inhibit transcription (575).

The addition of pure recombinant TBP restored pol III transcription to extracts preincubated with TATA box oligonucleotide competitor, suggesting that the sequestered TATA-binding factor required for transcription is TBP (575). The specific inactivation of TBP by mild heat treatment or its removal from extracts by fractionation also abolished transcription of TATA-less pol III templates (237, 575). Addition of recombinant TBP to replace that depleted was able to restore transcription, providing further evidence of a role for TBP in the transcription of all class III genes (237, 575). Similar biochemical experiments suggested that TBP is also required for transcription of pol I templates and some TATA-less, as well as TATA-containing, pol II templates (213, 434, 479, 574). In recent years it has become clear that TBP is not required by all class II genes however.

In vivo evidence for the involvement of TBP in transcription by all three nuclear RNA polymerases was provided by genetic analyses in yeast (105). Expression of both TATA-less and TATA-containing class III genes was rapidly inhibited in yeast strains with a temperature-sensitive mutation in TBP upon incubation at the non-permissive temperature (105). The inhibitory effect on pol III transcription of an inactivating mutation in the largest subunit of pol II has much slower kinetics, suggesting that this inhibition due to loss of TBP function was not an indirect consequence of pol II transcription also being inhibited. Pol I transcription was also dramatically reduced, suggesting an in vivo role for TBP in transcription by all three nuclear pols (105). Extracts prepared from mutant TBP strains following incubation at the non-permissive temperature were unable to support pol I, II or III transcription (464). The addition of recombinant TBP restored transcription by each pol, demonstrating that the devastating effect of the TBP mutation on in vivo transcription by each of the pols is a direct one and independent of its effects on transcription by the other two nuclear pols (420, 464).

TBP is the only classless transcription factor identified to date and as such may be regarded as the most general. It also constitutes an obvious target for the co-ordinated regulation of transcription by pols I, II and III. A general role for TBP in nuclear transcription has been shown both in yeast and human suggesting this is likely to be the case in many, if not all, eukaryotes. In support of this, TBP has been highly conserved through evolution (213). The N-terminal region of TBP is variable both in size and sequence, but the C-terminal region is extremely well conserved. The C-terminal 180 residues of TBP are 100% identical in human, mice and frogs

and at least 80% identical between human and yeast (146, 198, 509). Moreover, in yeast the conserved C-terminal region is sufficient for cell viability and mutations in TBP that affect basal transcription by pols I, II and III all map to this region (105, 420, 464). However, recent work from the Tjian laboratory suggests that TBP may not be required for pol III transcription or restricted cell-type specific pol II transcription in Drosophila (193, 508).

The Drosophila genome contains a single TBP gene but also has two distinct genes that encode proteins homologous to TBP (109, 429). One of these, TBP-related factor 1 (TRF1) binds specifically to TATA sequences and shows considerable sequence similarity to TBP, with ~60% overall identity in the conserved C-terminal domain of TBP (109). The immunodepletion of TRF1 from Drosophila extracts was found to specifically inhibit pol III transcription of tRNA, 5S and U6 genes (508). In contrast, depletion of TBP was observed to have no effect on the level of pol III transcription, suggesting that TRF1 and not TBP may be required for pol III transcription in Drosophila, at least in vitro (508). These results came as a surprise, as it had previously been shown that the overexpression of TBP in Drosophila tissue culture cells by transient transfection stimulates pol III transcription, both of TATA-less and TATA-containing class III genes, suggesting TBP is limiting for pol III transcription in these cells (527). One plausible explanation is that overexpressed TBP may be able to substitute for TRF1. However, it has yet to be demonstrated that in the context of a living cell, TRF1 has a role in pol III transcription in Drosophila. In support of this possibility, antibody staining of polytene chromosomes revealed that TRF1 colocalises at a number of sites that contain one or more tRNA genes (193). A subsequent immunolocalisation study using a different TRF1 antibody showed that TRF1 colocalises at the majority of sites at which the TFIIIB component BRF was detected (508). However, this TRF1 antibody appeared to have lower specificity than that used in the previous study and TRF1 staining was very extensive, raising the possibility that the observed colocalisation was due to non-specific TRF1 antibody staining or was coincidental (508). There does not appear to be a mammalian homologue of Drosophila TRF1, however, several orthologs of a TBP-like protein have recently been identified in humans, which conceivably might perform analogous functions (32, 380, 429).

TBP fractionates extremely heterogenously during column chromatography of cell extracts, suggesting that it may be part of several different protein complexes,

each with distinct chromatographic behaviour (33, 55, 91, 371, 421, 456, 507, 524). Indeed, gel filtration analysis of HeLa extract suggests that there is very little or no free TBP in the cell (524). TBP participates in transcription by the nuclear pols as part of three different pol-specific TBP-containing multisubunit protein complexes, SL1 (103, 133, 616), TFIID (130, 175, 425, 426, 524) and TFIIIB (237, 264, 340, 484, 507, 573), that are required for transcription by pol I, pol II and pol III respectively. The polypeptides that interact with TBP in each of these complexes, so-called TBP-associated factors (TAFs) appear to be different, suggesting it is the TAFs that confer the class specificity upon the different complexes (91, 103, 507). The extent to which TBP is shared between pols I, II and III therefore seems to be dictated by the distinct sets of TAFs with which it associates. In HeLa cells there does not seem to be much, if any, free TBP in the cell suggesting that there may be competition between the different class-specific TAFs for binding to TBP (434, 524). Nevertheless, in most mammalian cells investigated, TBP is not normally limiting for pol III transcription. In yeast, there are significant amounts of free TBP in the cell.

### 1.5.2 TFIIIB

The accurate initiation of transcription requires the formation of a functional preinitiation complex at the promoter of the gene, a precisely ordered complex of transcription factors assembled on the DNA that accurately recruits and positions the pol over the start site for initiation (40, 405). For TATA-containing pol II templates, TFIID nucleates preinitiation complex formation, the TBP contained within this complex enabling it to specifically recognise and bind to the TATA sequence located upstream of the start site (442). Similarly, the TBP contained within TFIIIB is thought to enable TFIIIB, in co-operation with a PSE-binding factor (PBP/SNAPc/PTF) that binds sequence-specifically to the PSE, to nucleate complex formation at class III genes such as the vertebrate U6 and 7SK genes that also have a TATA box (376). However, for TATA-less class III genes, TFIIIB is recruited to the promoter by protein-protein interaction with other transcription factors such as TFIIIA and/or TFIIIC that are able to specifically bind to the promoters of these genes (36, 318, 472). In yeast, once TFIIIB has been recruited to the promoter and is stably bound, TFIIIC and TFIIIA can be stripped away by high salt or heparin and

TFIIIB alone is able to support multiple rounds of accurate transcription by pol III (263). Thus, clearly TFIIIB is the central class III initiation factor. The essential role of this factor in pol III transcription is reflected by the considerable effort that has been devoted to its purification and elucidation of its composition, both in yeast and in mammals (568).

An early attempt by Klekamp and Weil to purify TFIIIB from yeast by ion-exchange chromatography yielded weakly active fractions highly enriched in a 60kDa polypeptide that constituted ~30% of the total protein in the most highly purified fraction (283). To investigate whether this abundant 60kDa polypeptide corresponds to TFIIIB, it was gel-purified and polyclonal antibodies were raised against it (283). The resulting antiserum inhibited tRNA and 5S transcription reconstituted either with yeast or human extracts, whereas the preimmune serum did not (283). Furthermore, preincubation of the antiserum with purified 60kDa protein used to raise it blocked its inhibitory effect on transcription. The authors thus concluded that this 60kDa polypeptide likely constitutes yeast TFIIIB (yTFIIIB) (283). However, whereas the partially purified TFIIIB fractions were capable of reconstituting tRNA transcription in the presence of partially purified TFIIIC and pol III, the gel-purified 60kDa polypeptide was unable to do so despite multiple attempts at renaturation (283), raising the possibility that additional proteins may be required for TFIIIB activity. In support of this, quantitative analyses of transcription reconstituted using the partially purified fractions estimated that 100-150 molecules of 60kDa polypeptide were required per template molecule (283). Although such a stoichiometry could simply reflect the inactivation of the 60kDa protein during purification, an equally plausible explanation is that other proteins are also required for TFIIIB activity that are present in much smaller quantities, the bulk of these having been removed during fractionation.

Kassavetis et al. later partially purified yeast TFIIIB by a fractionation scheme based on that of Klekamp and Weil but slightly modified (267). Their most highly purified TFIIIB fraction still contained at least 25 electrophoretically separable polypeptides after five chromatographic steps, but nevertheless had ~60-fold higher specific activity than that of Klekamp and Weil (267). Notably, a 60kDa species was not a major constituent of this fraction (267).

Bartholomew et al. used this partially purified fraction and an ingenious photocrosslinking approach (23) to probe the polypeptide composition of yTFIIIB.

DNase I footprinting analysis with crude extract had earlier shown that the assembly of active pol III transcription complexes on yeast 5S rRNA and $tRNA^{Tyr}$ genes caused protection of the entire transcription unit and ~45 bp of 5′ flanking sequence immediately upstream of the transcriptional start site (48). To dissect the interactions of the individual class III transcription factors with the DNA and their contributions to this extensive footprint, DNase I protection analyses with partially purified TFIIIA, TFIIIB and TFIIIC had then been performed (48). The activity providing protection of the upstream region of the $tRNA^{Tyr}$ gene was found to consistently copurify with TFIIIB activity (267). Furthermore, protection of this region was dependent on the prior interaction of TFIIIC with the B-block and was resistant to heparin, both properties of TFIIIB and its interactions with tRNA gene promoters (267). Together, these observations suggest that this upstream DNA-binding activity and TFIIIB are the same. Bartholomew et al. exploited this apparent ability of yTFIIIB to bind to the 5′ flanking sequence of these genes to search for putative components of the factor using a novel photocrosslinking method (24). In this method, a photoactive nucleotide, 5-[N-(p-azidobenzoyl)-3-aminoallyl]-deoxyuridine ($N_3$RdU), is incorporated into specific sites in the DNA, adjacent to radiolabelled nucleotides (23). The modified DNA is incubated with appropriate protein fractions for a short time to allow the assembly of proteins on the DNA probe and is then irradiated with ultraviolet light. Irradiation generates a reactive nitrene group from $N_3$RdU that is able to rapidly form covalent bonds with polypeptides in very close proximity to the photoactive nucleotide, thus "tagging" them with radiolabelled DNA. The probe is then incubated with DNase I and micrococcal nuclease so that those regions of the DNA not bound by protein are destroyed. The size of the "tagged" proteins can then be determined by SDS-PAGE.

The structure of $N_3$RdU places the reactive nitrene on a 0.9 to 1nm tether, thus ensuring only proteins in the space just outside the DNA helix, within a distinct spatial region, will be detected (23). This approach, using photoactive nucleotides incorporated into the 45bp upstream region protected from DNase I digestion in a crude extract, detected two polypeptides, of 70 and 90kDa in size, within the partially purified TFIIIB fraction (24). The specificity of their crosslinking to this region of DNA was demonstrated by its dependence both upon a functional promoter and the presence of TFIIIC (24). The crosslinked 70 and 90kDa polypeptides are

distinct and separate components of TFIIIB. In support of this, the two polypeptides were found to be preferentially crosslinked by photoactive nucleotides located on opposite sides of the DNA helix suggesting they have distinct spatial distributions within this upstream region (24). They also produce different V8 protease digestion patterns (24).

The 70 and 90kDa polypeptides are only minor components of the partially purified TFIIIB fraction of Kassavetis et al. used in these crosslinking experiments (267). Upon further purification of this fraction on Mono S, a cation exchanger, TFIIIB was split into two essential components, B′ that eluted at ~275mM NaCl in a 100mM-400mM NaCl gradient and B″ that eluted in the subsequent 1M NaCl step (261). Together, the B′ and B″ fractions were able to reconstitute both the transcriptional and photocrosslinking properties of TFIIIB (261). Photocrosslinking associated the B′ fraction with the 70kDa polypeptide of TFIIIB and the B″ fraction with the 90kDa polypeptide of TFIIIB (261). Indeed, the B′ fraction was found to be highly enriched in a 70kDa polypeptide that represented ~5% of the total protein, which was distributed among ~10 polypeptides (261).

Three different groups independently cloned the BRF1 gene (TDS4/PSF4) that encodes the 70kDa polypeptide component of yeast TFIIIB (64, 101, 342). Database searches revealed that the N-terminal half of this protein shares extensive sequence homology with the pol II general transcription factor TFIIB, which is much smaller (64, 101, 342). The protein was therefore named yBRF, short for yeast TFIIB-related-factor (101). The sequence similarity between TFIIB and yBRF suggested that yBRF may function in pol II transcription. However, despite this homology, in contrast to the rapid decline in tRNA synthesis observed, a reduction in the levels of yBRF was found to have no effect on the in vivo expression of either class II or class I genes (64, 342). Similarly, in vitro transcription of tRNA and 5S rRNA genes was found to be substantially diminished in extracts lacking functional yBRF, whereas transcription by pols I and II was as efficient as in wild type extracts (101). Recombinant yBRF was able to rescue transcription of tRNA and 5S rRNA genes in these BRF-deficient extracts and could also stimulate their expression in wild type extracts, but had no effect on transcription by pols I and II (101). These results demonstrate that yBRF is a pol III-specific factor and suggest that it is usually limiting for pol III transcription. Indeed, yeast strains with a dominant mutation in

the BRF1 gene that specifically increases the in vivo expression levels of yBRF were found to have higher pol III transcriptional activity than wild-type strains (342).

The bulk of the homology between yBRF and the various TFIIB proteins that have been cloned is within three distinct regions, a putative zinc finger at the extreme N-terminus and two imperfect direct repeats (64, 101, 342). The direct repeat region of TFIIB has been shown to interact directly both with TBP and pol II, consistent with the proposed function of TFIIB as a bridging factor between promoter-bound TBP and pol II during preinitiation complex formation (183). Since this region is well conserved in yBRF it suggested that yBRF might also interact with TBP and, perhaps, pol III since several subunits are shared between pols II and III. In support of this, two of the groups that cloned the BRF1 gene isolated the gene as an allele specific multi-copy suppressor of TBP mutations (64, 101). The allelic specificity of the suppression is suggestive of a direct interaction between TBP and yBRF. A combination of in vitro and in vivo experiments showed TBP to be essential for yeast pol III transcription (105, 464). However, the crosslinking experiments of Bartholomew et al. that detected the 70 and 90kDa subunits of TFIIIB did not identify a polypeptide of ~27kDa corresponding to the size of yeast TBP (24). Nonetheless, TBP was detected in the partially purified TFIIIB fraction used for crosslinking and a significant amount was found in the B′ fraction that is enriched in yBRF (264). In contrast, no TBP was detected by western blot analysis in highly purified fractions of TFIIIC and pol III (264). In addition, antibodies against TBP were found to specifically supershift a heparin-stripped TFIIIB-tDNA complex in an EMSA, demonstrating that TBP is part of this TFIIIB complex (264). Subsequently, it was shown that recombinant TBP and recombinant BRF directly interact (235, 271). GST-BRF pulldown assays were used to map the regions of BRF to which TBP binds (271). As expected, the conserved direct repeat region of BRF was able to specifically bind TBP. Rather surprisingly, however, the C-terminal region alone was also shown to bind TBP with even higher affinity (271). Moreover, three separate point mutations in TBP that specifically inhibit pol III transcription in vivo were found to prevent TBP from interacting with the C-terminal half of BRF yet had no effect on its interaction with the direct repeats (271). This strongly suggests that the interaction between TBP and the C-terminal half of BRF is of physiological significance. The N-terminal direct repeat region was also found to interact with a

subunit of pol III, consistent with the homologous region of TFIIB binding pol II (271). However, within this region the two pols interact with different repeats, moreover, the pol III subunit, C34, that directly binds to this region of BRF is unique to pol III (183, 271). In addition, the N-terminal half of BRF, but not the C-terminal half, was found to interact with the largest subunit of yeast TFIIIC (82). Thus, the N-terminal half of yBRF may perform an analogous function in pol III transcription to the highly homologous TFIIB since it participates in interactions both with the activity that recognises class III promoters (TFIIIC or TBP depending on promoter type) and the polymerase itself. Although there is significant conservation between TFIIB and the N-terminal half of BRF, these regions clearly actively participate in determining the differential pol specificity of TFIIB and BRF.

In contrast to the N-terminal region, the C-terminal half of yBRF has no obvious sequence similarity with any other known yeast protein. It is characterised by being highly charged, with 21% acidic residues (D or E), 17% basic residues (K or R) and 14% Ser and Thr residues (271). The high charge density of the C-terminal region of BRF has been well maintained through evolution (271). Sequence comparisons of yBRF cloned from three evolutionarily distant yeast species Saccharomyces cerevisiae, Kluveromyces lactis and Candida albicans, showed the N-terminal half of the protein to be highly conserved, especially the direct repeat region. The C-terminal half was found to be much less well conserved but it does contain three discreet areas of strong conservation that are likely to be essential for BRF function, yeast homology regions I, II and III (HI, HII and HIII) (271). In support of this, the deletion of the last 165 amino acid residues of yBRF, which includes the HII and HIII domain, prevented the binding of TBP (271). The HII domain is thought to mediate the interaction of TBP with the C-terminal half of BRF (11, 102, 262, 265, 271). The exact function of the other two homology domains have yet to be fully elucidated, but their high level of conservation suggests that they may mediate other important interactions of BRF.

The TFC5 gene encoding the 90kDa polypeptide of yTFIIIB has also been cloned (266, 439, 452). It actually encodes a 594 amino acid protein with a predicted molecular mass of ~68kDa that migrates anomalously as a ~90kDa protein on SDS PAGE (266, 439, 452). The protein, which has been called B″, has little or no sequence similarity with other known proteins except for a putative SANT domain

also found in N-CoR, SWI3 and ADA2 (1). This motif, which shares weak homology with the DNA-binding domain of the oncoprotein Myb, is implicated in protein-protein and protein-nucleic acid interactions (1).

TBP, BRF and B″ are all essential for yeast cell viability and, specifically, pol III transcription. Despite its indispensability, however, B″ is remarkably resistant to truncation (266, 297). Although B″ is essential for U6 transcription, extensive deletion analysis has failed to identify a single amino acid that is absolutely required for transcription with only four isolated amino acids yet to be tested (297). A core B″ protein of only 176 amino acids (out of 594) has been found to be sufficient for U6 transcription, although a larger domain is required for tRNA transcription (297). This is likely to reflect the TFIIIC dependency of tRNA synthesis. Two distinct domains (I and II), the first of which includes the SANT domain, that both seem to be necessary for tRNA transcription can function on an either-or basis for U6 snRNA synthesis (297). B″ makes multiple contacts within the preinitiation complex (24, 253, 261, 264, 266, 439, 452), which may explain why it is so tolerant of deletion mutagenesis, the loss of any individual contact being compensated by other interactions made by the protein (266).

The recombinant B″ protein is able to replace the Mono S B″ fraction in DNA binding and transcription of yeast tRNA and U6 genes, indicating that it is the sole essential component of the B″ fraction (266). Similarly, recombinant TBP and recombinant BRF together are sufficient to reconstitute all the properties of B′ TFIIIB activity (264). Indeed, it has been possible to reconstitute yTFIIIB from entirely recombinant components (266, 439, 452). However, although TFIIIB reconstituted from recombinant TBP, BRF and B″ displayed identical DNA binding properties to native yTFIIIB, the factor was found to be less active for transcription (266, 452). This may be because the recombinant polypeptides are not folded correctly or lack important post-translational modifications. Alternatively, some component(s) may be missing from the transcriptional system reconstituted with recombinant TFIIIB components. Perhaps there are additional subunits of yTFIIIB that have yet to be identified that are nonessential but stimulatory, analogous to the TFIID TAFs that are dispensable for basal transcription of TATA-containing class II promoters but are required for activated transcription. It is also plausible that the component is an essential part of TFIIIB but present in residual amounts as a

contaminant in the purified complementary fractions used to reconstitute transcription. Alternatively, the putative missing component may be distinct from TFIIIB. A prime candidate for such a component is TFIIIE, which has been detected in both native B′ and B″ fractions (121, 122). Ruth et al. found that transcription reconstituted with recombinant TBP, BRF, and B″ is stimulated by TFIIIE (452). Thus, yeast TFIIIB consists minimally of BRF, TBP and B″, but there may be additional, perhaps regulatory, subunits. However, the identification of any such subunits is made more difficult by its apparent lability (235, 261). Indeed, yTFIIIB has yet to be purified to anywhere near homogeneity without its resolution into at least two separate components. B″ is easily dissociated from TFIIIB by a variety of mild chromatographic procedures (235, 261). It has been suggested that yTFIIIB is not a stable molecular entity, except when in a DNA-bound state (83, 235).

The composition of mammalian TFIIIB is much less well characterised than that of yeast. The mammalian factor was first identified as a single polypeptide of 60kDa in size, as was originally reported for yeast TFIIIB by Klekamp and Weil (545). This followed chromatography of HeLa cell extracts on a series of ion-exchange columns in a fractionation scheme essentially the same as used by Klekamp and Weil. A 60kDa polypeptide was found to constitute ~90% of the protein in the most highly purified fraction which was capable of supporting transcription of tRNA, 5S and VA genes in the presence of partially purified complementing fractions providing a source of TFIIIA, TFIIIC and pol III (545). Additional support for TFIIIB being a 60 kDa protein was provided by the sedimentation of TFIIIB activity at ~60kDa following glycerol gradient centrifugation of either crude or partially purified fractions (545). However, it was subsequently demonstrated that TBP is an essential component of human TFIIIB, as in yeast (340, 371, 484, 507, 573). Thus, antibodies raised against TBP were found to specifically immunoprecipitate TFIIIB activity (91, 340, 371, 507, 573). Furthermore, a subpopulation of TBP molecules were found to consistently cofractionate with TFIIIB during a variety of chromatographic procedures (340, 371, 484, 507, 514, 573). The 60kDa protein reported by Seifart's group is therefore likely at best to be only one component of a larger TFIIIB complex.

Gradient chromatography on Mono Q, a strong anionic exchanger, splits human TFIIIB (hTFIIIB) into two components raising the possibility that hTFIIIB,

like yeast TFIIIB, might be labile (91, 340, 371). Lobo et al. found that these components eluted at 380mM and 480mM KCl and named them 0.38M-TFIIIB and 0.48M-TFIIIB, respectively (340). TBP was only found in the 0.38M-TFIIIB fraction (340). The active component of this fraction could be specifically immunodepleted using anti-TBP antibodies. Moreover, whereas recombinant TBP was unable to reconstitute transcription of 5S and $VA_I$ genes in a TBP-immunodepleted extract, the 0.38M-TFIIIB fraction was able to do so (340). This suggests that one or more TAFs exist in a complex with TBP in the 0.38M-TFIIIB fraction and that these are required for $VA_I$ and 5S transcription.

Using anti-TBP antibodies, a polypeptide of 88-90kDa was found to specifically coimmunoprecipitate with TBP and TFIIIB activity from 0.38M-TFIIIB fractions and other TFIIIB-containing fractions, suggesting that it might be a subunit of TFIIIB (374, 552). The cDNA for this protein was isolated and found to encode a 677 amino acid protein (374, 552). The N-terminal 280 residues are 41% identical to S.cerevisiae BRF and 24% identical to human TFIIB (374). The protein is therefore referred to as human TFIIB-related factor (hBRF) (374). Regions of extensive homology shared by all three proteins include a zinc finger motif and two imperfect direct repeats (374). This is also true of BRF homologues cloned from Caenorhabditis elegans (CeBRF) and Drosophila melanogaster (DmBRF) (316, 508). The C-terminal half of hBRF is highly divergent with little homology to the yBRFs except for yeast homology regions II and III, which are conserved (374). The conservation of yeast HII and HIII domains between metazoan and lower eukaryotic BRF species suggests that these two domains are likely to be required for core BRF function. The C-terminal half of hBRF also has a region of low homology to chicken HMG2, including an acidic C-terminal tail typical of HMG2 proteins (552). However, CeBRF lacks an HMG2-like motif, suggesting such a motif is not essential for metazoan BRF function (316). Like the yeast BRFs, hBRF has two TBP-binding sites, a weak one in the N-terminal half and a strong one in the C-terminal half (552).

Immunodepletion of hBRF from HeLa nuclear extracts was found to severely inhibit transcription of VA and tRNA genes, demonstrating its essential role in human pol III transcription (374, 552). Transcription could be restored by the addition of recombinant BRF and TBP but not by rBRF alone, consistent with the stable association of TBP and BRF within hTFIIIB (374, 552). That rTBP and rBRF were sufficient to reconstitute transcription suggests that any other components of

hTFIIIB are either loosely associated, displaced by the antibodies, or not required for basal expression. Gel filtration of 0.38M-TFIIIB suggests that the complex has a molecular weight of 300kDa, so it is unlikely that TBP and BRF are its sole constituents (340).

In addition to hBRF, a variety of other polypeptides were also found to coprecipitate with TBP following the immunoprecipitation of partially purified TFIIIB fractions with anti-TBP antibodies (91, 340, 374, 507). Taggart et al. identified a tightly-associated polypeptide of 172kDa (TAF172) and another more loosely-associated polypeptide of unreported size (TAF-L) (507). Lobo et al. detected polypeptides of 54, 83 and 150kDa (340), Chiang et al. detected polypeptides of 60, 87, 96 and 190kDa (91), and Mital et al. detected polypeptides of 27, 45, 67 and 70 kDa, as well as a 90kDa polypeptide that corresponds to hBRF (374). At present, however, hBRF is the only polypeptide identified by this approach to be confirmed as a subunit of hTFIIIB. Some of the polypeptides detected appeared substoichiometric to TBP and BRF, suggesting they may only be loosely associated or may bind TBP indirectly. This may partly explain some of the differences in the polypeptides detected by the different groups, the relationships between which are presently unclear (434). Although it remains to be seen which, if any, of these polypeptides are actually subunits of hTFIIIB, the coprecipitation of so many polypeptides suggests that the polypeptide composition of hTFIIIB might be complex.

Although the exact composition of hTFIIIB remains uncertain, significant progress has recently been made with the cloning of a human homologue of yeast B″ (463). Human B″ (hB″) is substantially larger than its yeast counterpart, with a predicted molecular mass of 156kDa (463). There are three major regions of sequence similarity, a putative SANT domain (αα 415-472) that is 43% identical with that of yeast B″, a 131-amino acid region immediately upstream of this domain of 21% identity and a 115-amino acid region immediately downstream of the SANT domain of 17% identity (463). In addition to human and Saccharomyces cerevisiae B″, sequences coding for putative B″ homologues have also been detected in mouse, Drosophila melanogaster, Caenorhabditis elegans, Schizosaccharomyces pombe and Arabidopsis thaliana genomes (463). In all of these organisms, the B″ SANT domain is extremely well conserved, suggesting that it may be essential for B″

function (463). The most notable feature of the large C-terminal extension of hB″ that is missing from yB″ is a stretch of 19 short repeats of 26-28 amino acids. These repeats are similar to sequences that form coiled-coil structures from proteins belonging to the myosin and intermediate filament families, but a similar structure is not predicted for this region of hB″ (463). An essential role for hB″ in human pol III transcription is suggested by its immunodepletion from cell extracts which was found to severely compromise class III gene expression but had no effect on pol II transcription from the adenovirus major late promoter or the human U1 snRNA promoter (463). Furthermore, the addition of bacterially expressed recombinant hB″ to depleted extracts was able to restore pol III transcription (463). TFIIIB therefore seems to be strongly conserved between yeast and humans, all three core yeast subunits, TBP, BRF and B″, having structural and functional homologues in human TFIIIB.

However, whereas in yeast there is a single form of TFIIIB that is necessary and sufficient for the transcription of all class III genes, in humans there appear to be multiple forms of TFIIIB that function at different class III promoters. There is now substantial evidence that the TFIIIB requirements for type III promoters are different to those of types I and II (340, 365, 374, 463, 514, 515, 552). There are several examples of the physical separation of these requirements by column chromatography (340, 514). Teichmann and Seifart separated two forms of hTFIIIB on EMD-DEAE-Fractogel (EDF) (514). One form, hTFIIIB-α, which eluted in 200mM KCl, was active for U6 but not $VA_I$ transcription. Conversely, the other form, hTFIIIB-β, which eluted with TBP and BRF in 300mM KCl, was capable of supporting $VA_I$ but not U6 transcription (514). hTFIIIB-α and hTFIIIB-β were also found to behave differently on Cibacron blue and on a Sephacryl-S300 HR gel filtration column (514). It is well established that hBRF is necessary for the transcription of type I and type II genes, but its requirement for transcription of type III genes has been controversial (374, 552). Whereas Wang and Roeder observed inhibition of U6 and 7SK gene expression following immunodepletion of extracts with anti-BRF antibodies (552), Mital et al. found that immunodepletion of hBRF inhibited $VA_I$ but had no effect on U6 transcription (374). A possible explanation of this discrepancy is suggested by the recent cloning by two different groups of a novel gene that encodes a protein, BRFU (463) (or hTFIIIB50) (515), that is highly related

to hBRF and hTFIIB. This protein is specifically required for transcription of human U6 and 7SK genes but not the adenoviral $VA_I$ gene (463, 515). It might be the anti-BRF antibodies of Wang and Roeder recognised both hBRF and BRFU, but those of Mital et al. only depleted extract of hBRF (374, 552). In addition to BRFU, several splice variants of hBRF (hBRFs 2, 3 and 4) have also recently been discovered (365). It is reported that the different splice variants function at different class III genes (365). Thus, hBRF, hereon referred to as hBRF1, is the most active variant in transcription of type I and II promoters whereas a different variant, hBRF2, a 139 amino acid protein that completely lacks the first N-terminal direct repeat of hBRF1, is implicated in U6 gene expression. The form of TFIIIB required for U6 transcription may therefore contain two distinct BRF species, BRFU and hBRF2. In contrast, BRF1 is required for the expression of genes with type I or type II promoters. The recently identified hB″ is an essential component of TFIIIB for the transcription of both $VA_I$ and U6 genes, suggesting that, like TBP, it is a common factor of the different forms of TFIIIB utilised by different class III genes (463). At present it is unclear whether the identity of the BRF species within the TFIIIB complex represents the only difference in the composition of TFIIIB utilised by type III promoters versus types I and II, or whether there are additional protein components that may be involved in conferring promoter specificity. Possible candidates include proteins of 54, 48, 42 and 40kDa that were found to stably associate with BRFU (TFIIIB50) during its immunopurification (515). That these proteins might constitute active components of TFIIIB is suggested by the ability of this immunopurified complex to restore U6 transcription to extracts depleted of BRFU (TFIIIB50), whereas recombinant BRFU alone was unable to do so (515). The resistance of this immunopurified complex to stringent washing with 500mM KCl provides support that these BRFU-associated proteins may be of physiological significance (515). A protein of similar size to BRF2 (365) was also observed in some preparations of the BRFU (TFIIIB50) complex (515).

### 1.5.3 TFIIIC

TFIIIB alone has little or no sequence-specific affinity for DNA. Its recruitment to class III genes with type I or type II promoters requires TFIIIC (318).

TFIIIC specifically recognises and binds to the A- and B- block intragenic promoter elements of type II promoters. This sequence-specific DNA-binding activity of TFIIIC has greatly facilitated its purification (71, 118, 156, 267, 291, 331, 408, 505, 608). The factor was first purified from yeast where it is also referred to as τ factor. Yeast TFIIIC comprises of six polypeptides of 138 (325), 131 (355, 431), 95 (407, 505), 91 (14), 60 (119) and 55 kDa (351) (referred to as τ138, τ131, τ95, τ91, τ60 and τ55 respectively). These polypeptides were found to tightly associate and copurify as a single entity during ion-exchange chromatography and affinity chromatography on specific tDNA or B-block columns (71, 156, 267, 408, 505). Limited protease treatment of TFIIIC generated a smaller form of the factor that retained its DNA-binding affinity but was only able to bind the B-block (360). This suggested that the A-block and B-block binding domains of TFIIIC are separate. In support of this, visualisation of yeast TFIIIC-tDNA complexes by electron microscopy revealed TFIIIC to consist of two linked globular domains, each of ~300 kDa and ~10 nm in diameter (465). The linker connecting the two DNA-binding domains appears flexible and able to stretch (67, 465), accounting for the remarkable feat of TFIIIC to bind to A- and B- blocks separated by a wide variety of distances (18, 138). All six polypeptides of TFIIIC have been cloned and have been shown to be essential for yeast cell viability (14, 119, 325, 351, 355, 407, 431, 505). Although several of the polypeptides contain sequences that closely resemble known DNA-binding motifs (326, 355, 505), none of the polypeptides seem to be able to bind specifically to DNA on their own, suggesting the A-block and B-block binding domains may be composite, involving more than one polypeptide. Photocrosslinking experiments showed τ131, τ95 and τ55 to be positioned in close vicinity to the A-block, suggesting that they may be involved in TFIIIC binding to the A-block (23, 24). Only τ138 is accessible to photocrosslinking from the B-block, suggesting it is likely to be directly involved in B-block DNA binding (23). TFIIIC fractions consisting primarily of just τ138 and τ95 retain a substantial proportion of TFIIIC DNA binding activity, suggesting that these polypeptides are the major quantitative determinants of tDNA recognition (155).

Human TFIIIC is less stable than the yeast factor (118, 607). It is resolved into two components, TFIIIC1 and TFIIIC2, during ion exchange chromatography on Mono Q (607). Both of these components are required for transcription of tRNA, 5S

and VA genes (118, 331, 553, 607) but only TFIIIC1 is required for U6 and 7SK transcription (307, 397, 604). The subunit composition of TFIIIC1 has yet to be determined. Sedimentation analysis suggested a mass of up to 200kDa for TFIIIC1, assuming that it is globular, however little further progress has been made towards its characterisation (607). TFIIIC2 has been highly purified and found to comprise of 5 polypeptides of 220, 110, 102, 90 and 63 kDa (α, β, γ, δ, ε, respectively) (291, 331, 608). TFIIIC2 binds specifically and with high affinity to the B-block region of $VA_I$ and tRNA genes (44, 553, 607). TFIIIC1 enhances the B-block footprint generated by TFIIIC2 and extends it both 3′ and 5′ to include the A-block (553, 607). However, it remains to be determined whether TFIIIC1 binds directly to the A-block or induces rearrangements in TFIIIC2 allowing the latter to do so. All five subunits of TFIIIC2 have been cloned (230, 231, 307, 331, 488). In contrast to TFIIIB and pol III, TFIIIC seems to be very poorly conserved between yeast and humans. Human TFIIICα and TFIIICβ, which together appear to be responsible for the specific B-block binding activity of TFIIIC2 (43, 44, 291, 481, 608), show no significant homology to any of the subunits of yeast TFIIIC. This is very surprising because the A- and B-block sequences are highly conserved between yeast and mammals. hTFIIICδ also lacks significant sequence similarity with any of the yeast TFIIIC subunits (230). However, the remaining two components of TFIIIC2 display weak homology to particular subunits of yeast TFIIIC; hTFIIICε shares 22% identity with τ95 and hTFIIICγ is 31% identical to τ131, both of which contain 11 copies of a tetratricopeptide repeat (TPR) (231).

### 1.5.4 TFIIIA

TFIIIB, TFIIIC and pol III are sufficient for basal transcription of class III genes with type II promoters, however, 5S transcription requires an additional factor called TFIIIA (134, 473). Like TFIIIC, TFIIIA is poorly conserved between species, for example frog and yeast TFIIIA share only 20% overall sequence identity (13). Despite the sequence divergence of TFIIIAs from different species, in every species the factor is characterised by its possession of nine tandemly arranged, zinc-dependent DNA binding domains, commonly referred to as zinc fingers (13, 373,

381). The TFIIIA zinc fingers have a single zinc ion coordinated by two cysteine and two histidine residues. The loop-like structure or "finger" created is thought to consist of an antiparallel β-sheet packed against an α-helix (31). The α-helix of such fingers typically make sequence-specific contacts in the major groove of DNA. TFIIIA is highly modular, with clusters of zinc fingers binding specifically to distinct regions of the 5S gene ICR. The N-terminal three fingers (fingers 1-3) bind the C-block, finger 5 binds the intermediate element, fingers 7-9 bind the A-block, whereas fingers 4 and 6 span these promoter regions, functioning primarily as spacer elements (98, 139, 200, 201). TFIIIA also specifically contacts TFIIIC, thus serving as a molecular adaptor between TFIIIC and the 5S gene promoter and allowing TFIIIC to recruit TFIIIB to 5S genes (318, 472).

### 1.5.5 PTF/SNAPc

Class III genes with a type III promoter such as 7SK and vertebrate U6 genes have distinct transcription factor requirements from genes with type I or II promoters (307, 365, 374, 432, 463, 514, 515, 546, 604). The lack of an internal control region (ICR) obviates the need for TFIIIA or TFIIIC2, however the poorly characterised TFIIIC1 is required for transcription (307, 604). Since type III promoters contain a TATA box, TFIIIB can bind to such promoters independently of other factors. However, recruitment of TFIIIB to the TATA box is greatly enhanced by a factor called SNAPc/PTF that binds the PSE of these promoters (604). This factor is essential for transcription of 7SK and vertebrate U6 genes (604). It has five subunits, all of which have been cloned (17, 208-210, 455, 592, 605). The largest subunit, SNAP190 (PTFα), contains a Myb DNA-binding domain and can be crosslinked to the PSE (592). The U6 and U2 PSEs are functionally interchangeable suggesting that the same PSE-binding factor may be employed by class II and class III snRNA genes (334, 338). Indeed, immunodepletion of extracts of SNAPc/PTF was found to inhibit not only U6 and 7SK transcription but also pol II transcription of U1 and U2 genes (17, 208, 210, 455, 605). Moreover, purified SNAPc/PTF was able to restore transcription of these genes; thus it seems that there is a single PSE-binding protein shared by pols II and III.

The very strict PSE-TATA box spacing requirement of type III promoters suggests that TFIIIB and SNAPc/PTF interact (167, 329, 339, 543). Indeed, subunits of SNAPc/PTF have been shown to bind directly to TBP (17, 208, 210, 455, 605). This may explain how SNAPc/PTF stimulates TFIIIB recruitment to the U6 promoter. Conversely, TBP enhances SNAPc/PTF binding to the PSE (376). It seems that SNAPc/PTF and TFIIIB cooperatively interact facilitating the recruitment of each other to the promoter. Despite this, their binding to the PSE and the TATA box respectively is relatively slow. SNAP190 contains a C-terminal extension that inhibits it from interacting with the PSE, perhaps by masking its DNA-binding domain (377). However, interaction with a pol II- and pol III- transcriptional activator called Oct1, which binds octamer motifs found in the DSE of these promoters and those of pol II-transcribed U snRNA genes, overcomes this autoinhibition (375). Thus, whereas the association of SNAPc with the PSE of the human U6 gene takes over one hour in the absence of Oct1 it is complete in 15-30 minutes in its presence (375).

## 1.6 Preinitiation complex formation on class III genes and interactions between class III transcription factors and with pol III

The formation of active transcription complexes on the different class III genes was initially investigated by means of the template exclusion assay. This approach monitors the ability of a gene that is preincubated with limiting amounts of factor(s) to exclude transcription of a second gene added subsequently; preferential transcription of the first gene indicates the stable interaction of a limiting component during the preincubation, thereby precluding its association with the second gene. Using this assay, ordered stepwise assembly pathways for the formation of preinitiation complexes on type I and type II promoters have been defined (Fig. 1.1). Thus, TFIIIB was found only able to bind to VA and tRNA genes after TFIIIC had bound (318). In the case of a 5S gene, TFIIIC was found to bind after TFIIIA and before TFIIIB (318). Using separated TFIIIC1 and TFIIIC2 it was found that TFIIIC2 is the first factor to bind to $VA_I$ or tRNA genes, consistent with its B-block binding activity (117). TFIIIC1 and TFIIIB can then interact in either order to form a functional preinitiation complex (117).

**Figure 1.1**

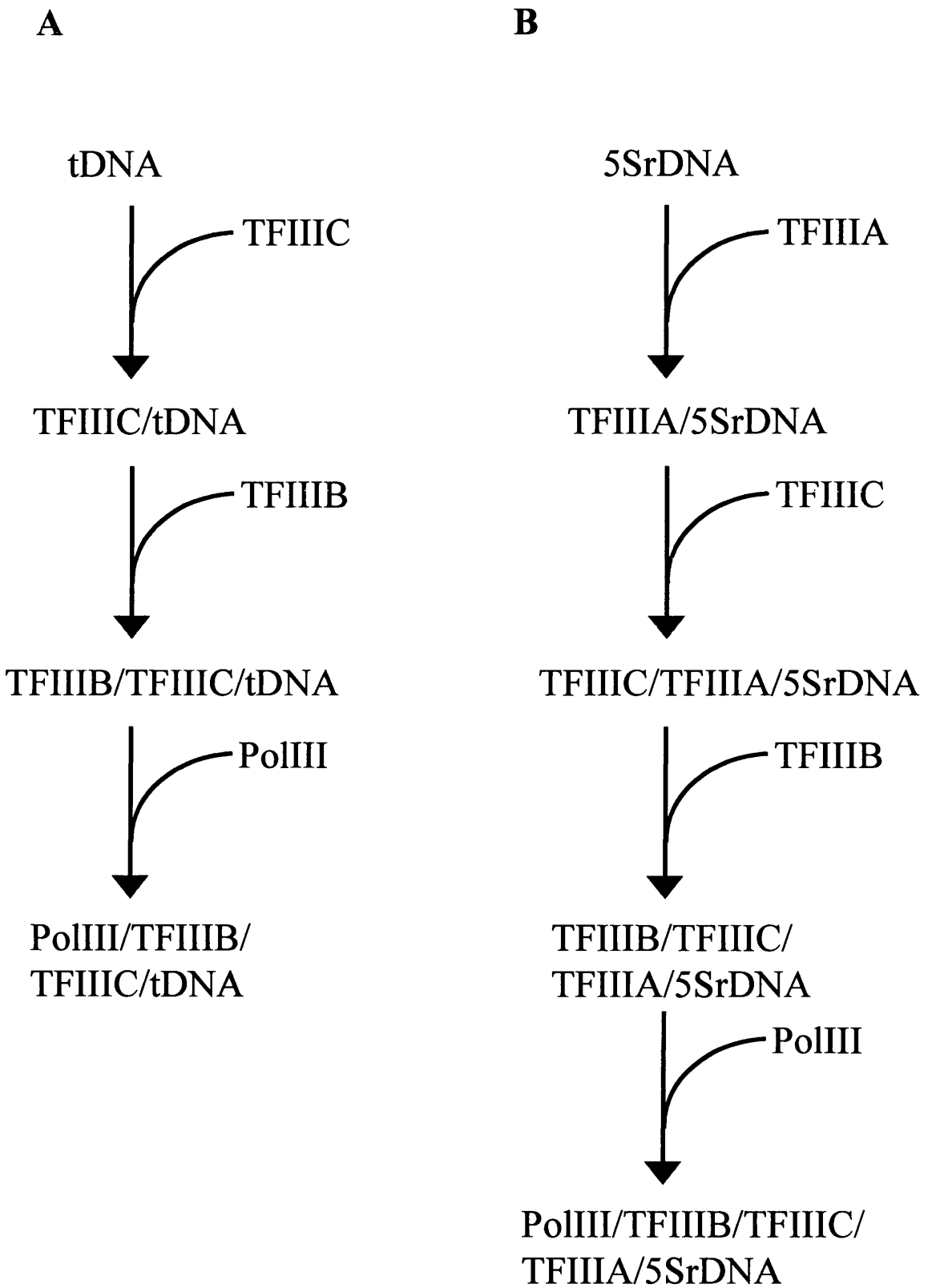
**Stepwise assembly of class III preinitiation complexes in vitro**

**A)**

Flow chart indicating the order of interaction of transcription factors and polymerase with a typical type II promoter such as that of a tRNA gene.

**B)**

Flow chart indicating the order of interaction of transcription factors and polymerase with the promoter of a 5S rRNA gene.


A
tDNA
TFIIIC
TFIIIC/tDNA
TFIIIB
TFIIIB/TFIIIC/tDNA
PolIII
PolIII/TFIIIB/
TFIIIC/tDNA
B
5SrDNA
TFIIIA
TFIIIA/5SrDNA
TFIIIC
TFIIIC/TFIIIA/5SrDNA
TFIIIB
TFIIIB/TFIIIC/
TFIIIA/5SrDNA
PolIII
PolIII/TFIIIB/TFIIIC/
TFIIIA/5SrDNA

Such assembly pathways defined in vitro with separated components clearly may differ from how functional preinitiation complexes are formed in vivo. Indeed, Wang et al. (551) recently reported the discovery of a human pol III holoenzyme, that is, a complex of the pol III machinery that is fully functional for accurate and specific pol III transcription that exists independently of DNA, suggesting that preinitiation complexes may be preassembled off the DNA. Nevertheless, these sequential assembly pathways are an indication of how transcription complex formation might occur in vivo and provide some information of interactions that likely occur between the transcription factors and with pol III that may be functionally important.

The current knowledge of the network of interactions between individual components of the pol III transcriptional machinery is most advanced in yeast where the majority of the components required for basal transcription have been cloned (83). Protein-DNA photocrosslinking has been used to map the positions of the various components of the pol III transcriptional machinery on a yeast tRNA promoter (23, 24). Thus, yBRF and B″ can be specifically crosslinked to sequences located ~40bp upstream of the transcriptional start site in a TFIIIC-dependent manner (24). τ131 is the only subunit of TFIIIC accessible for crosslinking with photoactive nucleotides located upstream of the start site (24, 25). This subunit is therefore an excellent candidate for interacting with TFIIIB and correctly positioning it on the DNA. Moreover, τ131 contains 11 tetratricopeptide repeats (TPRs) which are known to mediate protein-protein interactions (355). Two-hybrid analysis showed that τ131 interacts both with yBRF (82) and yB″ (452). In addition, recombinant yBRF has been shown to bind directly to τ131 (271). Significantly, τ131 is one of the two subunits of yTFIIIC that has sequence similarity with a subunit of human TFIIIC suggesting that these interactions may be conserved. Indeed, the human homologue of τ131, hTFIIICγ, directly binds hBRF (231). It has also been shown to directly associate with TBP (231). In yeast, τ60 interacts with TBP (119). In the vertebrate system interactions have also been described for hTFIIICε with hBRF and hTBP (231).

The assembly of TFIIIB on a yeast tRNA promoter can be reconstituted in vitro using isolated recombinant yeast TFIIIB subunits (266, 452). A precisely ordered series of interactions are required. yBRF is the first factor to associate with

the TFIIIC/tDNA complex, followed by TBP and then B″ (264). In the absence of BRF, TBP cannot be recruited, whereas recruitment of B″ requires both TBP and BRF (264, 266, 452). Each step in the sequential assembly of TFIIIB on DNA is accompanied by changes in the photocrosslinking efficiency of τ131 and yBRF (264). Such changes are suggestive of conformational rearrangements. This may explain the paradoxical ability of yeast TFIIIB, which alone has little or no affinity for DNA, to bind so tightly to DNA once fully assembled on the DNA by TFIIIC. It seems that yeast BRF contains a cryptic DNA-binding domain in its C-terminal half that becomes unmasked by the conformational changes that occur in the factor during the assembly of TFIIIB on the promoter (234). By hiding the DNA-binding domain, which is sequence independent, this prevents BRF from being randomly dispersed on irrelevant DNA sites through the genome.

In yeast, TFIIIA and TFIIIC are dispensable for transcription once they have recruited TFIIIB to the promoter (263). TFIIIB in turn is responsible for the recruitment of pol III to the promoter and correctly positioning it over the start site for transcription initiation. All three subunits of yeast TFIIIB are required for the stable recruitment of pol III however so far only yBRF has been shown to directly interact with the polymerase (22, 145, 271, 564). Prior to initiation, the C160, C128 and C34 subunits of pol III are all accessible to crosslinking upstream of the start site and may therefore be in the close vicinity of promoter-bound TFIIIB (21, 22, 415). As such, these three pol III subunits constitute excellent candidates for interacting with TFIIIB. One of these, C34, has been shown to directly bind yBRF (271). The human homologue of C34, C39, interacts directly with both hBRF and TBP (554). The functional conservation of an interaction between TFIIIB and this subunit of pol III through evolution suggests that their interaction may be essential (554). In support of this, C34/C39 appear to play a crucial role in the recruitment of pol III to class III genes, which is also dependent upon interaction with TFIIIB (236, 554). Thus, antibodies against C34 were found to potently inhibit tRNA synthesis in vitro but had little effect upon nonspecific transcription of poly(dA.dT) by pol III (236). Furthermore, mutations in C34 that impair its interaction with yBRF also inhibited pol III recruitment (61). A similar role for C39 is suggested by its selective dissociation from human pol III by sucrose sedimentation in 0.5M KCl or partially denaturing conditions as part of a stable subcomplex that also includes C62 and C32

(554). Human pol III that has been depleted of this subcomplex is fully competent for transcription elongation and termination but has lost the ability to support promoter-directed initiation (554). Accurate initiation can be restored to the depleted polymerase by adding back recombinant or natural subcomplex (554). The homologous yeast proteins C34, C82 and C31 also form a subcomplex, which dissociates from yeast pol III during native gel electrophoresis (535, 565). Interactions between the human and yeast pol III subcomplexes and TFIIIB have only been detected for C34/C39 (554, 564). Recently, however, a direct interaction has been described between the newly identified yeast pol III subunit C17 and yBRF (145). C17 also interacts with C31 (145). Thus, at least two subunits of yeast pol III, C34 and C17, may contribute to the specific recognition of TFIIIB by the enzyme. Both subunits are essential, suggesting that their roles in recruiting pol III to class III genes are not redundant.

The interaction site on yBRF for C34 has been mapped to the direct repeat region in the TFIIB-related N-terminal half of the protein (271). This region is highly conserved between BRF and TFIIB, raising the remote possibility that C34 might also bind TFIIB. However, GST-pulldown assays have failed to detect an interaction between TFIIB and C34 (271). TFIIB performs an analogous role in pol II transcription to BRF in the pol III system since it is involved in the specific recruitment of pol II to class II promoters. Moreover, it is the direct repeat region of TFIIB that binds pol II (183). Importantly, C34 and C17 are both unique to pol III and have no similarity to any pol I or pol II subunit (145, 437, 499). This, and the selectivity of the interaction between BRF and C34 and that between BRF and C17 may be essential in defining the pol specificity of class III gene preinitiation complexes, although other interactions involving pol III may also be important.

Direct interactions have recently been detected between pol III and TFIIIC (129, 148, 230, 231). In yeast, two-hybrid screening detected an interaction between τ131 and the ABC10α polymerase subunit that is found in all three nuclear pols (129). This has been confirmed in vitro using recombinant proteins (129). Additionally, a temperature sensitive mutation in ABC10α has been shown to be specifically suppressed by the overexpression of a mutant form of τ131 (129). Furthermore, the mutation in ABC10α was found to weaken the interaction with τ131 and the suppressive mutation in τ131 increased the interaction between the two

proteins (129). Thus, the interaction between ABC10α and τ131 appears to be functionally important. This is rather surprising because yeast TFIIIC is dispensable for transcription in vitro once TFIIIB has been recruited (263). Promoter-bound yTFIIIB is able to recruit pol III for multiple rounds of accurately initiated transcription with the same efficiency as a fully assembled transcription complex containing TFIIIC and, for 5S genes, TFIIIA (263). However, in a physiological context TFIIIB may not be sufficient and interactions between TFIIIC and pol III may be required. These may contribute to the recruitment of pol III or may be necessary for a post-recruitment function. Alternatively, in vivo, pol III may be recruited preassembled with TFIIIB and TFIIIC as part of a holoenzyme. The ABC10α-τ131 interaction may be involved in the formation and/or stability of such a complex. In support of the possible existence of a yeast pol III holoenzyme, Seifart's group has immunopurified yeast pol III enzyme that contains immunologically detectable amounts of TBP, BRF and TFIIIC (83). Interestingly, this potential holoenzyme form contained functional amounts of TFIIIC but had to be supplemented with TFIIIB components for transcriptional activity (83). An interaction has also been detected by two-hybrid analysis between the yeast pol III subunit C53 and a fragment of τ131 (148), however this interaction could not be detected using the entire τ131 protein (129). In humans, interactions of TFIIICδ with C39 and C62, and TFIIICε (homologous to τ95) with C62 (homologous to yeast C82), have been reported (231).

The assembly of a functional preinitiation complex on class III genes is a slow process. Once formed however the complex is extremely stable with its constituent components all remaining associated after transcription initiation (40, 154, 317, 318, 405). With pol III being the only factor that is recycled, once a preinitiation complex has formed on a class III gene the gene tends to be committed to multiple rounds of transcription (40, 154, 318). A very different situation exists for pol II, where most of the components of the transcription complex dissociate following initiation; only TFIID remains at the promoter, and the other factors must reassemble for each cycle (612). Clearly, the need of the class II factors to reassociate makes reinitiation slow and inefficient however it also provides considerable scope for regulating each round of transcription that is not available to pol III (123). The scope for regulation of transcription elongation and termination is

also less for pol III. Whereas pol III appears to be able to carry out these two processes accurately and efficiently on its own, pol I and pol II both require the assistance of dedicated elongation and termination factors (411). After the formation of a stable preinitiation complex, the transcription of class III genes can proceed extremely rapidly. Even the recycling of pol III has been optimised such that after the initial round of transcription, cycles are 5- to 10- fold more rapid than the first (123). The polymerase is recycled without actually being released from the template; consequently the slow initial step of polymerase recruitment is avoided (123).

Pol III transcription is therefore notably more efficient than that of pol II and the level of basal pol III transcription is relatively high (568). Such transcriptional efficiency may be necessary to ensure that sufficiently large quantities of 5S rRNA and tRNA are produced to sustain adequate levels of cellular protein synthesis. Clearly, however, the translational requirements of a cell can vary considerably. For example, a cell that is actively proliferating will require substantially higher levels of protein synthesis than a cell that has withdrawn from the cycle and is in a state of quiescence. Although absolutely essential, the levels of 5S rRNA and tRNA needed by the cell can therefore also alter drastically. Rather than synthesise a vast excess of 5S rRNA and tRNA to cater for variations in the amounts required of these two transcripts, it appears that their transcription by pol III is tightly regulated, thereby allowing their production to be tailored to the metabolic demands of the cell (568). This will reduce the energy load on the cell but also provides a potential point of control of the biosynthetic capacity of the cell. Pol III transcription of the other class III genes is also subject to regulation (568).

## 1.7 Regulation of RNA polymerase III transcription

A variety of proteins have been found to be able to modulate the rate of pol III transcription, either in a positive or a negative fashion (568). As such, these modulatory proteins are, potentially, regulators of pol III transcription. In support of this, pol III transcription has been shown to be regulated in response to changes in a number of different physiological conditions (569). The mechanisms which cells employ to regulate expression of class III genes under particular cellular and environmental conditions are slowly being elucidated. Many of the proteins shown

to modulate the level of pol III transcription have been implicated. Changes in the chromatin structure of class III genes can also influence the level of transcription and may have a role in the physiological regulation of pol III transcription (569).

### 1.7.1 Activities that reduce pol III transcription

#### Dr1

Dr1, a 19kDa nuclear phosphoprotein, was originally isolated from HeLa cells because of its ability to repress pol II transcription (242) but it has subsequently been shown to inhibit pol III transcription as well (576). The factor has been highly conserved through evolution, with 37% sequence identity between the human and yeast proteins, and is essential for yeast cell viability (276). Dr1 functions as a transcriptional repressor by directly binding TBP and blocking essential interactions made by the protein that are required for transcription (242, 276, 576, 602). For example, Dr1 inhibits pol II transcription by preventing promoter-bound TBP from interacting with the pol II–recruiting factor TFIIB (242). The exclusion of TFIIB binding to TBP is thought to result from conformational changes in the structure of TBP induced by the binding of Dr1 (370). Dr1 does not bind TFIIB (602) and contacts a surface of TBP that is spatially removed from the TFIIB-docking site (277). Using recombinant proteins, Dr1 has also been shown to interfere with the binding of TBP to the pol III-specific factor BRF (576). Thus, Dr1 appears to inhibit transcription by pols II and III by very similar mechanisms, in each case it prevents TBP from binding to a class-specific factor that is required for polymerase recruitment; TFIIB in the case of pol II and its homologue BRF in the case of pol III (242, 576). Unlike TFIIB though, two distinct regions of BRF contact TBP (82, 271, 552). By analogy to its effects on TFIIB, Dr1 may disrupt binding to the N-terminal direct repeats of BRF through conformational changes in TBP. However, whereas TFIIB and this region of BRF interact with TBP at a site distinct from that bound by Dr1, the C-terminal domain of BRF appears to make a pol III-specific high-affinity interaction with the basic repeat region of TBP, the very same region to which Dr1 binds (271). Point mutagenesis indicates that the binding sites of Dr1 and BRF in the

basic repeat region of TBP extensively overlap (64, 271, 277). Thus, BRF and Dr1 are in direct competition for binding this region of TBP.

By targeting TBP and being able to repress both pol II- and pol III-transcription Dr1 has the potential to coordinately regulate the transcriptional activities of these two polymerases (576). TBP is also required for pol I transcription as part of the basal factor SL1 (103). However, whereas the addition of highly purified recombinant or native Dr1 to human extracts was found to potently repress the expression of a variety of different class III genes including tRNA, VA and U6, pol I transcription of rRNA under the same conditions was unaffected (576). Similarly, the overexpression of Dr1 in yeast was found to inhibit tRNA synthesis but had no discernible effect on pol I transcription (276). In contrast to pols II and III, pol I transcription is seemingly immune to the repressive effects of Dr1. By selectively inhibiting pols II and III, Dr1 may be able to shift the balance of nuclear transcription in favour of pol I, which could be of considerable value when rRNA levels are limiting.

RB

The retinoblastoma protein, RB, a 105kDa nuclear phosphoprotein (324), was the first tumour suppressor to be identified, over a decade ago (153). The retinoblastoma susceptibility gene that encodes RB was initially cloned because of its association with an inherited predisposition to retinoblastoma, a rare paediatric tumour of the retina (153). Individuals who inherit a non-functional allele of the Rb gene have a roughly 90% chance of developing retinoblastoma at an early age (580). For retinoblastoma to actually arise, however, this requires the inactivation of the remaining allele of the Rb gene, since inactivating mutations in Rb are recessive (580). Loss of RB function appears to be a universal feature of both the familial and sporadic forms of this tumour and is likely to be the rate-limiting step in its initiation (226). Inactivating mutations in both alleles of the Rb gene have also been identified in a variety of other human cancers including many sarcomas, bladder and small cell lung carcinomas (226, 557, 580). In many other human malignancies a wild-type Rb allele is retained, but RB function is still disrupted, for example because of the

association of RB with viral oncoproteins or the hyperactivity of cyclin-dependent kinases that switch off RB (557).

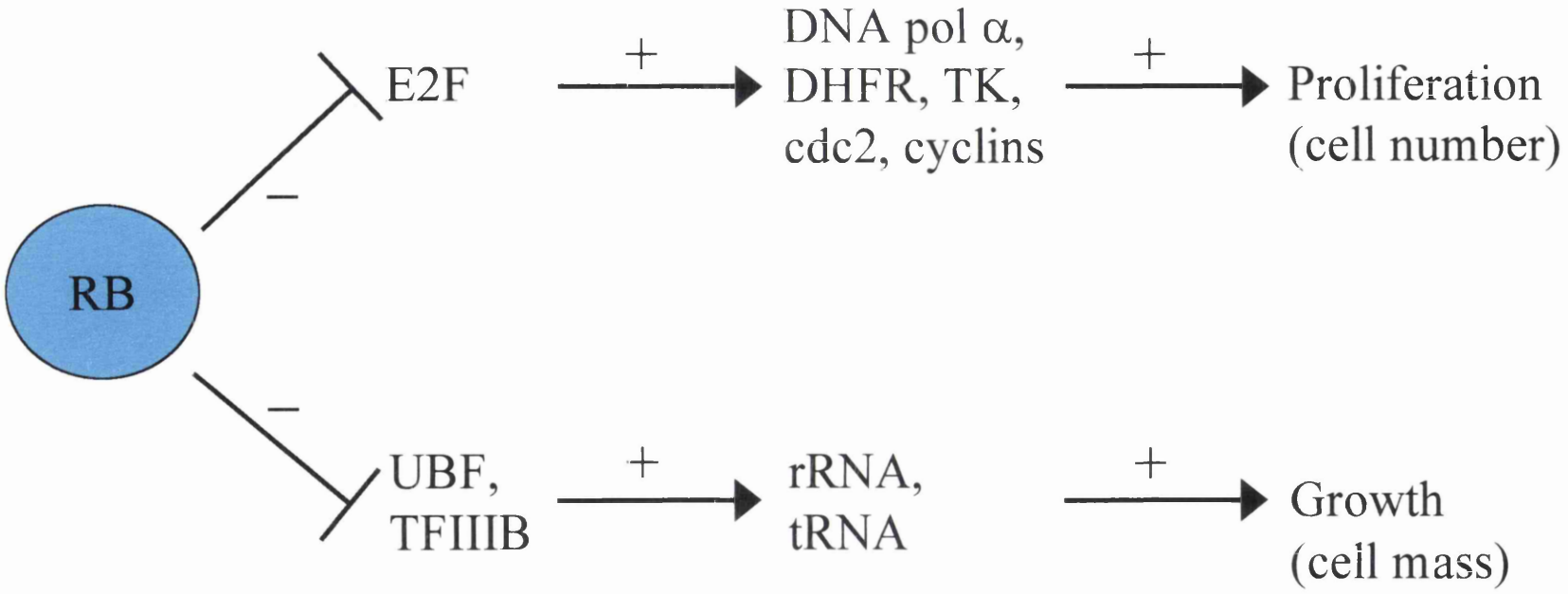
The ability of RB to function as a tumour suppressor can be explained by its normal roles in the cell. Thus, RB suppresses both the proliferation (increase in number) and the growth (increase in mass) of cells and is involved in ensuring that the cell does not divide and grow when conditions are unfavourable (557). When RB function is missing, the ability of cells to arrest proliferation and growth is severely compromised and the cell is less sensitive to normal regulatory signals; these changes constitute a major step towards carcinogenesis (482, 557, 580). RB is also implicated in promoting cell differentiation, which may also contribute to its tumour suppressor functions (95, 214, 245, 322). All of these functions of RB appear to be at least partly attributable to the effects of RB on gene expression (173, 214, 290). For example, RB is able to cooperate with certain transcription factors such as C-EBP family members and MyoD to activate the transcription of a number of pol II-transcribed genes involved in differentiation (179, 475).

RB is a highly abundant protein and can bind and regulate a variety of transcription factors (214, 290). One of the key targets of RB is the factor E2F (3, 131, 305, 310, 556). E2F activates the expression of a range of pol II-transcribed genes that are important for cell cycle progression (3, 131). These include several genes that are required for DNA replication, such as those encoding DNA polymerase α and the replication origin-binding protein HsOrc1, as well as genes that drive the cell cycle such as cdc2 and various cyclins. RB can bind to E2F, inactivating it, and can form a repressive complex that actively inhibits the transcription of these E2F-responsive genes that are required for passage through the cell cycle, thereby providing a possible explanation as to how RB is able to constrain cell proliferation (3, 131). The effects of RB on gene expression are not restricted to pol II-transcribed genes, however. RB can directly inhibit transcription by all three nuclear RNA polymerases (Fig. 1.2) (313, 566, 567). Whereas only a very restricted number of genes transcribed by pol II are inhibited by RB, it appears to be a general repressor of pol III transcription (314, 579). In a growing cell, pols I and III are responsible for ~80% of total RNA synthesis (411). The bulk of this is devoted to the production of rRNA and tRNA, which are essential components of the translational machinery. By limiting the production of these, RB may be able to

**Figure 1.2**

**Suppression of cell growth and proliferation by RB**

The pol II transcription factor E2F promotes the expression of a variety of genes required for cell cycle progression. These include the genes that encode for DNA polymerase α (DNA pol α), dihydrofolate reductase (DHFR), thymidine kinase (TK), cdc2 and various cyclins. RB, by inhibiting E2F, can suppress transcription of these E2F-responsive genes thereby providing a brake on cellular proliferation. In addition, RB also represses the pol I factor UBF and the pol III factor TFIIIB thereby reducing the synthesis of rRNA and tRNA, important determinants of the biosynthetic capacity of the cell. By restraining protein synthesis, this may provide a mechanism for RB to suppress cell growth.


RB
E2F
+
DNA pol α,
DHFR, TK,
cdc2, cyclins
+
Proliferation
(cell number)
–
–
UBF,
TFIIIB
+
rRNA,
tRNA
+
Growth
(cell mass)

suppress the level of protein synthesis, which could provide a brake on cellular growth (313, 567). Careful measurements in animal cells have demonstrated that the rate of growth is directly proportional to the rate of protein accumulation, the main determinant of which is protein synthesis, although turnover also makes a contribution (27).

The ability of RB to repress pol III transcription was initially demonstrated in vivo by transient transfection experiments (579). The overexpression of RB was found to reduce transcription of the adenoviral $VA_I$ gene whereas transcription of a cotransfected CAT gene under the control of the pol II-transcribed human immunodeficiency virus promoter was unaffected (579). Clearly, however, this inhibitory effect of RB on pol III transcription observed in vivo could be very indirect, perhaps resulting from cell cycle changes. It was therefore tested whether RB could repress pol III transcription when reconstituted in vitro using partially purified fractions (579). This was indeed shown to be the case, suggesting that one or more components of the pol III transcriptional apparatus may be a direct target for regulation by RB.

These results showed for the first time that high levels of RB can inhibit pol III transcription. However, the possibility existed that the repression observed in vitro and in vivo is simply an artefact of overexpression. It was therefore very important to determine whether RB can influence pol III transcription when present at physiological concentrations within a cell. To address this, the pol III activity of two human osteosarcoma cell lines was compared; U2OS, which contain functional wild-type RB, and SAOS2, which only express a truncated non-functional form of RB (579). SAOS2 cells were found to express a transfected $VA_I$ gene ~5-fold more actively than U2OS (579). The higher pol III activity of the RB-deficient SAOS2 cells was confirmed in vitro using cell extracts (579). In addition, primary fibroblasts from knockout mice in which the Rb gene had been inactivated by site-directed mutagenesis were shown by nuclear run-on assays to synthesise tRNA and 5S rRNA at ~5-fold higher rates than equivalent cells from wild-type mice (579). In contrast, the overall level of pol II transcription was unchanged. Extracts prepared from the Rb-negative cells also displayed higher levels of pol III activity, providing independent confirmation that the pol III transcriptional apparatus is more active in the absence of RB (579). Since the only genetic difference between the $Rb^{+/+}$ and $Rb^{-/-}$ fibroblasts is the presence or absence of the Rb gene, it can be concluded from

these results that endogenous RB plays a major role in suppressing pol III transcription in vivo. What is less clear is to what extent this may contribute to growth control by RB. However, multiple components of the translational apparatus are frequently deregulated in cancer cells, suggesting that regulation of protein synthesis is an important aspect of growth control (445, 494). There is also a correlation between the ability of RB to function as a tumour suppressor and its ability to affect pol III transcription (579). Thus, the minimal region of RB that is necessary to regulate cell growth and proliferation is also sufficient to repress pol III transcription (579). Moreover, four naturally occurring and highly localised mutations that inactivate RB in human cancers were each found to prevent RB from regulating pol III transcription (59, 579). However, it has yet to be demonstrated that tRNA and rRNA levels are ever limiting for protein synthesis in mammalian cells under normal physiological conditions. Nevertheless, the production of tRNA and rRNA does increase following mitogenic stimulation and decreases when cells quiesce, suggesting that their levels are closely regulated in accordance with the biosynthetic requirements of the cell (96, 255, 363, 445). In the yeast Saccharomyces cerevisiae, tRNA levels clearly can be limiting; a two-fold reduction in the level of initiator tRNA was found to result in a three-fold increase in cell doubling time (152). If similar is true of a mammalian cell, the 5-fold decrease in tRNA levels imposed by endogenous RB (579) could be a major constraint on cellular growth.

Apart from tRNA, 5S rRNA and $VA_I$ genes, RB has also been shown to inhibit transcription of B2, U6, EBER2, 7SK, and Alu genes (93, 215, 314, 503, 579). In fact, every pol III template tested has been found to be inhibited by RB. Clearly, RB is effective in repressing transcription from all categories of pol III promoter, suggesting that it may target one of the general pol III transcription factors, or pol III itself, in order to achieve repression (314).

To try and determine which component(s) of the pol III transcriptional apparatus are targeted by RB, add-back experiments were performed to see if any of these could restore pol III transcription reconstituted in vitro in the presence of RB (314). Indeed, the addition of increasing amounts of a partially purified TFIIIB fraction was found to relieve inhibition of $VA_I$ transcription by recombinant RB in a dose-dependent manner (314). In contrast, the addition of partially purified pol III or TFIIIC, the latter of which is limiting in the absence of RB, had no effect (314).

These results suggested that RB specifically targets TFIIIB for repression. In support of this, in pull-down assays GST-RB was shown to bind TBP and BRF (314). Furthermore, immunoprecipitation and cofractionation experiments have shown that endogenous RB and TFIIIB interact stably (314). This association is diminished or abolished in SAOS2 cells, which contain only a nonfunctional mutant form of RB (314). In addition, complementation assays have found that TFIIIB activity is significantly elevated in primary fibroblasts from $Rb^{-/-}$ mice (314). In contrast, little or no difference is detected in TFIIIC2 B-block-binding activity between the Rb-positive and Rb-negative cells (314). Thus, endogenous RB also specifically inhibits TFIIIB. Since TFIIIB is required for the transcription of all class III genes, this provides a possible explanation as to how RB may act as a general repressor of pol III transcription, although the mechanism by which RB inactivates TFIIIB has yet to be determined (314). However, whereas the majority of class III genes utilise the same form of TFIIIB as $VA_I$, recent data indicates that those templates with a type III promoter, such as the vertebrate U6 snRNA gene, utilise a different form of TFIIIB (365, 463, 515). At present it is unclear whether RB inhibits this form of TFIIIB as well. Alternatively, RB might repress transcription of these genes by targeting a different factor(s).

Hirsch et al. recently reported that RB and SNAPc physically interact (215). Moreover, they showed that TBP, or more effectively, a combination of TBP and SNAPc, is able to restore pol III transcription of the human U6 snRNA gene to RB-treated extracts (215). These results suggest that RB represses transcription of class III genes with external promoters, such as U6, by targeting TBP and/or SNAPc (215). However, the possibility remains that RB may also inhibit the form of TFIIIB utilised by type III promoters and that this also contributes to RB repression of these genes. Nevertheless, the fact that RB can inhibit SNAPc function, which is not required for transcription of pol III templates with type I or type II promoters, suggests that one or more class III genes with a type III promoter may be an important target for RB function. This is very unlikely to be true for all the different class III genes repressed by RB. Instead, this blanket repression of pol III transcription may simply be the indirect consequence of RB targeting a general component of the pol III transcriptional apparatus in order to repress functionally important targets such as 5S rRNA and tRNA genes.

Recently, it has also been demonstrated that p107 and p130, which are structurally and functionally related to RB, also inhibit pol III transcription when overexpressed, both in vitro and in vivo (504). In addition, primary fibroblasts derived from p107 p130 double knockout mice were found to display elevated pol III transcriptional activity (504).

## p53

Another important tumour suppressor, p53, which is unrelated to RB, also represses pol III transcription (70, 90). The gene that encodes p53 is highly conserved amongst vertebrate species; it also holds distinction as the most frequently mutated gene in human cancers (284). In fact, p53 is lost or mutated in over half of all human malignancies (222, 223). Like RB, wild-type p53 can arrest cell growth and proliferation (108, 358, 369, 372). However, whereas RB function is required every cell cycle as part of the normal proliferative and growth control, p53 function is more that of an emergency checkpoint control against aberrant growth and neoplastic transformation that is only occasionally needed (284). That p53 is not an essential cell cycle regulator is demonstrated by the viability and normal development of $p53^{-/-}$ mice (127). However, these mice have a propensity to cancer, such that 74% develop tumours by the age of six months (127). Clearly, the inactivation of p53 can play a crucial role in carcinogenesis. Li-Fraumeni individuals, who carry a germ-line mutation in p53, are highly cancer-prone (284).

Under normal conditions, very little p53 protein is found in most of the cells of the body and much of it is in a latent form (332). However, on exposure of cells to certain stress stimuli such as DNA damage, cellular or viral oncogene activity, or hypoxia, active p53 protein rapidly accumulates and will either induce a growth arrest or apoptosis (284, 332). p53 is activated in response to such stimuli because cells that are exposed to these stresses commonly contain potentially carcinogenic mutations and exhibit abnormal cell behaviour, and are more likely to become cancerous. The ability of p53 to arrest cell growth or trigger apoptosis, by analogy to RB tumour suppressor functions, appears to be mediated, to a large extent, by changes in gene expression (284, 332).

p53 is a transcription factor and can regulate the expression of a variety of genes (284, 332). It can bind DNA both in a sequence-specific manner and non-specifically, and is capable of both activating and repressing transcription depending on the gene (284, 332). Genes activated by p53 contain p53-binding sites in their promoters. These include pol II-transcribed genes encoding proteins that are involved in inhibiting cell cycle progression such as the cyclin-dependent kinase inhibitor $p21/^{WAF1}$, which can block both the $G_1$/S and the $G_2$/M cell cycle transitions, and pro-apoptotic genes such as Bax (284, 332). In addition, p53 can specifically repress a number of pol II-transcribed genes devoid of p53 recognition sequences, including those that encode c-fos, PCNA, and cyclin A, all of which are involved in promoting cell cycle progression (284). Both the transcriptional activation and transcriptional repression activities of p53 are therefore likely to contribute to the ability of p53 to function as a tumour suppressor. The vast majority of p53 mutations found in human cancers map to the sequence-specific DNA binding domain of p53 (284). The ability of these mutants to activate transcription is severely impaired (284). However, several tumour-derived mutants have also been found that can still stimulate transcription but are incapable of transcriptional repression (453). Furthermore, two oncoproteins have been shown to block p53-mediated transcriptional repression but have no effect on transcriptional activation by p53 (108). These results suggest that both transcriptional activities of p53 are targeted in carcinogenesis.

Transcriptional repression by p53 is not restricted to a few select genes transcribed by pol II however; p53 can also inhibit genes transcribed by pols I and III (62, 70, 90). The overexpression of p53 has been shown to repress transcription of a variety of class III genes both in vitro and in transfected cells (70, 90). Using gene knockout technology to disrupt the p53 gene, a physiological role for p53 in suppressing pol III transcription has also been demonstrated (70). Thus, a comparison of the rates of transcription in intact nuclei from $p53^{+/+}$ and $p53^{-/-}$ mouse embryonic fibroblasts revealed that the rate of synthesis of tRNA and 5S rRNA is 4- and 6- fold higher, respectively, in the p53-negative cells (70). Endogenous p53 has also been shown to inhibit transcription of various other pol III templates and appears to be a general repressor of pol III transcription (70). However, the class III genes display differential sensitivity to the repressive effects of p53 (70, 90). This has been observed both in vitro using recombinant p53 and when extracts from $p53^{+/+}$ and

$p53^{-/-}$ cells are compared (70). There appears to be an inverse correlation between sensitivity to p53 and promoter strength. In vivo, this may enable p53 to differentially regulate pol III transcription, with genes such as Alu and U6 that have particularly weak promoters becoming inhibited at a lower p53 threshold than other genes.

Like RB, p53 appears to repress pol III transcription by binding and inactivating TFIIIB (70, 90). Thus, TFIIIB activity is specifically elevated in fibroblasts derived from p53 knockout mice (70). Moreover, add-back experiments revealed that TFIIIB is limiting in extracts from the equivalent wild-type cells so the rise in TFIIIB activity in response to disruption of the p53 gene should stimulate transcription (70). In addition, the inhibition of pol III transcription in vitro by recombinant p53 has been shown to be specifically relieved by the addition of excess TFIIIB (70). However, it remains possible that other components of the pol III transcriptional apparatus are additionally targeted and contribute to the repressive effects of p53. Endogenous TFIIIB and p53 have also been shown to stably associate, providing further support for regarding TFIIIB as a bona fide target of p53 (70). Pull-down assays using GST-p53 fusion proteins have shown that the N-terminal 73 amino acids of p53 are sufficient to bind TFIIIB (90). This N-terminal region of p53 contains a TBP binding domain, suggesting that p53 directly binds TBP within TFIIIB (337, 528). In support of this, point mutations within this N-terminal region that abolish the binding of free TBP also abolish TFIIIB binding (90). Significantly, these same mutations also abrogate the ability of p53 to repress pol III transcription (90).

The inhibition of pol III transcription, by reducing the levels of tRNA and 5S rRNA, may contribute to growth suppression by p53 (70). However, although p53 is frequently mutated in human cancers, it remains to be determined whether or not the regulation of pol III transcription by p53 is compromised by these mutations and therefore the extent to which this control may contribute to the tumour suppressor function of p53. In addition, it is presently unknown how the regulation of pol III transcription by p53 is affected by the various stress stimuli that are known to influence p53 activity.

p34cdc2/cyclin B1

The cyclin-dependent kinase p34cdc2/cyclin B1 has been immuno- and affinity- purified from mitotic Xenopus extracts (169, 587). Both purified sources of p34cdc2 have been shown to potently inhibit 5S rRNA transcription (169, 587); in contrast, immunopurified MAP kinase had no discernible effect on transcription (587). In addition, the affinity-purified p34cdc2 has been shown to specifically inactivate TFIIIB when incubated with fractionated Xenopus pol III factors (169). This is dependent upon phosphorylation, since repression could be blocked by the general kinase inhibitor DMAP and reversed by alkaline phosphatase (169).

### 1.7.2 Activities that stimulate pol III transcription

Staf

Staf, like Oct1, can activate both pol II- and pol III transcription (375, 392, 393, 458, 466). It contains seven tandemly repeated zinc fingers; these enable Staf to bind specifically to DNA (466). Staf recognition sites are found in the DSEs of vertebrate U snRNA and 7SK genes, commonly located close to an octamer motif, which binds Oct1 (458). It has been suggested that Staf may function co-operatively with Oct1 because their respective binding sites can activate transcription synergistically when appropriately spaced (458).

In addition to Staf and Oct1, several other pol II factors that bind DNA in a sequence-specific manner have been implicated as potential regulators of pol III transcription on the basis of sequence motifs found in the promoters of class III genes. For example, GC boxes that resemble Sp1 binding sites are found in the promoters of the EBER2, mammalian 5S and Xenopus U6 genes (228, 330, 495). Sp1 is one member of a family of related factors that share similar DNA-binding specificities. It remains to be determined which, if any, of these factors can influence the expression of these genes with Sp1-like binding sites. Nevertheless, two tandem copies of a Sp1 activation domain have been shown to activate U6 transcription when fused to a heterologous DNA-binding domain (113). In the pol II system, the

response to upstream activators is commonly mediated by specific TAFs in the TFIID complex that are otherwise dispensable for transcription. For example, activation by VP16 requires direct contact with $TAF_{II}40$ (166); similarly, Sp1 activation is dependent upon a direct interaction with $TAF_{II}110$ (220). Perhaps TFIIIB contains a functional homologue of $TAF_{II}110$; alternatively, the activation domain of Sp1 may contact different component(s) of the pol III transcriptional apparatus (568).

Another example of pol II factors that may be involved in pol III transcription are members of the ATF and CREB family that bind ATF sites (54). The promoters of EBER2 and 7SL genes contain several ATF sites, which are important for high levels of expression of these genes (51, 228). In support of this possibility, extracts from cells treated with forskolin, which can activate a subset of these factors through activation of the cAMP signal transduction pathway, were found to support increased levels of 7SL transcription, but to have no effect on 7SK, whose promoter is devoid of ATF sites (51).

Protein phosphatase 2A

The substrate specificity of protein phosphatase 2A (PP2A) is thought to be defined by its regulatory subunits (100). The TPD3 gene encodes the regulatory subunit A of PP2A (537). Strains of Saccharomyces cerevisiae that lack the TPD3 gene are severely compromised for growth (537). A temperature sensitive tpd3 strain ($tpd3^{ts}$) was found to stop synthesising tRNA, an important determinant of the biosynthetic capacity of the cell, at the nonpermissive temperature; similarly, extracts prepared from these cells were also unable to transcribe tRNA (537). Extracts from $tpd3^{ts}$ cells grown at the permissive temperature were 2- to 4- fold less active for tRNA synthesis than wild-type cells. Transcription could be fully restored to wild-type levels in these extracts by the addition of partially purified TFIIIB, whereas pol III was partially stimulatory and TFIIIC had no effect (537). Mixing experiments indicate that the defect in $tpd3^{ts}$ cells is due to an inhibitory activity rather than the loss of a transcription component (537). These results suggest that PP2A positively regulates pol III transcription in yeast and that it does so by stimulating TFIIIB activity and, to a lesser extent, pol III (537). The inhibitory activity in the ts extracts

is probably a kinase that phosphorylates and inactivates TFIIIB and perhaps pol III, whose repressive influence is normally antagonised by PP2A.

## CKII

The highly conserved Ser/Thr protein kinase CKII has also been shown to stimulate pol III transcription in the yeast Saccharomyces cerevisiae (163, 164, 216). CKII functions primarily as a tetramer, consisting of two isozymic catalytic subunits (α and/or α′) and two regulatory subunits (β) (7, 419). The regulatory subunits of CKII are required for optimal kinase activity and also regulate substrate specificity. As for PP2A, a positive role for CKII in yeast pol III transcription was initially suggested by the phenotype of a yeast strain with a temperature sensitive lesion in one of the subunits of the protein (216). Thus, when a yeast mutant with a ts defect in the catalytic α′ subunit of CKII ($cka2^{ts}$) was shifted to the nonpermissive temperature, tRNA synthesis and growth both declined, whereas production of large rRNA was unaffected (216). Similarly, extracts prepared from $cka2^{ts}$ cells were found to be severely compromised for transcription of tRNA and 5S rRNA genes, whereas pol I and basal pol II transcription was as efficient as in extracts from wild-type cells (216). Moreover, the addition of increasing amounts of purified wild-type CKII to $cka2^{ts}$ extracts stimulated tRNA and 5S rRNA transcription, providing direct evidence of a positive role for CKII in pol III transcription (216). Add-back experiments using purified pol III factors from wild-type cells showed that transcription could also be restored to $cka2^{ts}$ extracts by the addition of TFIIIB (163, 164). However, pretreatment of TFIIIB with phosphatase abolished its ability to rescue transcription (164). These results suggest that CKII stimulates pol III transcription by phosphorylating TFIIIB. In vitro phosphorylation of a partially purified TFIIIB fraction with recombinant CKII suggested that the TBP subunit of TFIIIB is the preferred substrate of CKII (163, 164). CKII was also found to efficiently phosphorylate recombinant TBP (163, 164). Moreover, limiting amounts of CKII and recombinant TBP were found to synergistically increase pol III transcriptional activity in $cka2^{ts}$ extracts, suggesting that the stimulatory effect of CKII on yeast pol III transcription is mediated, at least in part, by the

phosphorylation of the TBP subunit of TFIIIB (163, 164). However, the phosphorylation of other components of TFIIIB, or indeed other components of the pol III transcriptional apparatus, may also contribute to the stimulation of pol III transcription by CKII (163, 164).

### 1.7.3 Repression by chromatin

A human diploid cell contains ~6.4 x $10^9$ base pairs of DNA. In a molecule of DNA adjacent base pairs are separated by 3.4Å, so if all 6.4 x $10^9$ bp were part of the same DNA molecule it would stretch a staggering 2.2 metres in length. Since the nucleus is only ~10μm in diameter, clearly the DNA must be highly compacted so as to actually physically fit it inside the nucleus! In order to stably bend the DNA, however, the negatively charged phosphates of the DNA phosphodiester backbone must be neutralised so as to overcome charge repulsion when different regions of the polyanionic DNA are brought close together. In the eukaryotic cell this is achieved by the association of the DNA with highly conserved, small basic proteins called histones. Histones are unique to eukaryotes and are amongst the most invariant proteins known. They bind DNA essentially without sequence specificity enabling the packaging of the entire genome into a highly condensed structure called chromatin.

The non-specific binding of histones to DNA is not random, as revealed by the 'beads on a string' chromatin structure observed by electron microscopy when chromatin is released from nuclei at low ionic strength (520). This is the first level of packing of DNA (~6-7 fold), the 10nm filament. The 'beads', which are regularly spaced, represent nucleosomes. The nucleosome is the basic unit of structure in chromatin (288). It consists of a wedge-shaped histone octamer around which two turns of DNA are organised. The crystal structure of the nucleosome has recently been determined at a resolution of 2.8Å, revealing details of the histone-histone and histone-DNA interactions (345). The histone octamer consists of two molecules each of the four core histones, H2A, H2B, H3 and H4. A central $(H3/H4)_2$ tetramer is flanked on either side by a H2A/H2B dimer (288, 520). Each of these histones has a very similar C-terminal domain structure, the "histone fold", consisting of a long

central α helix that forms the dimerisation interface flanked by shorter helices and loops (345). Histone dimerisation creates the DNA-binding surfaces. The N-terminal "tails" protrude outside the nucleosome and interact with both nucleosomal and linker DNA, and adjacent nucleosomes (345). In addition, there is a fifth type of histone molecule associated with the nucleosome, the so-called linker histone, which is the least conserved of the histones (520). The majority of cells contain the histone H1. However, there are a number of H1 variants which tend to be cell- or developmental stage-specific; these include $H1^0$ found only in differentiating mammalian cells, sea urchin sperm-specific H1, and H5 which is unique to avian erythrocytes (520). All the variants share the same basic structure, consisting of a central globular domain flanked on either side by a highly basic tail. The central globular domain is thought to seal the two turns of DNA around the nucleosome, accounting for the protection of DNA at the dyad axis of the nucleosome from digestion by DNase I (520). Recently two DNA binding sites on the globular domain of H5 have been identified (172), this explains the preference of this linker histone for four-way junction DNA (520). The highly basic tails of the linker histone are thought to interact with the linker DNA between nucleosomes, thereby allowing the linker DNA to be easily bent.

The majority of interphase chromatin exists not as the 10nm filament but in a more condensed state, the so-called 30nm filament or solenoid (DNA packing ratio ~40 fold) (520). There has been some controversy over the exact structure, but most of the evidence supports the coiling of the 10nm filament into a helix with ~ 6 nucleosomes per turn (520). It has been demonstrated that H1 or one of its variants is essential for the formation of the 30nm filament. Chemical cross-linking and neutron diffraction studies suggested formation of a H1 'polymer' within the solenoid (520). There is also evidence for several further levels of chromatin organisation above that of the 30nm filament, finally culminating in the structure of the metaphase chromosome, the most condensed state of chromatin (520).

The packaging of DNA into chromatin is a major obstacle to transcription, severely restricting the access of the transcriptional machinery to the DNA (568, 588). Thus, class III genes assembled into solenoid-like structures in vitro are incapable of supporting significant levels of transcription. In vivo, both transcription and DNA replication are thought to require the decondensation of chromatin to the

10nm filament (520). At this level of chromatin organisation, the susceptibility of different genes to nucleosomal repression can vary considerably. To a large extent this depends on the positioning of nucleosomes over the gene (203, 588). Thus, a gene whose promoter is located within the centre of a nucleosome is more likely to be repressed than one whose promoter is located at the edge of a nucleosome or is nucleosome-free. Although histones bind DNA non-specifically and nucleosomes are regularly spaced along the DNA, there is considerable evidence for sequence-directed positioning of nucleosomes on DNA. This is related to the intrinsic bendability of the DNA sequence, that is the energy of bending DNA, which can vary substantially between different sequences (203). In addition, the locations of nucleosomes are sometimes constrained by "boundary" effects such as the binding of sequence-specific factors on the DNA that can compete effectively with histones (203). The extent to which the transcription factors of a gene can compete with histones is also a major determinant of susceptibility to chromatin repression.

5S rRNA genes

Transcription complex formation on a 5S rRNA gene is nucleated by the sequence-specific binding of TFIIIA (568). The surface of interaction between TFIIIA and the 5S rRNA gene is extensive. TFIIIA protects ~1 turn of DNA at each end of the 5S ICR, wrapping around all faces of the helix at the A- and C- block with the intervening 20 bp protected only on one face (203, 568). Clearly, this wrapping of TFIIIA around the DNA is incompatible with the simultaneous wrapping of the same region of DNA around a histone octamer. Significantly, however, the sequences of Xenopus 5S genes direct the positioning of nucleosomes on or near the ICR (202, 321). Thus, TFIIIA and histone octamers may directly compete for binding the ICR. The outcome of this competition will have a major effect on the potential transcriptional activity of these genes.

Once an active transcription complex or repressive chromatin structure has been established on a class III gene, either can be extremely stable. However, the passage of a replication fork displaces all bound factors (590). Therefore, at each cell cycle there is the opportunity to either reestablish or alter the set of genes that are actively expressed. Although the core histones have been shown to be sufficient to

establish a repressed transcriptional state of 5S rRNA genes refractory to the subsequent addition of transcription factors in vitro, under certain circumstances stable repression may also require the linker histone (460). The linker histone inhibits nucleosome sliding, which can allow transcription factors to gain access to promoters (533). It can also repress transcription by directly occluding regions of DNA and promoting nucleosome compaction (591). For human 5S rRNA genes, binding of TFIIIA has been shown to be sufficient to prevent incorporation into a repressive chromatin state (501).

tRNA genes

The assembly of nucleosomes on tRNA genes in vitro has been shown to potently repress transcription (298). However, in vivo tRNA genes are highly resistant to repression by histones (45, 189, 384). For example, manipulating the level of histone H1 in Xenopus embryos has no effect on tRNA expression (45). Similarly, a nucleosome deficiency in yeast that activates several class II genes does not affect tRNA synthesis (189). Moreover, a wild type yeast tRNA gene fused to nucleosome positioning signals capable of suppressing pol II transcription and the initiation of DNA replication was found to remain transcriptionally active in yeast cells (384). However, the ability of the gene to override these signals was lost on mutation of the B-block (384). This is highly significant in light of recent in vitro data from the human pol III system. Thus, Roeder and colleagues have shown that elevated amounts of highly purified hTFIIIC, the major B-block binding activity, can effectively overcome nucleosome-mediated repression of a human $tRNA^{Met}$ gene in vitro (298). Moreover, they also report that three subunits of hTFIIIC possess weak histone acetyltransferase (HAT) activity (231, 298). Histone acetyltransferases catalyse the acetylation of specific lysine residues in the N-terminal tails of the core histones (177). This reduces the positive charge of these highly basic tails and has been shown to weaken histone-DNA interactions and also interactions between nucleosomes (591). The acetylation of core histones assembled onto 5S rRNA genes in vitro overcomes the ability of these histones to exclude the binding of TFIIIA, thereby allowing transcription to proceed (321, 534). Thus, histone acetylation can facilitate the binding of transcription factors to their promoter elements in chromatin

(321). However, it has yet to be demonstrated that the reported HAT activity of hTFIIIC contributes to its ability to relieve the chromatin-mediated repression of a human $tRNA^{Met}$ gene in vitro or has any role in vivo.

Yeast U6 genes

The Saccharomyces cerevisiae U6 gene is also highly resistant to the repressive effects of chromatin (357). Thus, the deletion of the histone H4 gene has no effect upon the level of U6 snRNA expression (357). However, mutant U6 genes with weakened promoters are susceptible to repression (357). The promoter of yeast U6 gene is highly unusual, with a TATA box at –30 and a B-block downstream of the transcription termination site (57). Significantly, a functional B-block and TFIIIC are only required for U6 transcription in the presence of histones, suggesting a dominant role of this interaction in protecting the U6 gene from chromatin-mediated repression (66). The assembly of nucleosomes on the yeast U6 gene in vitro by a Xenopus egg extract represses transcription (66). However, TFIIIC can compete effectively with histones even after the incorporation of the gene into chromatin and transcription is restored by the subsequent addition of affinity-purified TFIIIC (66). Thus, both human and yeast TFIIIC have major roles in relieving chromatin-mediated repression (66, 298). Preliminary investigations by Sentenac's group have failed to detect any histone acetyltransferase activity associated with yeast TFIIIC (83). It may be that yeast TFIIIC and human TFIIIC counteract chromatin repression by different mechanisms. Alternatively, the reported HAT activity of hTFIIIC may not be involved in alleviating the repressive effects of chromatin.

5S rRNA genes also utilise TFIIIC; however, these genes are much more susceptible to nucleosomal repression than tRNA or yeast U6 genes. The addition of TFIIIA and TFIIIC makes no difference once a 5S rRNA gene has been incorporated into a chromatin structure (568). However, these genes lack a B-block and TFIIIC does not bind DNA directly, but depends on the prior binding of TFIIIA.

SINEs

In chromatin isolated from interphase HeLa cells, the majority of tRNA and 5S rRNA genes are accessible to transcription factors, whereas ~99% of Alu genes with functional promoters are silenced and inaccessible to exogenous factors (451). The majority of potentially active copies of SINEs are thought to be constitutively masked by chromatin. This may be of considerable importance to the cell. The genomes of higher eukaryotes contain a vast number of SINEs. For example, there are ~500,000 Alu elements in the haploid human genome, 5% of the total genetic material of the cell (251). Such a huge number of templates are potentially an enormous sink for transcription factors. Competition between the SINEs and essential class III genes for a limiting number of transcription factors might therefore be highly detrimental to the cell; hence the packaging of SINEs into repressive chromatin structures inaccessible to transcription factors. SINEs are extremely susceptible to nucleosomal repression. Silencing of Alu elements by histone octamers is so efficient that the depletion of H1 from chromatin of HeLa cells raises Alu expression by only 2-fold (451). The Alu sequence directly positions a histone octamer over the start site and A-block, potently repressing transcription (135, 136).

DNA methylation may also be involved in chromatin-mediated repression of SINEs. Transcriptional repression by CpG methylation is closely correlated with alterations in chromatin structure (367, 510). Alu sequences contain an unusually high CpG density, and account for ~ one-third of all potential methylation sites in human DNA (136). The methylation of Alu genes has been shown to repress their transcription in vitro (285). Most Alu genes are highly methylated in vivo (285, 461). Moreover, a reduction in the methylation of Alu genes in HeLa cells, stimulated by the treatment of cells with 5-azacytidine which cannot be methylated, was accompanied by a 5- to 8- fold increase in the abundance of Alu transcripts (335). The mechanism by which methylation of Alu genes inhibits their transcription is poorly understood. However, repression in vitro can be relieved by the presence of methylated competitor DNA, suggesting that the inhibition is due to proteins that specifically bind to methylated DNA, such as MeCP1 or MeCP2 (37, 336, 367, 510). It also involves changes in chromatin structure, causing a 20-fold increase in the efficiency with which a histone tetramer can block transcription factor access to an Alu promoter and repress transcription (136).

Clearly, the chromatin structure of a gene can be a major determinant of its transcriptional activity and is therefore an obvious target for regulation. Although chromatin exerts a general repressive effect upon pol III transcription, the class III genes display differential sensitivity to these effects providing the opportunity for the differential regulation of class III genes by subtle changes in chromatin structure. The structure of chromatin in the cell is dynamic; moreover, it can be modified locally. A variety of chromatin remodelling factors have been identified. One major group is the ATP-dependent remodelling factors that include NURF, SWI-SNF, ACF and CHRAC (591). These are thought to facilitate transcription factor access by destabilising the nucleosome or altering its position. The other major group is the histone acetyltransferases (HATs) and their enzymatic antagonists the histone deacetylases (177, 500, 591). In recent years, a growing number of coactivators involved in pol II transcription have been found to possess HAT activity. Moreover, the HAT activity of two of these factors has been shown to be an essential part of their coactivator function (303, 357). Conversely, several transcriptional corepressors have been found to exist in complexes with histone deacetylases (5, 52, 53, 144, 308, 349, 388). These findings suggest that the chromatin remodelling machinery can be targeted to particular genes and that alteration of chromatin structure has a major role in regulating pol II transcription. Interestingly, TFIIIC has recently been reported as a novel HAT, suggesting that similar chromatin remodelling activities may play an important part in the regulation of pol III transcription (230, 298).

## 1.8 Physiological regulation of pol III transcription

In higher eukaryotes, pol III transcription has been shown to be strongly regulated in response to a variety of important physiological stimuli such as growth and differentiation, the cell cycle, viral infection and transformation (568, 569). For some of the stimuli, in particular systems, the mechanisms responsible for regulating pol III transcription have been partially elucidated. However, invariably it has yet to be determined whether the mechanistic bases for regulation are peculiar to the system(s) in which it was examined or a general phenomenon for the particular physiological stimulus. In other cases it can be inferred that particular proteins

previously shown to modulate pol III transcription might be involved based upon what is known regarding their physiological regulation.

### 1.8.1 Developmental regulation during Xenopus embryogenesis

There are two families of 5S genes in Xenopus laevis, the somatic 5S genes, of which there are ~ 400 copies per haploid genome, and the oocyte 5S genes, of which there are ~ 20,000 copies (568). During oogenesis, both somatic and oocyte 5S genes are strongly expressed. However, transcription of both types of genes, and indeed transcription in general, is potently inhibited following meiosis. This repression continues post-fertilisation and through the first twelve cleavage divisions up until the mid-blastula transition (MBT). This blanket repression of transcription appears to be caused by the inaccessibility of genes to the transcriptional machinery resulting from the accumulation of a huge excess of core histones by the oocyte that gain access to the DNA when the nucleus breaks down during oocyte maturation (395, 595). At the MBT transcription resumes; equal amounts of somatic and oocyte 5S RNA are produced indicating a 50-fold transcriptional preference for the somatic 5S genes (544, 597). Several cell divisions later transcription ceases completely from the oocyte 5S genes, establishing the state found in all adult somatic cells in which 5S transcription proceeds solely from the less abundant somatic 5S genes (544, 597).

A variety of mechanisms appear to contribute to this developmental regulation of 5S transcription in Xenopus, which shall now be discussed. In the oocyte nucleus there are ~70,000 molecules of TFIIIA per 5S gene, allowing high levels of transcription from both somatic and oocyte 5S genes (480). However, as development continues TFIIIA levels rapidly decline and become limiting; in adult somatic cells there are five 5S genes per molecule of TFIIIA (480). A difference in affinity for TFIIIA, or the stability of its association, between the somatic and oocyte 5S genes might therefore contribute to the transcriptional bias towards the somatic 5S genes after the MBT. In support of this, the injection of purified TFIIIA protein into Xenopus embryos resulted in a dramatic increase in 5S rRNA at the blastula stage and this was mostly due to transcription from oocyte 5S genes (58). However, this elevation in the expression of oocyte 5S genes relative to somatic 5S genes in

response to artificially raised levels of TFIIIA is transient (12). By neurulation, the effect of increased levels of TFIIIA upon 5S transcription from the oocyte 5S genes is lost and replaced by the situation found in adult somatic cells in which these genes are transcriptionally inactive (12). This may be due to changes in chromatin structure that make oocyte 5S genes inaccessible or perhaps a different factor becomes limiting.

Footprinting and template challenge experiments have shown that transcription complexes formed on oocyte 5S genes are less stable than those assembled on somatic 5S genes (589). Significantly, transcription directed by unstable transcription complexes is more susceptible to changes in the concentration of limiting factors because factors in such complexes are in equilibrium with free factors (589). A difference of 3 nucleotides in the upstream part of the ICR results in TFIIIC having a 5-fold higher affinity for the TFIIIA/somatic 5S complex than for the TFIIIA/oocyte 5S complex (269). Moreover, the instability of the transcription complex formed on oocyte 5S genes provides more opportunity for the formation of a repressive chromatin structure that can exclude transcription factor binding. Indeed, clear differences are detected in the chromatin structure of somatic and oocyte 5S genes in adult somatic cells and oocyte 5S genes are found to be much less accessible to exogenous factors than somatic 5S genes (92). The removal of the linker histone H1 by high salt or ion exchange chromatography restores accessibility to the oocyte 5S genes (92, 460). The readdition of H1 represses transcription of oocyte 5S genes but has no effect on somatic 5S genes (460). H1 has a preference for oocyte 5S genes; this is related to the spacer sequence, which has a higher A/T content than that of somatic 5S genes (287). A/T richness has been shown to strongly promote H1 binding (433). Significantly, linker histone synthesis has also been shown to be developmentally regulated with the accumulation of H1 correlating temporally with the decreased accessibility and transcription of oocyte 5S genes (125). In early stages of development H1 is replaced by an embryonic variant, B4, which binds 6-fold less tightly to nucleosomes.

The developmental regulation of 5S transcription thus appears to be achieved by a multiplicity of effects. Factors contributing to the ~50-fold transcriptional preference towards somatic 5S genes that occurs shortly after the MBT likely include the lower stability of transcription complexes that form on oocyte 5S genes, decreased amounts of transcription factor(s) and increasing amounts of H1, which

binds preferentially to oocyte 5S genes. In adult somatic cells, the somatic 5S genes are replicated earlier than the oocyte 5S genes and therefore have a competitive advantage in binding limiting amounts of transcription factors and forming an active transcription complex rather than a repressive chromatin structure (165, 180). This, and further reductions in the levels of TFIIIA, likely plays an important role in the complete repression of pol III transcription from the oocyte 5S genes (568).

### 1.8.2 Regulation in response to differentiation

RNA pol III transcription is also strongly regulated during early mouse development (538). In situ hybridisation has been used to follow changes in pol III transcript levels and reveal a dramatic decrease when cells differentiate into endoderm (538). High expression is maintained in ectoderm and mesoderm at this stage of development (7.5 days post coitum) (538). The decrease in pol III transcription during differentiation into endoderm can be accurately reproduced in culture using embryonal carcinoma (EC) cells (10, 577). The F9 EC cell line can be induced to differentiate into parietal endoderm (PE) by exposure to retinoic acid and cAMP, mimicking events in the early embryo (577). The rate of pol III transcription decreases ~9-fold when F9 cells differentiate (577). This was found to be accompanied by a specific decrease in TFIIIB activity (10). The abundance of BRF is substantially reduced in PE cells; furthermore, add-back experiments indicate that TFIIIB TAF activity is limiting in PE cells, suggesting that the decline in BRF levels may be sufficient to account for the decrease in pol III activity upon differentiation (10).

### 1.8.3 Mitotic regulation

In higher eukaryotes all nuclear transcription is repressed during mitosis. RNA pol III transcription is inhibited by the phosphorylation and inactivation of TFIIIB (169, 327, 572). Thus, TFIIIB isolated from metaphase-arrested Xenopus eggs is unable to support transcription unless first treated with phosphatase (169). The predominant kinase at mitosis, cyclin B/cdc2, can inactivate affinity purified

Xenopus TFIIIB (169). However, mitotic extracts depleted of cyclin B/cdc2 still repress pol III transcription (196). This inhibition is sensitive to the kinase inhibitor DMAP, suggesting there may be one or more additional kinases that can also inhibit pol III activity in metaphase-arrested frog eggs (196).

TFIIIB is also found to be specifically inhibited in mitotic extracts from HeLa cells (572). The TBP subunit of TFIIIB becomes hyperphosphorylated at mitosis, both in Xenopus and HeLa cells (327, 572). However, the consequences of this phosphorylation are unclear. Add-back experiments demonstrate that recombinant TBP is unable to restore expression to mitotic extracts (572). However, affinity-purified TFIIIB TAF fractions are able to reconstitute transcription (572). Thus, it appears that TFIIIB TAF activity is limiting in mitotic extracts. It may be the BRF subunit of TFIIIB is specifically inactivated since it is also found to be hyperphosphorylated in mitotic HeLa extracts (366).

Xenopus egg extracts can be shifted to the mitotic state by the addition of cyclin B (196). Preincubation of extracts with non-specific DNA to titrate out histones or a topoisomerase II inhibitor to block mitotic chromosome condensation made no difference to the extent of repression of pol III transcription (196). These results indicate that nucleosome formation or chromatin condensation is not required for the inhibition of pol III transcription by mitotic extracts, at least in Xenopus. However, in vivo the repression of TFIIIB by phosphorylation may not be sufficient to account for the complete silencing of pol III transcription that occurs during mitosis (572). The highly condensed state of the chromosomes that characterise mitosis are also likely to contribute to the inhibition of pol III transcription (572).

### 1.8.4 Regulation during interphase

On exit from mitosis, TFIIIB is rapidly dephosphorylated and pol III transcription resumes albeit at low levels (571). Transcription increases gradually as cells progress through G1, with a sharp rise in late G1 around the G1/S transition, resulting in high levels of expression in S and G2 (571). Thus, the rate of pol III transcription is 2- to 3- fold higher in S and G2 than it is in early G1 (571). Extracts from cells synchronised in G1, S or G2 phase were analysed for changes in the activity of the different components of the general pol III transcriptional machinery.

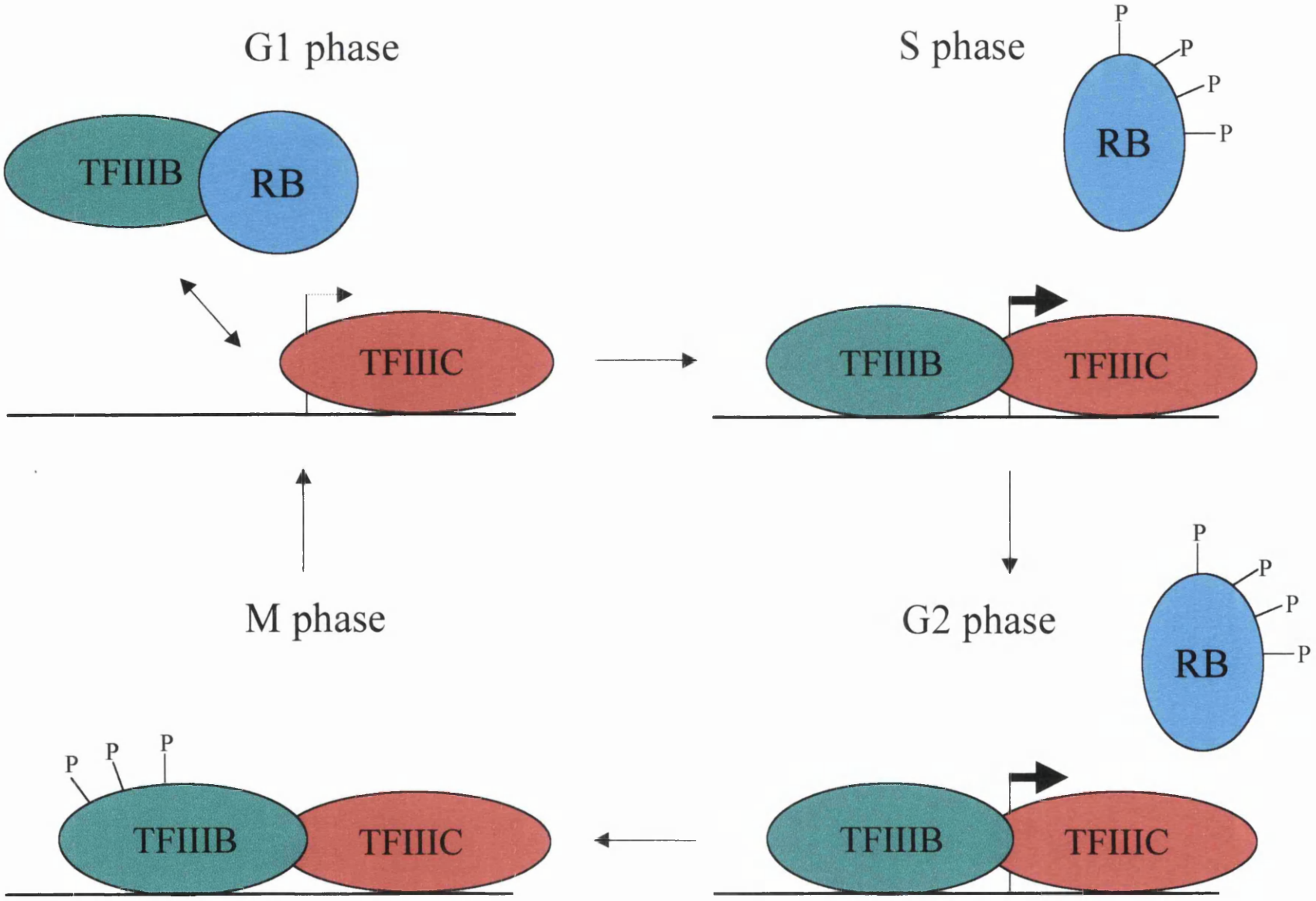
Whereas TFIIIC- and pol III- activity remain relatively constant, TFIIIB activity was found to be severely compromised in G1 phase extracts (571). Complementation assays revealed that the TAF component of TFIIIB is 6- to 8- fold less active in early G1 phase than it is in S or G2 (571). The addition of affinity-purified TFIIIB TAF fractions to early G1 phase extracts restored transcription to levels found in S and G2 phase extracts, suggesting that the deficiency in TFIIIB TAF activity may be sufficient to account for the low pol III transcriptional activity in early G1 phase (571). In contrast, these TAFs have little or no effect when added to S or G2 phase extracts, indicating that TFIIIB TAF activity is no longer limiting in these phases of the cell cycle. Indeed, TFIIIB activity increases to such an extent in late G1 that by S phase it is in relative excess and TFIIIC is limiting for transcription in S and G2 (571).

Detailed time courses have shown that the dramatic rise in pol III transcription and TFIIIB activity in late G1 phase closely corresponds to the time when RB is switched off by its hyperphosphorylation by the cyclin D- and cyclin E-dependent kinases around the restriction point (255, 363). Since RB is a potent repressor of pol III transcription and, moreover, is known to specifically inactivate TFIIIB, this suggests that the inactivation of RB by the cyclin-dependent kinases may be at least partly responsible for the increase in pol III transcription in late G1 phase (93, 314, 571, 579). In support of this, the hyperphosphorylation of RB appears to prevent it from binding to TFIIIB (471). Moreover, coimmunoprecipitation experiments show that RB dissociates from TFIIIB shortly before S phase entry (471). In addition, the overexpression of cyclin D/cdk4 and cyclin E/cdk2 in vivo by transient transfection is found to specifically stimulate pol III transcription (471). Together these data strongly suggest that the hyperphosphorylation of RB by the cyclin D- and cyclin E- dependent kinases prevents RB from inactivating TFIIIB and contributes to the rise in pol III transcription in late G1 phase. In early G1 phase RB is underphosphorylated and can bind and inactivate TFIIIB and is likely to be involved in maintaining a low level of pol III transcription during this part of the cell cycle (Fig. 1.3) (471). Thus, RB appears to have a major role in the cell cycle regulation of pol III transcription. However it is extremely likely that additional control mechanisms are also involved. Indeed, the gradual increase in pol III transcriptional activity through G1 prior to the

**Figure 1.3**

**Cell cycle regulation of pol III transcription**

Schematic diagram illustrating some mechanisms that are thought to contribute to the cell cycle regulation of the mammalian pol III transcriptional apparatus. In G1 phase RB is in an active underphosphorylated form and can bind to and repress TFIIIB, resulting in only low levels of basal pol III transcription. However, in late G1 phase RB becomes inactivated due to its hyperphosphorylation by the cyclin D- and E-dependent kinases and TFIIIB is released enabling it to interact with TFIIIC at the class III promoter and recruit pol III into an active preinitiation complex. This is thought to account for the rapid rise in pol III transcription at the G1/S transition. RB remains in a hyperphosphorylated state until the end of M phase when it is dephosphorylated by protein phosphatase 1, which may explain the high levels of pol III transcription observed during the S and G2 phases of the cell cycle. During mitosis, pol III transcription ceases completely. This results from the direct phosphorylation and inactivation of TFIIIB. The packaging of the DNA into a more condensed chromatin structure might also contribute to the mitotic silencing of pol III activity.


G1 phase
TFIIIB
RB
TFIIIC
S phase
P
P
P
P
RB
TFIIIB
TFIIIC
G2 phase
P
P
P
P
RB
TFIIIB
TFIIIC
M phase
P
P
P
TFIIIB
TFIIIC

abrupt rise at the R point cannot be readily explained by the cell cycle regulation of RB; however, it is presently unclear what may be responsible for this effect.

### 1.8.5 Regulation in response to quiescence

The principle determinant of proliferation rate in mammalian cells is the decision whether to quiesce; that is, to exit the cell cycle and growth arrest, or continue to cycle. The synthesis of tRNA and 5S rRNA is significantly reduced during quiescence (74, 96, 217, 255, 363, 471, 477, 525). This likely reflects the substantially diminished requirements for protein production in quiescent cells. Cells grown in culture can be induced to quiesce by serum withdrawal. The downregulation of pol III transcription in untransformed mouse fibroblasts in response to serum starvation has been studied in detail. Add-back and complementation experiments show that this is due to a specific reduction in TFIIIB activity (471). Analysis of the abundance of TBP and BRF by western blotting reveal little or no change in response to serum deprivation, suggesting that a decrease in the amount of TFIIIB is not responsible for the reduction in TFIIIB activity in these growth-arrested cells (471). However, the possibility cannot be excluded that unidentified components of TFIIIB become less abundant and contribute to the low TFIIIB activity of these cells. Significantly, quiescent cells express very little cyclin D or E, and RB is present in these cells in its active underphosphorylated form, raising the possibility that RB may contribute to the repression of TFIIIB during quiescence (173, 471). Indeed, fibroblasts derived from RB knockout mice are compromised in their ability to down-regulate pol III transcription in response to serum withdrawal relative to equivalent cells from wild type mice (471). Thus, Northern blot analysis revealed a 2.4-fold decrease in tRNA levels when $RB^{+/+}$ cells were made quiescent, whereas $RB^{-/-}$ cells show only a 1.3-fold decrease (471). In contrast, the levels of the pol II transcript ARPP P0 did not change. Similar results were obtained when rates of pol III transcription were assayed directly by nuclear run-ons (471). Some decrease, albeit less substantial, was still observed in the $RB^{-/-}$ cells in response to quiescence, however, indicating that there are additional mechanisms that contribute to the downregulation of pol III transcription following exit from the cell cycle (471). The RB-related pocket proteins p107 and p130 have

both recently been shown to repress pol III transcription both in vitro and in vivo (504). Moreover, p107 p130 double knockout fibroblasts showed much less of a decrease in pol III transcript levels in response to serum starvation than the corresponding wild-type cells, indicating that p107 and/or p130 are also part of the regulatory control by which pol III transcription is repressed during quiescence (504). Serum-starved mouse fibroblasts contain very little p107, but p130 is relatively abundant and in an active form (173).

### 1.8.6 Regulation in response to adenovirus infection

A variety of viruses have been shown to infect mammalian cells and stimulate pol III transcription; these include adenovirus, simian virus 40 (SV40), human T-cell leukaemia virus type 1 (HTLV-1), hepatitis B virus (HBV) and polyomavirus (143, 569). Following infection, the viruses subvert the host cell's translational and replication machinery towards mass production of viral proteins and the viral genome (569). The stimulation of pol III transcriptional activity therefore likely reflects a requirement for increased biosynthetic capacity. In addition, several viral genomes are found to contain class III genes, which are required for viral replication (568). Indeed, the adenovirus genome encodes two pol III products, $VA_I$ and $VA_{II}$ (492, 559); these small RNAs (~160nt) are synthesised at very high levels late in infection and are involved in the corruption of the host cell's translational apparatus to ensure the synthesis of viral proteins (519).

The activation of pol III transcription in response to adenovirus infection appears to be achieved, to a large extent, by the activities of the E1A oncoprotein of adenovirus (16, 341, 488, 569). Thus, a transfected E1A gene alone is sufficient to activate transcription of the $VA_I$ gene in human cells (16, 341). Furthermore, the addition of purified recombinant E1A protein to HeLa cell extracts can stimulate $VA_I$ transcription up to 50-fold (114, 409). HeLa cells infected with wild-type adenovirus have elevated TFIIIC activity (217, 218, 488, 606). This appears to result from the conversion of a transcriptionally inactive form of TFIIIC2 (TFIIICb) that lacks the TFIIICβ subunit into an active form (TFIIICa) that has the β subunit (114, 217, 488). E1A induces an increase in TFIIICβ mRNA and infected cells display a selective rise

in the levels of TFIIICβ protein (488). However, E1A can also stimulate pol III transcription in vitro, suggesting that there are additional mechanisms by which adenovirus can activate pol III transcription that do not require de novo protein synthesis (114, 409). Indeed, E1A can bind and inactivate RB and has been shown to relieve repression of pol III transcription by RB, both in vitro and in transfected cells (579). In addition, E1A can disrupt the interaction of Dr1 with TBP, thereby releasing TFIIIB from Dr1-mediated repression (293). Another transforming protein of adenovirus, E1B, can bind and inactivate p53, suggesting that adenovirus infection may also overcome the suppressive effects of p53 on pol III transcription (284). In support of this, full induction of Alu gene expression by adenovirus requires E1B (403). This also involves changes in the chromatin structure of Alu genes with an increase in the proportion of these templates that are accessible to transcription factors (451). Thus, multiple mechanisms may contribute to the activation of pol III transcription in response to adenovirus infection (Fig. 1.4).

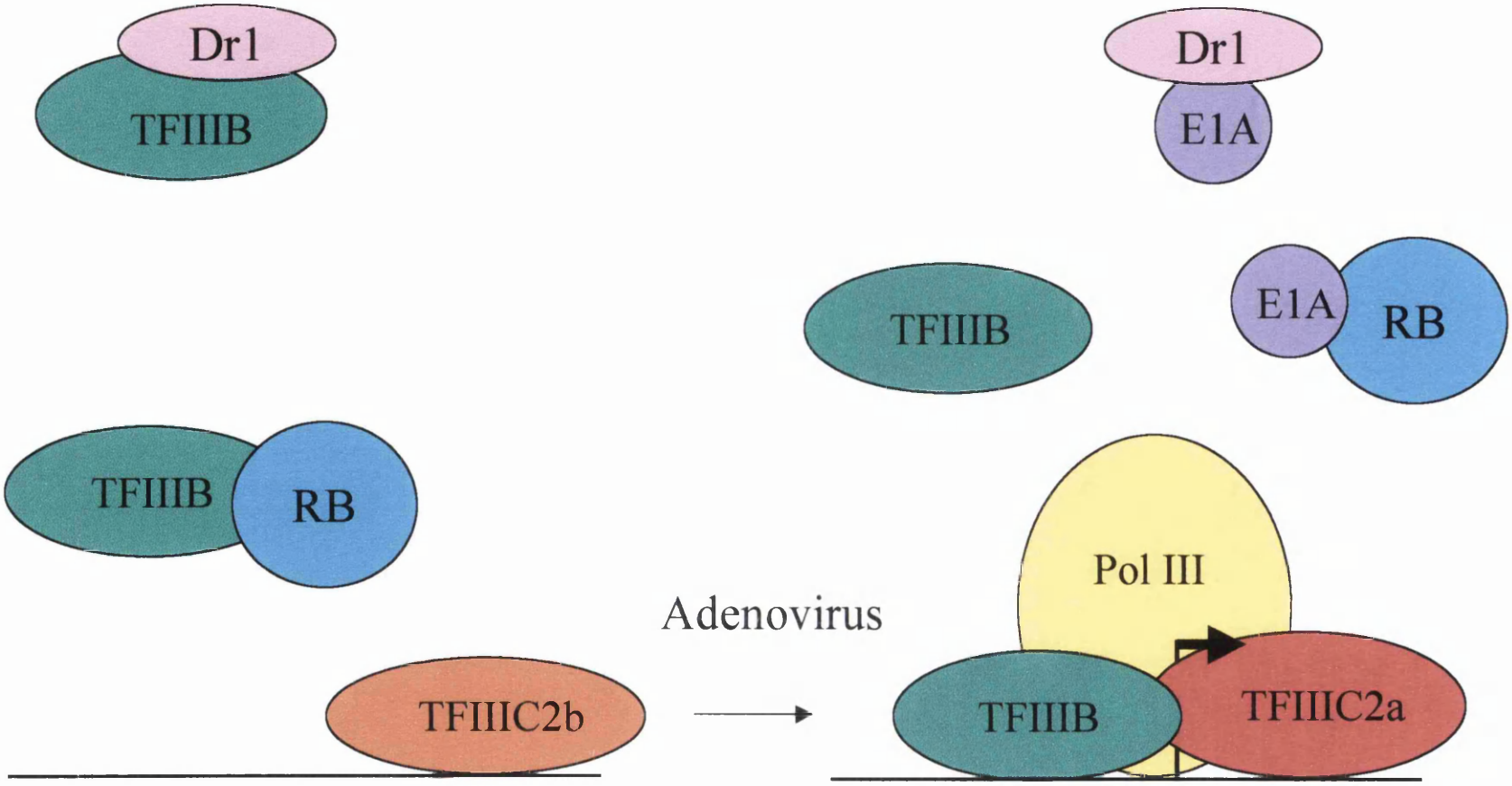
### 1.8.7 Regulation in response to transformation by the DNA tumour virus SV40

Rodent fibroblasts that have been transformed by the DNA tumour virus SV40 display abnormally elevated levels of pol III transcription (75, 315, 469, 487, 578). Like adenovirus, SV40 appears to activate pol III transcription by multiple mechanisms, since the activity of both TFIIIB and TFIIIC2 is significantly higher in extracts from SV40-transformed cell lines than in extracts from the untransformed parental cell line (315). The TFIIICα and TFIIICβ subunits of TFIIIC2 are much more abundant in extracts from SV40-transformed cells, suggesting that the rise in TFIIIC2 activity may result from the overexpression of TFIIIC2 (315). However, there is also evidence for an increase in the proportion of cellular TFIIIC2 that is in a transcriptionally active form, as was observed in response to adenovirus infection (578). In contrast to the α and β subunits of TFIIIC2, there is little or no change in the protein levels of TBP or BRF in response to SV40 transformation (315). Instead, the increase in TFIIIB activity appears to be due to the release of TFIIIB from repression by RB (315). This is dependent on the large T antigen of SV40 which, like E1A, can bind and inactivate RB (315). The large T antigen also interacts with

**Figure 1.4**

**Subversion of normal regulatory controls of pol III transcriptional activity by adenovirus**

Schematic diagram illustrating some of the mechanisms that may contribute to the activation of pol III transcription following adenovirus infection. Adenovirus stimulates the conversion of TFIIIC2 from the inactive TFIIIC2b form to the active TFIIIC2a form. This is thought to result from a selective increase in the levels of TFIIICβ mRNA and protein induced by the E1A oncoprotein of adenovirus. E1A can also overcome the repressive effects of RB and Dr1 on TFIIIB.


Dr1
TFIIIB
Dr1
E1A
TFIIIB
E1A
RB
TFIIIB
RB
Pol III
Adenovirus
TFIIIC2b
TFIIIB
TFIIIC2a

and inhibits p53 (284). However, the effect of this interaction on the ability of p53 to repress pol III transcription has not yet been tested.

Adenovirus and SV40 appear to upregulate pol III transcription by very similar mechanisms (569). However, other viruses may stimulate pol III transcription differently. HBV and HTLV-1 both appear to be more restrictive in their effects, specifically targeting only one component of the basal pol III transcriptional machinery (168, 549). HBV induces a specific increase in TFIIIB activity; this is achieved via activation of Ras signalling (548, 549).

### 1.8.8 Regulation in response to other transforming agents and tumorigenesis

A broad range of transforming agents have been found to induce the overexpression of pol III products (168, 469, 487, 548, 568, 578). In addition to DNA tumour viruses such as SV40 (469, 487, 578) and RNA tumour viruses such as HTLV-1 (168), a variety of chemical carcinogens have also been shown to stimulate pol III transcription (161, 162, 469). An increase in the abundance of pol III transcripts is a very common feature of transformed cell lines (568). However, in most cases the mechanistic basis of this effect has not been studied. It has also recently been shown that pol III products are abnormally elevated in actual tumours (87, 88, 586). A possible explanation for the frequent overexpression of pol III transcripts in transformed and tumour cells is suggested by the very high incidence that RB and p53 become inactivated during neoplastic transformation. In untransformed cells these two key tumour suppressors play a major role in repressing pol III transcription (70, 579).

Although only a limited study, four different point mutations in RB that have been detected in human tumours have each been tested and found to prevent the tumour suppressor from repressing pol III transcription (59, 579). This provides direct evidence that the inactivation of RB in tumours can contribute to the elevation of pol III activity consistently observed in transformed and tumour cells. In normal cells the activity of RB is regulated by phosphorylation by the cyclin D- and cyclin E- dependent protein kinases (173). In many human cancers in which RB is wild-type, the cyclin D-dependent kinases are hyperactive providing an alternative mechanism by which RB function is lost. Indeed, cyclin D1 is overexpressed in 30-

40% of primary breast tumours. In many oesophageal, bladder, lung and pancreatic carcinomas the gene for p16, a specific inhibitor of the cyclin D-dependent kinases, is deleted. The overexpression of cyclin D- and E- dependent kinases in an untransformed mouse fibroblast cell line has been shown to stimulate pol III transcription (471). Moreover, the transcriptional response is significantly impaired in an SV40-transformed derivative of this cell line, consistent with the possibility that the stimulatory effect of the CDKs is achieved through the release of TFIIIB from repression by RB (471). Similar results were also obtained using a p16 ribozyme to lower the concentration of p16 in the cell and release endogenous cyclin D-CDK4/6 from repression. Moreover, hyperphosphorylated RB and TFIIIB are unable to interact (471). Thus, the inactivation of RB by hyperphosphorylation by the CDKs also appears to relieve pol III from its suppressive effects, providing another mechanism as to how pol III activity may become elevated in response to transformation. A third mechanism by which RB function can be lost is by its association with viral oncoproteins such as adenovirus E1A or SV40 large T antigen (541). The E7 oncoprotein of human papillomavirus (HPV) has also been shown to bind and inactivate RB (132). HPVs have an etiological role in most cervical neoplasias (541). Significantly, the E7 proteins from the more malignant HPV strains, such as HPV-16 and –18, bind RB with higher affinity (206). Like E1A and large T antigen, HPV E7 has also been shown to activate pol III transcription (504). Furthermore, analysis of several E7 mutants showed that this is dependent on the integrity of the LXCXE motif, which is the RB-binding site within E7 (504).

Since all three mechanisms by which RB is inactivated in cancers can also release pol III transcription from repression by the tumour suppressor, it is highly probable that the loss of RB function contributes to the elevated pol III activity found in a large variety of tumours and transformed cell types. However, it remains to be determined how the frequent missense mutations in p53 found in approximately half of all the major forms of human cancer affect its ability to inhibit pol III. In addition, constitutively active Ras has recently been shown to stimulate pol III transcription in vivo (471, 548). Activating mutations in Ras are also very frequently found in human malignancies, raising the possibility that Ras may also contribute to the overexpression of pol III transcripts in transformed cells and tumours (344).

## 1.9 Objectives

The overall purpose of this project was to gain a better molecular understanding of how the mammalian pol III transcriptional apparatus is normally regulated and how it might become deregulated in cancer. For each of the instances of regulation where it has been investigated which component(s) of the basal pol III transcriptional apparatus are targeted, a change in the activity of TFIIIB or TFIIIC is implicated; in no case has the polymerase itself been found to be directly controlled. However, rather crude sources of TFIIIB and TFIIIC have often been used. A major objective was therefore to obtain more purified sources of active TFIIIB and TFIIIC to allow regulatory studies to be conducted with more confidence. Another aim of this approach was to help identify the transcriptional target of novel regulators through their consistent copurification with one of these two basal factors. The most highly purified fractions were used to investigate the deregulation of pol III transcription in ovarian cancer. This was of particular importance because although pol III transcript levels are frequently found to be abnormally elevated in transformed and tumour cells, in most cases the mechanistic basis for this has not been elucidated.

In a healthy cell the level of pol III transcription is restrained by two key tumour suppressors, RB and p53. The loss of these constraints on pol III activity may also contribute to the overexpression of pol III products consistently observed in transformed cells. Another aim of this research was to further investigate the regulation of pol III activity by the pocket proteins, especially why the binding of RB to TFIIIB inhibits pol III transcription.

A common mechanism of cellular regulation whose study has been largely neglected in the case of pol III transcription is that of phosphorylation. Moreover, several subunits of pol III components have previously been shown to be phosphorylated in vivo. The final aspect of this research investigated the candidacy of the protein kinases CKII and GSK-3 as novel kinase regulators of mammalian pol III transcription. CKII had previously been shown to stimulate pol III transcription in yeast and was also of particular interest because it is a putative cellular oncogene and has uncharacterised roles in stimulating growth and proliferation. GSK-3 phosphorylates and inhibits the translation elongation factor eIF2B. The possibility existed therefore that it might also inhibit tRNA and 5S rRNA transcription, which

would enable it to coordinately regulate various components of the translational machinery.

# Chapter 2.

# Materials and Methods

## 2.1 Cell culture

All cell culture was performed in a class II hood using aseptic technique and sterile equipment and reagents.

Balb/c 3T3 (A31), NIH 3T3 and HeLa cells, and the rat ovarian epithelial cell lines ROSE 199-*βgal* and ROSE 199-*neu* (115), were all grown in DMEM supplemented with 10% fetal calf serum, 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin at $37^0$C in a 5% $CO_2$ atmosphere. ROSE 199-*βgal* and ROSE 199-*neu*, kindly provided by Bruce A. J. Ponder, were generated from the untransformed parental cell line ROSE 199 by transduction with retrovirus vector expressing the *β-galactosidase* gene or vector expressing the *neu* oncogene with an activating point mutation (V664E), respectively (115). The culture media of these two stably transfected cell lines additionally contained 0.6mg/ml of the antibiotic G418 to maintain selection.

All cells were appropriately passaged every 3 days (3T3s 1:5, HeLa and *B-gal* 1:10, *neu* 1:15) to maintain in log phase growth. To harvest a monolayer, cells were washed briefly with buffered trypsin-EDTA solution to remove serum that inactivates the trypsin and fresh solution was applied. Flasks were then incubated for 1-2 minutes at $37^0$C to allow trypsinisation to occur. To facilitate detachment of cells, flasks can be firmly tapped on the side. Once adherent cells have dissociated to a single cell suspension, DMEM that contains serum was immediately added to neutralise the trypsin.

## 2.2 Preparation of whole cell extracts

Cultured cells were harvested for extract preparation when subconfluent. This procedure was performed on ice and as quickly as possible so as to minimise loss of activity during extract preparation. Approximately 0.5-3 x $10^7$ cells were

required for preparing extracts by the freeze-thaw method described below. First each 10cm petri dish of adherent cells was washed twice with 5ml of ice cold phosphate buffered saline (PBS) and then the cells were harvested into 10ml of ice cold PBS by scraping with a plastic spatula. The suspended cells were then decanted to a pre-cooled 50ml Falcon tube and gently pelleted by centrifugation at 1200 rpm for 8 min at $4^0$C. The cells were gently resuspended in 1ml of fresh ice cold PBS, transferred to an eppendorf tube and pelleted again by pulse centrifugation. The volume of the cell pellet was determined by comparison with measured volumes of water and should be between 50-150µl. The volume of the cell pellet is critical to this technique and larger pellets were subdivided. The pellet was resuspended in an equal volume of freshly made pre-cooled extraction buffer (20mM Hepes pH 7.8, 450mM NaCl, 50mM NaF, 25% glycerol, 0.2mM EDTA, 1mM DTT, 0.5mM PMSF, 0.5µg/ml leupeptin, 0.7µg/ml pepstatin, 1µg/ml trypsin inhibitor, 0.5µg/ml aprotinin, 40µg/ml ubenimex [Bestatin]). This was snap-frozen on dry ice and then placed at $30^0$C until thawed and then immediately snap-frozen again. This sequential freeze-thaw cycle was repeated twice more to ensure efficient lysis of cells. After the third thaw, the suspension was microcentrifuged for 7 min at $4^0$C to pellet the cell debris. The supernatant was carefully removed to a fresh tube, aliquoted into pre-cooled eppendorf tubes and snap frozen. The frozen extracts were stored at -$70^0$C.

## 2.3 Isolation of RNA from cultured cells

Appropriate precautions were taken to avoid problems of contamination by RNase. Solutions were treated with diethylpyrocarbonate (DEPC) to inactivate ribonuclease (0.1% DEPC, shaken vigorously to get the DEPC in solution, left overnight at room temperature, then autoclaved to inactivate remaining DEPC).

Total cellular RNA was extracted using TRI reagent (Sigma), according to the manufacturer's instructions. First, medium was aspirated from sub-confluent cultures in 10cm petri dishes and cells were gently washed twice with 5ml PBS to remove residual serum in case the same samples were also to be used for isolation of cellular protein. 1ml of TRI reagent was added to each 10cm petri dish of cells and dishes were rigorously scraped to detach and help lyse cells. This mixture was

pipetted up-and-down several times to ensure formation of a homogenous lysate and transferred to a sterile eppendorf tube. The TRI reagent, a mono-phase solution of guanidine thiocyanate and phenol, effectively dissolves DNA, RNA and protein released on cell lysis. Samples were left to stand for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes. 0.2ml of chloroform was then added to each tube and the samples were vortexed vigorously for 15 seconds. After allowing to stand at room temperature for 15 min, the samples were then centrifuged at 13,000g for 15 min at $4^0$C. This separates the mixture into three layers: an upper aqueous phase containing the RNA, which should be colourless and clear, a middle interphase that contains precipitated DNA and is whitish in colour, and a lower organic phase containing protein, which is the colour of the TRI reagent. The upper aqueous phases were removed to fresh eppendorf tubes taking particular care to avoid taking up any of the next layer. 0.5ml of isopropanol was added to each tube of aqueous RNA and mixed by inverting several times. The samples were left at room temperature for 5-10 min to ensure maximal precipitation of RNA before centrifugation at 13,000g for 10 min at $4^0$C to pellet the RNA. The supernatant was removed and the RNA pellet was washed with 1ml of 75% ethanol (made up with DEPC-$H_20$). Following a brief re-centrifugation (7500g, 5 min at $4^0$C), the supernatant was aspirated, pulse spun and a P20 pipette was used to remove residual amounts of ethanol. The pellet was redissolved in an appropriate volume of DEPC-$H_20$ (10-30µl). To facilitate this, samples were heated at 60-$65^0$C for 10-15 min and occasionally pipetted up-and-down. RNA samples were stored at -$70^0$C. The concentration of RNA was quantified by UV spectrophotometry (absorbance at 260nm x 40 x dilution factor = RNA concentration (µg/ml). The ratio of absorbance at 260nm to that at 280nm should be >1.8 and <2 for a sample relatively free of DNA or protein).

## 2.4 Northern blot analysis of total cellular RNA

For each sample analysed 10µg of RNA was used, made up to a volume of 10µl with DEPC-$H_20$ and mixed with an equal volume of 2 x RNA sample buffer (1 x MOPS {20mM MOPS pH 7.0, 8mM sodium acetate, 1mM EDTA pH 8.0}, 4.4M

formaldehyde, 54% formamide). The samples were heated at 65$^0$C for 15 min to denature the secondary structure of RNA and then rapidly cooled on ice to prevent any renaturation. 2µl of 10 x RNA loading buffer (50% glycerol, 1mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF) and 2µl of 1mg/ml ethidium bromide was added to the chilled samples. The different species of RNA in each sample were separated according to size by electrophoresis through a denaturing formaldehyde 1% agarose gel (1% agarose, 2.2M formaldehyde, 1 x MOPS). After pre-running the gel in 1 x MOPS for 20 min at 40V, the samples were loaded and the gel was run for a further ~5 hours at 40V. The fractionated RNA was visualised under a UV transilluminator and photographed before soaking the gel in several changes of DEPC-$H_20$ to leach out the formaldehyde. The gel was then immersed in 20 x SCC (3M NaCl, 0.3M sodium citrate pH 7.0) for 20 min. This was the buffer used for capillary transfer of RNA to Hybond N nylon filters (Amersham).

The gel was set up for capillary transfer as described by Maniatis et al. (352) and this was allowed to proceed overnight. Thus, the 20 x SCC-soaked gel was placed inverted on a wick of Whatmann 3MM chromatography paper fed from a reservoir of 20 x SCC onto which pre-wetted nylon membrane cut to the size of the gel was placed ensuring that there were no air bubbles between the layers. Two pieces of pre-soaked Whatmann paper cut to size were placed on top of the membrane followed by a stack of paper towels to provide the capillary action. This was weighed down with a 500g ballast ensuring a tight connection between layers. Pieces of autoradiography film were placed at the edges of the gel to block the towels from directly contacting the wick which would provide a short-circuit and would inevitably result in poor RNA transfer.

Following overnight transfer, the RNA was fixed to the nylon membrane by UV-crosslinking (1200µjoules), washed with DEPC-$H_20$ and stored in Saran wrap. To locate the RNA of interest, hybridisation was performed with a high specific activity radiolabelled DNA probe complementary in sequence to that particular RNA. The B2 gene probe was a 240 bp *Eco*RI-*Pst*I fragment from pTB14 (577). The ARPP P0 probe was a 1kb *Eco*RI-*Hind*III fragment from the mouse cDNA (240). The probes were labelled using the random oligonucleotide priming method of Feinberg and Vogelstein (142) and the Megaprime Random Priming Kit (Amersham). Thus, purified DNA template (25ng) was denatured by heating at 95$^0$C

for 5 min in the presence of random hexamer oligonucleotide sequences that can anneal to the DNA on the slow cooling of the mixture to room temperature. The annealed hexamer primes DNA synthesis. To initiate labelling, Klenow fragment of E. coli DNA polymerase I (1U), dATP, dGTP, dTTP (50μM each) and 40μCi of $\alpha^{32}$P-dCTP (10mCi/ml, 3000 Ci/mmol) were added and the reaction was allowed to proceed for 1h at 37$^0$C. The labelled DNA was denatured by heating at 95$^0$C and was then chilled on ice until ready for use. First, the nylon filter with bound RNA was prehybridised by rotation in a hybridisation oven for 30 min at 45$^0$C in hybridisation buffer (0.2M sodium phosphate buffer pH 7.2, 1mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS, 45% (w/v) formamide in DEPC-$H_20$). The radiolabelled probe was then added to fresh hybridisation buffer and incubated with rotation at 45$^0$C overnight. To remove nonspecific radioactivity, the filter was rotated for 2 min at room temperature and then twice for 10-15 min at 68$^0$C in wash solution (40mM sodium phosphate buffer pH 7.2, 1mM EDTA and 1% w/v SDS). The filter was then exposed to autoradiography film overnight at -70$^0$C. To strip the filter for reprobing, it was soaked in boiling water for 5-10 min.

## 2.5 Transient transfection

Cells were transiently transfected using the calcium phosphate precipitation method. Expression vectors coding for wild-type GSK-3β (pJ3M-GSK-3β) and kinase-inactive GSK-3β (pJ3M-GSK-3βkd) were kindly provided by Charles J. Sherr. To construct "empty" pJ3M expression vector, the insert in pJ3M-GSK-3β was excised by carrying out a double restriction enzyme digest with *Bam*HI and *Bgl*II. The linearised vector was then separated from the insert by electophoresis through a 1% low melting point agarose gel and gel-purified using the QIAquick gel extraction kit (Qiagen). Re-ligation of vector was carried out overnight at 15$^0$C using T4 DNA ligase. Ligation product was used to transform E. coli competent cells. Mini-preparations of plasmid DNA were performed from 2ml overnight cultures (in LB broth containing 50μg/ml ampicillin) of a number of isolated colonies and used to verify that the cells contain the correct vector and that it lacks the insert before carrying out a large scale plasmid preparation. All the plasmid

DNA used for transfection or transcription assays was purified using the QIAGEN Plasmid Maxi Protocol and was verified to be of very high quality and purity by UV spectrophotometry and electrophoretic analysis. Supercoiled plasmid DNA to be used for transfection was additionally ethanol-precipitated twice and then resuspended in a small volume of sterile water.

24 h prior to transfection, cells were split and seeded at ~ 4-6 x $10^5$ cells per 10cm dish (depending on cell type) resulting in a confluency of ~55-70% at the time of transfection. To improve transfection efficiency, 4 h before transfection the cells were refed with fresh growth medium. 45 min before transfection, for each dish the total plasmid DNA to be transfected was diluted to a volume of 450µl in filter-sterilised 0.1 x TE buffer, pH 8.0 (1mM Tris pH 8.0, 0.1mM EDTA pH 8.0) and 50µl of 2.5M $CaCl_2$ was added. To form calcium phosphate-DNA precipitates, the DNA-$CaCl_2$ mixture was added dropwise to 500µl of 2 x HEPES-buffered saline solution, pH 7.1 (280mM NaCl, 1.5mM $Na_2HPO_4.2H_2O$, 50mM HEPES, pH 7.1 with NaOH), with continuous swirling to mix. This was then vortexed for 15 seconds and left to stand for 30 min at room temperature before adding to cells and swirling to mix evenly. DNA precipitates were left on the plates overnight before removing and washing the cells twice with warm PBS. Fresh medium was then added and the cells were incubated at $37^0$C for a further 48 h to allow expression of transfected DNA. Cells were harvested and total RNA extracted for analysis by primer extension.

## 2.6 Primer extension

Primer extension was used to analyse the expression levels of transfected pol III template $VA_I$ and also a cotransfected CAT gene used as an internal control for transfection efficiency. For each primer extension reaction, 1µg of total RNA in 10µl of DEPC-$H_2O$ was mixed with 10µl of 5 x First Strand Buffer (Life Technologies) and 1µl (2.5ng) of $\gamma$-$^{32}$P end-labelled $VA_I$ (5′-CACGCGGGCGGTAA CCGCATG-3′) or CAT (5′-CGATGCCATTGGGATATATCA-3′) oligonucleotide as primer. This mixture was incubated at $80^0$C for 10 min followed by 2h at $50^0$C. 30µl of RT mix (23µl DEPC-$H_2O$, 0.5µl 1M DTT, 5µl 5mM dNTP mix, 0.5µl

4mg/ml actinomycin D, 0.5µl RNasin, 0.5µl (100U) Superscript II Reverse Transcriptase (Life Technologies)) was then added to the samples to initiate reverse transcription which was allowed to proceed for 1 h at $42^0$C. Reaction products were ethanol precipitated overnight and washed with 70% ethanol before electrophoresis through a 7M urea 7% polyacrylamide gel.

## 2.7 RNA pol III in vitro transcription assay

The in vitro transcription of class III genes was reconstituted using either crude extracts or fractionated factors as a source of the basal pol III transcription components and 250ng of plasmid DNA containing a particular pol III template. Transcription reactions were performed in a volume of 25µl in a final concentration of 12mM HEPES pH 7.9, 60mM KCl, 7.2mM $MgCl_2$, 0.14mM EDTA, 1.2mM DTT, 10% (v/v) glycerol, 1mM creatine phosphate, 0.5mM each of rATP, rGTP and rCTP and 10µCi [$\alpha$-$^{32}$P] UTP (400 mCi/mmol) (Amersham). Components for transcription were assembled on ice. For transcription reactions reconstituted with fractionated factors, these were preincubated for 15 min at $30^0$C with the pol III template before the addition of nucleotides required to initiate transcription. After 1 h incubation at $30^0$C, transcription was stopped by the addition of 250µl of 1M ammonium acetate/0.1% SDS containing 20µg of yeast tRNA that stabilises the synthesised RNA. The samples were then phenol-chloroform extracted to remove protein and DNA. The aqueous layer (250µl) was carefully transferred to a fresh eppendorf tube and 750µl of 96% ethanol was added to precipitate the RNA. The samples were mixed by inversion and left overnight at -$20^0$C. The precipitated RNA was pelleted by centrifugation at 13,000g for 20 min. After washing with 70% ethanol (750µl), as much supernatant as possible was removed, taking care not to dislodge the pellet. The samples were then placed at $47^0$C for 5-10 min to dry. The RNA was redissolved in 4µl of formamide loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue) by vortexing for 20 min. Transcription products were resolved by electrophoresis through a 7% polyacrylamide sequencing gel containing 7M urea and 1 x TBE (44.5mM Tris base, 44.5mM boric acid, 1mM EDTA pH 8.0). The gel was pre-run in 1 x TBE for 30 min at 40W and the samples

were boiled for 2 min at 95$^0$C and quenched on ice. 2μl of each sample was loaded and the gel was run for a further 1 hour at 40W. The gel was then vacuum-dried for 1 h at 80$^0$C and radiolabelled transcripts were detected by autoradiography.

Plasmid templates used for in vitro transcription assays were as follows. pVA$_I$ is a 221 bp *SalI-Ba*lI fragment of adenovirus 2 DNA containing the VA$_I$ gene subcloned into pUC18 (117). pLeu is a 240 bp *Eco*RI-*Hin*dIII fragment of genomic DNA containing a human tRNA$^{Leu}$ gene subcloned into pAT153 (577). pArg contains a human tRNA$^{Arg}$ gene. pHu5S3.1 is a 638-bp *Bam*HI-*Sac*I fragment of human genomic DNA containing a 5S gene subcloned into pBluescript SK+. pU6/Hae/RA2 contains the promoter of the human U6 gene followed by a 137 bp fragment of the rabbit β-globin gene (338).

## 2.8 Electrophoretic mobility shift assay (EMSA)

To assay for TFIIIC2 DNA-binding activity, EMSAs were performed using γ-$^{32}$P labelled oligonucleotide containing a B-block consensus (5′-AGAGGTCCTGAGTTCAAATCCCAG-3′ (RJW1) annealed to the complementary 3′ to 5′ strand (RJW2)). Sp1 binding was assayed using an oligonucleotide that contains a consensus Sp1 binding site (5′-ACTTGATTAACTGGGCGGAGTTAT GATTGA-3′ (M1) annealed to the complementary 3′ to 5′ strand (M2)). Oligonucleotides were 5′ end-labelled using T4 polynucleotide kinase (PNK). Thus, 40ng of RJW1 or M1 was incubated for 1 h at 37$^0$C with 10 units of PNK and 20μCi of [γ-$^{32}$P]-rATP (10mCi/ml, 3000Ci/mmol) in 1 x PNK buffer (Promega). The labelling reaction was stopped by heating at 65$^0$C for 10 min. PNK enzyme was phenol-chloroform extracted and oligonucleotide was ethanol precipitated. Unincorporated label was removed by multiple washes of precipitated oligonucleotide with 70% ethanol, before resuspending in TE buffer, pH 8.0 (10mM Tris pH 8.0, 1mM EDTA). Unlabelled complementary oligonucleotide was then added in 2.5 fold excess and the mixture was heated in a hot block at 90$^0$C for 2 min. The hot block was then turned off and the samples were left to cool slowly overnight. The annealed oligonucleotide was stored at 4$^0$C until ready to use.

The binding reactions were performed in a final volume of 10µl and an optimal salt concentration of 60mM KCl, as for in vitro transcription assays. Each reaction contained 1µg of polydIdC (2µl), 100ng of non-specific or specific competitor oligonucleotide (2µl), 0.25-0.5ng of labelled probe (2µl) and typically 1-4µl of protein extract or fractionated factors. Extract or protein factors were preincubated with unlabelled competitor DNA for 15 min at $30^0$C before the addition of labelled probe and a further 15 min incubation at $30^0$C. The formation of protein-DNA complexes was analysed by electrophoresis of samples on a prerun 4% nondenaturing polyacrylamide gel in 1 x TAE buffer (40mM Tris acetate, 1mM EDTA pH 8.0) for 1½-2 h at $4^0$C. The gel was quickly dried to avoid dissociation of complexes formed and exposed to X-ray film overnight at $-70^0$C.

## 2.9 In vitro transcription and translation of BRF

To transcribe BRF, 1µg of pCITE vector (Novagen) containing the BRF sequence subcloned downstream of the T7 promoter was incubated at $37^0$C for 90 min with 80 units of T7 phage RNA polymerase. RNA synthesis was carried out in T7 transcription buffer (40mM Tris pH 8.0, 25mM NaCl, 8mM $MgCl_2$, 2mM spermidine) supplemented with 5mM DTT, 1mM of each rNTP and 20 units of RNase inhibitor (RNasin). Following phenol-chloroform extraction and ethanol precipitation, the synthesised BRF RNA was then washed with 70% ethanol and resuspended in 50µl DEPC-$H_2O$.

Translation of the RNA was carried out using rabbit reticulocyte lysate (Promega), according to the manufacturer's specifications. Translation reactions were performed at $30^0$C for 90 min in a final volume of 50µl. Reaction mixtures typically contained 25µl of reticulocyte lysate, 1µl of amino acid mixture that lacks methionine and cysteine (Promega), 2µl of synthesised RNA, 1µl of RNasin (20 units), 7µl of DEPC-$H_2O$ and 4µl of a mixture of [$^{35}$S]-methionine and [$^{35}$S]-leucine (14.3mCi/ml, 1000Ci/mM) (Amersham). To check translation products, a small aliquot was resolved on a SDS 7.8% polyacrylamide gel and visualised by autoradiography.

## 2.10 Immunoprecipitation

Antibodies were coupled to protein A-Sepharose by incubating LDB-washed beads with crude antiserum or purified antibody on an orbital shaker for 1 h at $4^0$C in the presence of a cocktail of protease inhibitors (0.9μg/ml leupeptin, 1.2μg/ml pepstatin, 1.75μg/ml trypsin inhibitor, 0.9μg/ml aprotinin, 70μg/ml ubenimex [Bestatin]). The beads were then washed twice with LDB buffer (100mM KCl, 20mM Hepes.Cl pH 7.9, 12mM $MgCl_2$, 0.1mM EDTA, 17% glycerol, 2mM DTT) to remove unbound antibody and other factors such as proteases present in the sera.

Immunoprecipitation reactions were typically carried out using 150μg of HeLa nuclear extract (20μl) incubated for 3 h at $4^0$C on an orbital shaker with 20μl of protein A-Sepharose beads carrying equivalent amounts of prebound immunoglobulin (IgG) for the different reactions. The beads were then gently pelleted by pulse centrifugation, supernatants were carefully removed and the beads were washed five times with 500μl of LDB. The bound material was released by adding an equal volume of 2 x protein sample buffer (12.5mM Tris pH 6.8, 1% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.25% bromophenol blue) and was analysed by Western blotting. For other immunoprecipitations, PC-B, PC-C or $^{35}$S-labelled in vitro translated proteins was used instead of nuclear extract. In the latter case, precipitated material and supernatant was analysed by autoradiography.

## 2.11 Separation of proteins by gel electrophoresis

Proteins were resolved by electrophoresis on denaturing (SDS) polyacrylamide gels. The gel recipes were based on the SDS discontinuous buffer system of Laemmli (306). Typically, proteins were electrophoresed on 7.8% polyacrylamide resolving minigels (containing 375mM Tris pH 8.8, 0.1% SDS). A 4% polyacrylamide gel containing 125mM Tris pH 6.8 and 0.1% SDS was routinely cast as the stack. Before loading samples on the gel, they were boiled for 5 min in 1 x protein sample buffer (6.25mM Tris pH 6.8, 0.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.125% bromophenol blue). Electrophoresis was carried out in 1 x protein running buffer (0.1% SDS, 76.8mM glycine, 10mM Tris, pH 8.3) at 70V

initially until the bromophenol dye front had entered the resolving gel and then at 140V for ~ 1½ h until the dye had reached the bottom of the gel.

## 2.12 Western blotting

Following resolution by SDS-PAGE, proteins were transferred to a nitrocellulose filter by electroblotting in 20mM sodium phosphate (pH 6.7) at 37V for 30 min using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell. The filter was then washed for 10 min in 1 x TBS (25mM Tris pH 7.6, 150mM NaCl) and blocked for 1 h at room temperature in milk buffer (1 x TBS, 0.5% Tween-20, 4% skimmed milk powder (Marvel)). The blot was incubated with diluted primary antibody (typically a 1:1000 or 1:2000 dilution) overnight at $4^0$C. To remove excess primary antibody, the blot was washed for 2 min with milk buffer three times before incubating for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody. The blot was then washed extensively to remove excess secondary antibody (3 x 2 min and 2 x 15 min with milk buffer followed by a 5 min wash with TBS). The blot was developed by the enhanced chemiluminescence method (ECL, Amersham), according to the manufacturer's instructions.

## 2.13 Transformation

For plasmid storage and propagation, E. coli XL-1 Blue supercompetent cells (Stratagene) were transformed. For the expression of recombinant proteins in bacteria, E.coli BL21 (DE3) pLys competent cells were used as host. For both E.coli strains, competent cells were thawed on ice. 50µl of XL1-Blue cells or 20µl of BL21 (DE3) pLys cells were used per transformation reaction. For each transformation, 10-20ng (1µl) of plasmid DNA was added to the chilled aliquot of thawed cells and the contents of the tube were gently mixed by stirring with a pipette tip. After incubating on ice for 30 min, cells were heat shocked at $42^0$C for 45 seconds and then placed on ice for a further 2 min. 450µl of SOC medium (2% bacto-tryptone, 0.5% yeast extract, 20mM glucose, 10mM NaCl, 10mM $MgSO_4$, 10mM $MgCl_2$) was

then added and the cells were incubated at $37^0$C for 1 h on an orbital shaker (~200rpm). A variety of small volumes (200µl or less) of the transformation mixture were then plated on LB agar plates containing 50µg/ml ampicillin (Amp). Plates were incubated overnight at $37^0$C to allow transformed cells to grow and form colonies.

## 2.14 Plasmid DNA preparation

For large scale plasmid DNA preparation, a single isolated bacterial colony was first picked from a freshly streaked plate and used to inoculate 10ml of LB medium (10g/l bactotryptone, 5g/l yeast extract, 10g/l NaCl) containing appropriate selective antibiotic (50µg/ml ampicillin). After 6 h shaking at $37^0$C, 3ml of this mini-culture was used to inoculate 250ml of LB medium containing ampicillin. This larger bacterial culture was grown overnight at $37^0$C on an orbital shaker. Cells were then harvested by centrifugation at 6000g for 15 min at $4^0$C and plasmid DNA was prepared using the QIAGEN Plasmid Maxi Kit.

Following resuspension in 10ml of Buffer P1 (50mM Tris pH 8.0, 10mM EDTA, 100µg/ml RNase A), cells were subjected to alkaline lysis by incubating with 10ml of Buffer P2 (200mM NaOH, 1% SDS) for 5 min at room temperature. The lysate was then quickly neutralised by adding 10ml of chilled Buffer P3 (3M potassium acetate, pH 5.5) and mixing immediately causing a precipitate of potassium dodecyl sulphate to form. The SDS-denatured proteins and chromosomal DNA were co-precipitated with detergent whereas plasmid DNA, which lacks any close protein associations, remained in solution. After 20 min incubation on ice, the precipitate was pelleted by centrifuging at 20,000g for 30 min at $4^0$C and the supernatant was removed and applied to a QIAGEN tip 500 pre-equilibrated with QBT buffer (750mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100). This is an anion-exchange resin to which plasmid DNA was able to tightly bind. The resin was then washed twice with 20ml of Buffer QC (1M NaCl, 50mM MOPS pH 7.0, 15% isopropanol) before elution of purified plasmid DNA in QF buffer (1.25M NaCl, 50mM Tris pH 8.5, 15% isopropanol) and its precipitation using 0.7 volumes of isopropanol. After centrifugation at 15000g for 30 min and a

70% ethanol wash, plasmid DNA was resuspended in a small quantity of sterile water or TE buffer pH 8.0.

## 2.15 Expression and purification of glutathione S transferase (GST)-fusion proteins

A glycerol stock of BL21 (DE3) pLys bacteria transformed with the appropriate GST-fusion protein expression vector was thawed on ice. A loopful of cells was used to inoculate 20ml of LB medium containing appropriate selective antibiotics (GST-RB(379-928) expression vector: 50μg/ml ampicillin, 10μg/ml chloramphenicol; GST expression vector: 50μg/ml ampicillin) and grown overnight at 37$^0$C. This culture was then used to inoculate 200ml of LB medium which was grown for ~ 1 h at 37$^0$C to mid-logarithmic phase (optical density ~0.6). The culture was then transferred to an orbital shaker at room temperature and incubated with shaking (200rpm) for 30 min. 100μM isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to induce the expression of recombinant protein and cultures were incubated for a further 16 h at room temperature. Bacteria were harvested by centrifugation and resuspended in 9ml of ice-cold PBS containing 1% Triton X-100 and 1% PMSF. Cells were lysed by sonicating briefly on ice (2 x 20 secs on 60-80W). The cell debris was pelleted by centrifugation (12,000 rpm for 10 min at 4$^0$C in a Sorvall High Speed Centrifuge). The supernatant, which should be clear but coloured, was carefully decanted to a fresh tube containing 200μl of glutathione-agarose beads and mixed by constant rotation for 30 min at 4$^0$C. The beads were then washed extensively to remove unbound proteins (2 x 50ml ice cold PBS/1% Triton X-100, 2 x 50ml ice cold PBS with no Triton X-100). To elute bound GST-fusion protein from the glutathione agarose beads, the beads were mixed by rotation with an equal volume of elution buffer (50mM Tris, 25mM glutathione, pH 8.0) for 10 min at room temperature. This was repeated three times to ensure efficient elution. Samples were then dialysed in LDB for 5-6 h at 4$^0$C, aliquoted and stored at -70$^0$C.

## 2.16 Expression and purification of 6 x Histidine (His)-tagged RB

A single colony of BL21 (DE3) pLys cells freshly transformed with $His_6$Rb (379-928) construct was used to inoculate 50ml of LB medium. This culture was grown overnight at 37$^0$C and used to inoculate 500ml LB. After 1-1½ h further growth at 37$^0$C (optical density of 0.6-0.8), the 500ml culture was placed in an orbital shaker at room temperature and immediately induced (150μM IPTG). Cells were incubated for a further 16-24 h to allow expression of His-tagged protein. Bacterial pellets were resuspended in TBS/1% Triton/1% PMSF. Sonication and the clearing of the lysate were performed as for GST-fusion protein preparations. Supernatant was incubated with 300μl of nickel-agarose beads (QIAGEN) in a final concentration of 10mM imidazole for 30 min at 4$^0$C. The agarose beads were washed 5 x with 15ml of TBS containing 10mM imidazole followed by a further five washes with 15ml of TBS containing 40mM imidazole. His-tagged protein was batch eluted from the Ni-NTA (nitrilo-triacetic acid) resin by three sequential 10 min incubations at room temperature with 1 bead volume of TBS containing 150mM imidazole. Eluates were dialysed extensively in LDB as for purified GST-fusion proteins and stored in small aliquots at -70$^0$C.

## 2.17 Coomassie Blue staining of protein gels

The purity and yield of bacterially expressed recombinant proteins was analysed by running small volumes (<5μl) of purified protein on a SDS polyacylamide gel that was then stained with Coomassie Blue R250. First, the gel was fixed for 15 min in a 5:1:5 mixture of methanol, glacial acetic acid and water. To stain the gel, it was incubated in fixative containing 0.1% (w/v) Coomassie Blue R250 for ~30 min. Excess dye was removed by destaining in an aqueous solution of 5% methanol and 7.5% acetic acid. The concentrations of purified recombinant proteins were estimated by comparison of the intensity of staining with that of known amounts of bovine serum albumin that were run on the same gel.

## 2.18 Column chromatography

All chromatographic procedures were performed in a cold room at $4^0$C using the Biorad Econo System except for DNA- and immuno- affinity purification that was carried out by hand in Biorad Polyprep® disposable columns. Buffers were all prepared using ultrapure deionised water obtained from a Millipore $MilliQ_{plus}$ system and were autoclaved or filter-sterilised before use and pre-chilled at $4^0$C. The starting material for fractionation, nuclear extract from exponentially growing HeLa cells, was purchased from the Computer Cell Culture Centre (Mons, Belgium).

### 2.18.1 Fractionation of HeLa nuclear extract on phosphocellulose

Phosphocellulose chromatography was carried out by the method of Segall et al. (473). The day before fractionation, the phosphocellulose resin was prepared. The phosphocellulose (P11, Whatman), which is purchased as an anhydrous powder, was activated for use as a cation exchanger by washing first with base and then acid. A suitable amount of phosphocellulose was weighed out and stirred into 25 volumes of 0.5M NaOH. The phosphocellulose was left to settle for 5 min before decanting off the supernatant. It was then washed extensively with deionised water in a Buchner funnel until the filtrate pH was 11.0 or below. The phosphocellulose was then mixed with 25 volumes of 0.5M HCl and left for 5 min. To avoid excessive hydrolysis and swelling, it was important that the phosphocellulose was not exposed to 0.5M NaOH or 0.5M HCl for any longer than 5 min. After the acid wash, the phosphocellulose was washed with deionised water as previously until the filtrate pH was within several pH units of that of the starting buffer, i.e. pH 7.9. The phosphocellulose was then mixed with several changes of 1M Hepes.Cl pH 7.9 until the pH was within 0.5 units of the desired pH. The phosphocellulose was then left to equilibrate overnight in starting buffer, PC-A (0.1) (20mM Hepes.Cl pH 7.9, 20% glycerol, 0.2mM EDTA, 100mM KCl, 0.5mM DTT).

The following morning the pH was checked and an empty column of suitable volume was packed with equilibrated phosphocellulose resin. The column was then attached to the Biorad Econo System and the gradient monitor was calibrated by washing with several volumes of low salt buffer (PC-A (0.1)) followed by several

volumes of high salt buffer (PC-A (1.2)). The column was then reequilibrated in low salt buffer (PC-A(0.1)) ready for the application of extract.

The maximum protein binding capacity of phosphocellulose is ~10mg per ml. To avoid overloading the column, 5mg of nuclear extract (7.5mg/ml) was loaded per ml of resin. To allow the equilibration of applied protein with the column and adsorption to occur, extract was loaded slowly at a flow rate of 1 column volume per hour. PC-A (0.1) was applied at the same flow rate until protein was detected in the flow-through at which point the flow rate was increased to 2.5 column volumes per hour. A UV monitor measured absorbance at 280nm enabling the elution of protein from the column to be closely monitored. The Econo system was programmed to collect 1ml fractions. Individual fractions were directly tested for protein content using a colourimetric method based on the differential colour change of Bradford's reagent, an acidic solution of Coomassie Brilliant Blue G-250, in response to various concentrations of protein. 10µl of fractions were mixed with 100µl of a 1:10 dilution of Bradford's reagent (Biorad) in a 96-well plate. The binding of protein to dye resulted in an immediate colour change. The column was washed with PC-A (0.1) until the flow-through peak had returned to near zero and little or no protein was detectable in eluted fractions. These flow-through fractions (PC-A) contain TFIIIA. Bound protein was then step-eluted by sequential washing with PC-A buffer (20mM Hepes.Cl pH 7.9, 20% glycerol, 0.2mM EDTA, 0.5mM DTT) containing 350mM KCl (PC-A (0.35)), 600mM KCl (PC-A (0.6)) and 1.2M KCl (PC-A (1.2)). Fractions eluted by the 0.1 to 0.35M KCl step constitute PC-B and contain both TFIIIB and pol III. PC-C constitutes fractions eluted by the 0.35 to 0.6M KCl step and contains TFIIIC, pol III and SNAPc. PC-D is the 0.6 to 1.2M KCl step fraction and contains the pol II factor TFIID and the pol I factor SL1.

Before aliquoting and snap freezing, PC-B and PC-C fractions were extensively dialysed to lower the salt concentration. Dialysis was carried out at $4^0$C using the Gibco BRL Microdialysis System in accordance with the manufacturer's specifications. The dialysis membrane, stored in 20% ethanol, was prepared for dialysis by washing carefully with 4-5 changes of deionised water. The sheets of dialysis membrane have an exclusion limit of 12 to 14 kDa ensuring retention of the pol III factors but allowing exchange of ions. Fractions were dialysed against LDBO (20mM Hepes.Cl pH 7.9, 12mM $MgCl_2$, 0.1mM EDTA, 17% glycerol, 2mM DTT)

to a final salt concentration of ~100mM. The progress of dialysis was monitored by measuring the salt conductivity of fractions. Typically, PC-B fractions were dialysed for ~4-6 h and PC-C fractions were dialysed for ~8-10 h.

To reduce the likelihood of protein degradation, freshly prepared PMSF (0.1mM final concentration) was added to chromatographic and dialysis buffers. 0.1mM PMSF was also added to the starting material along with a cocktail of other protease inhibitors (0.9μg/ml leupeptin, 1.2μg/ml pepstatin, 1.75μg/ml trypsin inhibitor, 0.9μg/ml aprotinin, 70μg/ml ubenimex [Bestatin]) which together can inhibit the major classes of cellular proteases. Fractions obtained by chromatography were stored at -70$^0$C.

### 2.18.2 Heparin- and SP- Sepharose chromatography

Gradient chromatography on heparin-Sepharose was carried out using pre-packed 1ml Hi-Trap Heparin columns obtained from Pharmacia Biotech. The column was equilibrated by washing with 5 column volumes of Hep buffer (20mM Hepes.Cl pH 7.9, 20% glycerol, 0.2mM EDTA, 5mM $MgCl_2$, 0.1mM DTT, 0.1 mM PMSF, 0.1mM sodium metabisulphate) containing 100mM KCl (Hep100). Typically, 15mg of HeLa nuclear extract (2ml) or ~4mg PC-B (~4mls) were fractionated. Samples were loaded onto the column at a flow rate of 0.05ml/min (3 cvs/h). After extensive washing with Hep100, bound protein was gradient eluted. The Econo System pump has two separate buffer inlets with buffer uptake from either inlet controlled by a proportioning valve enabling the formation of a gradient by the appropriate mixing of two different buffers. The system was programmed to form a linear 100mM to 1M KCl gradient in 1 h. The gradient was typically run in 3 or 6 column volumes at a flow rate of 0.1ml/min. Eluted fractions (200μl) were dialysed against LDBO to a final KCl concentration of 100mM, as previously.

Gradient chromatography on SP-Sepharose was also carried out using a prepacked 1ml HiTrap column (Pharmacia Biotech). Chromatographic conditions and buffers were identical to those used for fractionation on heparin.

### 2.18.3 Fractionation of PC-B on Cibacron Blue

A 1ml prepacked HiTrap Sepharose column (HiTrap Blue, Pharmacia Biotech) was also used for chromatography of fractions on Cibacron blue 3GA. The column was equilibrated by washing with 5 cvs of CB buffer (20mM Tris.HCl pH 7.9, 5mM $MgCl_2$, 10% glycerol, 3mM DTT, 0.1mM PMSF) containing 100mM NaCl (CB100). 1mg of PC-B was then loaded at a flow rate of 0.05ml/min (3cvs/h). To remove unbound protein, the column was washed at 0.1ml/min (6cvs/h) with ~10 column volumes of CB100. Several column volumes of CB buffer containing 1M NaCl were then applied until little or no more protein was being eluted (CB1000). CB buffer containing 2M NaCl and 5M urea (CB2000U) was then applied to elute even more tightly bound protein. 0.2ml fractions were collected, as programmed by the Econo System. Protein-containing fractions were dialysed against LDBO for ~ 6-7 h until of approximately the same salt conductivity as LDB. The fractions were then dialysed for a further 3 h against LDB to ensure removal of any residual urea still present in the samples.

### 2.18.4 Fractionation of PC-B on hydroxyapatite

PC-B (1mg) was fractionated on hydroxyapatite using a prepacked 1ml Econo-Pac CHT-II column obtained from Biorad. The column was equilibrated by washing with 5 cvs of HAP buffer (10% glycerol, 60mM KCl, 5mM $MgCl_2$, 1.5mM DTT, 0.1mM PMSF) containing 10mM potassium phosphate pH 7.0 (HAP10). PC-B, diluted 2-fold in HAP10 buffer, was applied to the resin at a flow rate of 0.02ml/min. The flow rate was then increased to 0.04ml/min. After washing extensively with HAP10 buffer, bound protein was eluted with a 6ml linear gradient from 10mM to 500mM potassium phosphate pH 7.0 in HAP buffer. Fractions were dialysed into LDB as previously.

### 2.18.5 Carboxymethyl chromatography

Fractionation on carboxymethyl was carried out using a prepacked 1ml carboxymethyl column obtained from Biorad. Typically, less than 1mg of protein was applied to this column because of its low protein binding capacity. The column was equilibrated by washing with PC-A (0.1). Protein was applied at a flow rate of 0.1ml/min (6cvs/h). The column was washed at the same flow rate with PC-A (0.1). It had previously been shown that TFIIIB does not bind to this weak cation exchanger under these conditions, thereby avoiding the need for dialysis. 0.2ml flowthrough fractions were collected, aliquoted and snap-frozen immediately. These fractions were subsequently assayed for TFIIIB activity. TFIIIC and pol III were step-eluted with PC-A (1.2) and dialysed into LDB before aliquoting and snap freezing.

### 2.18.6 Purification of antisera for use in immunoaffinity chromatography

Antisera were purified on protein A-Sepharose beads (Pharmacia Biotech) by the low salt method of Harlow and Lane (195). 2mls of protein-A Sepharose beads were transferred to a disposable polypropylene column (Polyprep®, Biorad) and washed with several volumes of 100mM Tris pH 8.0. To ensure the tight binding of immunoglobulins to the protein A, one-tenth volume of 1M Tris pH 8.0 was added to the thawed sample of antiserum. The antiserum (4ml) was slowly recirculated through the column of protein A-Sepharose for ~1h (four passes). The protein A-Sepharose column was then washed with 10 column volumes of 100mM Tris pH 8.0 (20mls, 1 h) followed by a further 10 column volumes of 10mM Tris pH 8.0. Purified immunoglobulin was eluted by lowering the pH by applying 100mM glycine pH 3.0. To preserve the structure and activity of immunoglobulins, samples were immediately neutralised on elution by collecting in 0.5ml fractions in eppendorf tubes that already contained 50µl of 1M Tris pH 8.0 and mixing gently. Protein-containing fractions were pooled (4mls) and dialysed into LDB.

### 2.18.7 Immunoaffinity purification of TFIIIB or TFIIIC

TFIIIC was immunopurified using the polyclonal antiserum 4286, which was raised by immunising rabbits with synthetic peptide RPGFSPTSHRLLPTP (human TFIIICβ residues 897-911) coupled to keyhole limpet haemocyanin. The corresponding preimmune serum was used in control immunopurifications that were carried out in parallel. Prior to use, the sera were purified on protein A-Sepharose beads. Eluted antibodies were dialysed into LDB. 2 mls of PC-C fraction (~0.7mg/ml) was incubated with 300μl of purified antibody (~10mg/ml) on ice for 1 h. This mixture was recirculated slowly through 350μl of protein A-Sepharose at $4^0$C for ~2 h. The column was washed with 25 column volumes of CB buffer containing 100mM NaCl (CB100). Immunoaffinity-purified TFIIIC was then eluted using CB buffer containing 2M NaCl and 2M urea. Fractions were dialysed into LDB buffer.

TFIIIB was immunopurified using either the 128 or 330 antiserum, which were raised against residues 533-547 and 664-677 of human BRF, respectively. The immunopurification procedure was identical to that for TFIIIC except that PC-B (2ml) or TFIIIB-containing heparin fractions (2ml) were used as starting material.

### 2.18.8 DNA-affinity purification of TFIIIC

TFIIIC was DNA-affinity purified using a B-block oligonucleotide resin carrying the B-block promoter sequence from the adenovirus $VA_I$ gene. The starting material for DNA-affinity chromatography, PC-C or immunoaffinity-purified TFIIIC in LDB, was diluted into BL70 buffer (70mM KCl, 0.1% IGEPAL CA-630 (Sigma), 20mM Hepes.KOH pH 7.9, 12mM $MgCl_2$, 0.1mM EDTA, 17% glycerol, 9mM DTT, 0.1mM PMSF, 0.5μg/ml leupeptin, 0.7μg/ml pepstatin, 1μg/ml trypsin inhibitor, 0.5μg/ml aprotinin, 40μg/ml ubenimex [Bestatin]). Prior to loading onto the column, the diluted starting material was incubated on ice for 10 min with 30μg poly(dI.dC) per mg of protein. This protein-DNA mixture (1mg of protein) was slowly recirculated through 350μl of affinity resin for ~75 min at $4^0$C. The column was washed with 20 column volumes of LDB. 5 column volumes of PC-A(0.35) were

then sequentially applied, collecting 1 column volume fractions. 1 column volume of PC-A buffer (minus the EDTA) containing 2M KCl, 0.1% IGEPAL CA-630, and 0.2mg/ml insulin was then applied and left for 10 min. The fraction was then collected and this was repeated with a further 5 column volumes of this high salt PC-A buffer. Fractions were dialysed into LDB, as previously. Dialysed fractions were then extensively aliquoted to avoid the need for multiple freeze-thaw cycles and immediately snap frozen on dry ice.

## 2.19 Measuring protein concentration

The protein concentration of samples were accurately estimated by quantitating the colour change in Bradford's reagent (Biorad) on mixing 1ml of diluted reagent (1:10 in distilled water) with a small volume of the sample being assayed. The absorbance of this mixture at 595nm was measured in a UV spectrophotometer. For a narrow range of protein amounts (~1-12μg), the change in absorbance at 595nm in response to increasing amounts of protein is approximately linear. A standard curve was constructed by measuring absorbance at 595nm for known amounts of bovine serum albumin (BSA). For absorbances in the linear range of the standard curve, the protein concentration of samples was estimated by reading from the plotted graph the amount of protein that this absorbance equates to when using BSA as a standard. This was repeated using several different volumes or dilutions of the sample to obtain an average and a more accurate measurement of protein concentration. For absorbances outside the linear range of the standard curve, samples were appropriately diluted to try and obtain a reading within the linear range, which should give a more accurate estimation of protein concentration.

## 2.20 Silver staining

For very dilute protein samples, the use of Bradford's reagent for estimating protein concentration was unsuitable. Instead, the protein concentration of samples was estimated by running small volumes of sample on a SDS-polyacrylamide gel,

silver staining to visualise protein, and comparing the intensity of staining with that of known amounts of BSA run alongside the samples on the gel. This method of estimating protein concentration was identical to that used for bacterially-expressed recombinant proteins except that the polyacrylamide gel was stained with silver rather than Coomassie Blue. Silver staining is about 10-20 times more sensitive than using Coomassie Blue and enabled the detection of nanogram amounts of protein (detection limit ~2-5 ng per protein band).

To silver stain, first the gel was fixed by soaking in 250ml 50% methanol twice for 15 min. This was followed by a 15 min incubation in 5% methanol. The gel was then incubated in 250ml of deionised water containing 8µl of 1M DTT for 15 min. After washing briefly with deionised water twice, the gel was then gently agitated for 15 min in a freshly prepared 0.1% solution of silver nitrate. To remove excess silver nitrate solution, the gel was washed twice briefly with deionised water followed by two quick washes with a small amount of freshly made developing solution (3% (w/v) sodium carbonate, 0.5ml 37% formaldehyde per litre, in distilled, deionised water). The remainder of the developing solution (~200ml) was then added and incubated with the gel until the silver stained protein bands were of the desired intensity. Staining was stopped by pouring off most of the developing solution and sprinkling solid citric acid into the remaining solution containing the gel with swirling. Citric acid was slowly added until the fizzing ceased, a little water was added and the gel was gently agitated in this solution for 15 min. The silver stained gel was then washed three times for 15 min in water. The gel was soaked in 5% glycerol before drying.

## 2.21 Kinase assays

Protein fractions were assayed for the presence of particular kinases by incubating with micromolar amounts of specific peptide substrates and radiolabelled phosphate donor (2µCi $\gamma$-$^{32}$P ATP, 3000mCi/mmol) thereby enabling the detection of phosphorylated peptide. Phosphorylation reactions were allowed to proceed for 20 min at 30$^0$C and were stopped by pipetting samples onto 2cm$^2$ phosphocellulose paper discs (P81, Whatman). The discs were then immersed in ~200ml 75mM

phosphoric acid and incubated on a shaking platform for 5 min. This washing step in 75mM phosphoric acid, to remove unincorporated label, was repeated a further four times, followed by a 5 min wash with 70% ethanol. The discs were then left to air-dry and liquid scintillation counting was used to quantitate bound radioactivity.

## 2.22 Polymerase assays

Assays of RNA polymerisation activity were adapted from the method of Roeder (440). Reactions were carried out in a volume of 50µl in a final concentration of 6mM HEPES pH 7.9, 30mM KCl, 3.6mM $MgCl_2$, 6mM Tris pH 7.9, 200µM EDTA, 7.5mM ammonium sulphate, 800µM manganese chloride, 600µM of rATP, rCTP and rGTP, 50µM UTP, 0.6mM DTT and 5% glycerol. Each reaction also contained 5µg of poly(dA.dT) (2µl) as non-specific template, 20µg BSA, 10µCi [$\alpha$-$^{32}$P] UTP (400Ci/mmol), and up to 15µl of protein fraction. After 20 min incubation at 30$^0$C, reactions were stopped by pipetting samples onto 2cm$^2$ discs of Whatman DE51 paper. Discs were washed 6 x 5 min in 0.5M $Na_2HPO_4$, twice in distilled water, twice in 96% ethanol and finally once in ether. As for kinase assays, levels of incorporated radioactivity were measured in a scintillation counter.

## 2.23 HAT assays

HAT reactions were carried out in 1 x HAT buffer (50mM Tris pH 8.0, 5% glycerol, 0.1mM EDTA, 50mM KCl, 1mM DTT, 1mM PMSF) in a final reaction volume of 30µl containing 1µg of purified chicken core histones (1mg/ml) and 0.25µCi of $^{14}$C acetyl CoA. Reactions were initiated by the addition of protein sample and were incubated at 30$^0$C for 30 min. 10µl of 4 x protein sample buffer (25mM Tris pH 6.8, 2% SDS, 20% β-mercaptoethanol, 40% glycerol, 0.5% bromophenol blue) was added to stop the reactions. To resolve the core histones, samples were run on a 12% polyacylamide-SDS gel. The gel was fixed for 30 min and then impregnated with fluor by soaking in Amplify™ (Amersham) for 15-30

min. The gel was then dried and exposed to BIOMAX X-ray film (Kodak) at $-80^0$C for 2-4 days.

# Chapter 3.

# Partial purification of the mammalian RNA polymerase III general transcription factors TFIIIB and TFIIIC

## 3.1 Introduction

RNA polymerase III, with an aggregate molecular weight of 600-700 kDa and 16-17 different subunits, is the largest and most complex of the three nuclear RNA polymerases (83, 523, 554). Despite its complexity, in the absence of other proteins, the polymerase initiates transcription randomly (106, 188, 247, 555). Accurate and specific initiation of transcription requires the assistance of transcription factors that recruit the polymerase to the appropriate start sites of the appropriate sets of genes (405).

The transcription factors participate in highly specific protein-protein and protein-DNA interactions that are essential for the accurate recruitment of polymerase to the appropriate genes. For most genes, interaction is required between a transcription factor that specifically recognises the promoter elements and another transcription factor that specifically interacts with the appropriate polymerase. Although it is not inconceivable that the specificity of the three nuclear RNA polymerases for particular sets of genes could be achieved by utilising mutually exclusive, pol-specific domains of the same set of transcription factors, the utilisation of different transcription factors seems a much more attractive and simpler situation.

In 1980, Segall and co-workers demonstrated that the transcription factor requirements for specific pol II and pol III transcription are different (361, 473). Human cell-free extracts were fractionated on a phosphocellulose column and the ability of the various fractions, step-eluted with increasing concentrations of KCl, to reconstitute specific transcription of pol II and pol III templates was tested. No single fraction alone was able to reconstitute transcription of any pol II or pol III template tested. The PC-B fraction (0.1-0.35M KCl) in combination with the PC-C

fraction (0.35-0.6M KCl) effectively reconstituted transcription of tRNA and $VA_I$ genes (473). Reconstitution of pol II transcription required three of the four step-eluted fractions, the PC-A fraction (0.1M KCl), the PC-C fraction (0.35-0.6M KCl) and the PC-D fraction (0.6-1.0M KCl) (361).

The activities in the PC-B and PC-C fractions required to reconstitute specific transcription of tRNA and VA genes were designated TFIIIB and TFIIIC, respectively (473). The expression of many other class III templates is also supported by these two fractions, suggesting that the basal factor requirements are similar for many class III genes (75, 272, 487). TFIIIB and TFIIIC are thus regarded as general pol III transcription factors, unlike TFIIIA which elutes in the PC-A fraction and is specifically required for 5S gene transcription (134, 473).

The phosphocellulose fractions, resulting from the first step fractionation of cell-free extracts, are inevitably crude and it is clearly possible that the PC-B and PC-C fractions contain multiple factors that are required for pol III transcription. It is also worth noting that the activities in the PC-B and PC-C fractions that are required for one class III gene may be different for another class III gene. Reconstitution of pol II and pol III transcription both required the PC-C fraction, raising the possibility of a common factor (473). Further chromatography of this fraction on DEAE-cellulose separated the activities required for pol II and pol III transcription, lending support to the idea that there may be multiple transcription components in each of the PC fractions (473).

In the last twenty years there has been a considerable effort by a number of laboratories to purify TFIIIB and TFIIIC and determine their molecular composition. In the simple eukaryote *Saccharomyces cerevisiae*, a combined biochemical and genetic approach has enabled the identification of the polypeptide composition of both TFIIIB and TFIIIC. TFIIIB consists of three essential components, the TATA-binding protein (TBP) (264), the TFIIB-related factor (BRF) and a 90kD polypeptide, B″ (24, 261). The genes for TBP (77, 146, 185, 221, 225, 462), BRF (64, 101, 342) and B″ have all been cloned and TFIIIB activity has now been reconstituted using entirely recombinant subunits (266, 439, 452). Yeast TFIIIC consists of six subunits (23, 47, 156, 408, 505), which have also been cloned (325, 355, 407, 505).

The composition of TFIIIB and TFIIIC in higher eukaryotes is much less well defined. Two components of human TFIIIB have been unequivocally established; human homologues of yeast TBP and BRF (340, 371, 374, 484, 507, 552, 573). Very recently, a human homologue of yeast B″ has also been cloned (463). Furthermore, it has been shown to be required for pol III transcription in vitro and chromatin immunoprecipitations indicate that it associates with the U6 promoter in vivo (463). These results, and the fact that it is a homologue to an essential component of yeast TFIIIB, suggest that human B″ is a bona fide component of human TFIIIB. However, it is likely that human TFIIIB has additional subunits that have yet to be identified, these may be essential or may perform non-essential stimulatory functions. This may also be true for yeast TFIIIB, as that reconstituted from recombinant TBP, BRF and B″ is significantly less active than native yTFIIIB, suggesting there may be other unidentified subunits that are missing, or perhaps important post-translational modifications are lacking (266, 452).

hBRF was first identified as a potential component of hTFIIIB following its specific immunoprecipitation from partially purified TFIIIB fractions using anti-TBP antibodies (374, 552). Using this approach, a variety of other candidate TBP-associated factors (TAFs) were identified that may be part of TFIIIB (91, 340, 374, 507, 514). However, the functional significance of these TAFs is unclear. Moreover, a PC-B fraction contains at least two distinct TBP-containing complexes, TFIIIB and B-TFIID (524). The partially purified TFIIIB fractions used in these immunoprecipitations may have contained some B-TFIID. It has been established that the 172kD TAF described by Taggart *et al.* (507) as an essential component of TFIIIB is in fact the 170kD TAF that forms part of B-TFIID (371, 552). In addition, some of the TAFs appeared substoichiometric to TBP and hBRF. This may indicate that they are loosely associated or that they interact with TBP indirectly. The uncertainty as to the composition of hTFIIIB (434) is compounded by other evidence suggesting that it is a labile protein and also that the requirements may differ for different class III templates (91, 340, 365, 371, 463, 513-515).

In contrast to TFIIIB, which shows substantial conservation between yeast and humans (213, 316, 374, 552), hTFIIIC seems to have diverged significantly from its yeast counterpart (230, 231, 307, 331, 488). Fractionation of hTFIIIC on a variety of different columns splits it into two components, TFIIIC1 and TFIIIC2 (118, 607).

TFIIIC2 consists of five subunits, all of which have been cloned and partially characterised (230, 231, 291, 331, 488, 608). The composition of TFIIIC1 is unknown.

In yeast, where the pol III transcriptional apparatus is best characterised, there has been very little work carried out on the regulation of pol III transcription. The bulk of the regulatory studies have been in the mammalian system. Unfortunately, a lot of this work has, necessarily, utilised rather impure and ill-defined fractions as sources of the major pol III components.

In this chapter, I describe the partial purification of hTFIIIB and hTFIIIC for use in dissecting the role of these two essential transcription factors in the regulation of RNA pol III transcription. hTFIIIB and hTFIIIC were partially purified using a combination of conventional and affinity chromatography. I also describe the use of my partially purified TFIIIC fractions to investigate further recent reports suggesting that several components of TFIIIC2 possess intrinsic histone acetyltransferase (HAT) activity (230, 298).

## 3.2 Results

### 3.2.1 Screening of cation exchangers for effective separation of hTFIIIB and hTFIIIC

Since the early fractionations of Segall and co-workers (361, 473), chromatography on phosphocellulose has traditionally been used as the first fractionation step in any purification scheme of mammalian pol II or pol III transcription factors. To my knowledge, at least as far as the pol III transcription factors are concerned, the suitability of other columns as possible alternatives to phosphocellulose has not been investigated.

Although phosphocellulose is very effective at separating hTFIIIB and hTFIIIC, there are several disadvantages of using this particular resin compared to others. In addition to being time consuming, the need to activate the phosphocellulose the day prior to use, and to hand-pack the column, are two potential sources of considerable variation in the reproducibility of this chromatographic step. Indeed, inconsistency has been reported in the composition of the PC-B and PC-C fractions. TFIIIC1 elutes from phosphocellulose between 280 and 390 mM KCl (553). It can therefore be found both in the PC-B and PC-C fractions. The extent to which TFIIIC1 is localised in each of these fractions seems prone to variability.

Phosphocellulose is a strong cation exchanger. The stronger binding of TFIIIC than TFIIIB to phosphocellulose may reflect the high affinity of TFIIIC for A- and B-block DNA, whereas TFIIIB has little or no affinity for DNA. I decided to test the ability of some other cation exchangers for their effectiveness at separating TFIIIB and TFIIIC.

Figure 3.1 shows the results of fractionation of HeLa nuclear extract on a heparin-Sepharose gradient. Gradient chromatography was performed as the elution properties of TFIIIB and TFIIIC were unknown. Heparin belongs to a family of negatively charged polysaccharides called glycoaminoglycans. The disaccharide repeating unit of heparin has four negatively charged functional groups, one carboxylate and three sulphate groups. Like phosphocellulose, heparin functions as a strong cation exchanger. In addition, heparin has pseudo-affinity properties, as it mimics nucleic acids (468). Given that TFIIIC has DNA-binding properties whereas TFIIIB does not, this latter property of heparin potentially offered improved

resolution of TFIIIB and TFIIIC, compared with a cation exchanger separating solely by the criterion of charge.

Assaying of eluted fractions for TFIIIB activity and TFIIIC activity revealed that the recovery of active TFIIIB and TFIIIC is relatively good. However, there is a considerable overlap of the two activities (Fig 3.1, compare **A** & **B**). Indeed, all of the fractions containing TFIIIC activity, (fractions 48-58), also contained substantial TFIIIB activity (compare lanes 7-17 of Fig.3.1**B** with lanes 8-18 of Fig.3.1**A**). In addition, the peak of TFIIIB activity and the peak of TFIIIC activity closely cofractionated (fractions 50 & 51, compare lanes 10 & 11 of Fig.3.1**A** and lanes 9 & 10 of Fig.3.1**B**). I concluded that heparin-Sepharose, at least using the elution conditions that I adopted here, is not suitable as a replacement for phosphocellulose in the initial fractionation of HeLa nuclear extract.

A similar fractionation was obtained using a sulphopropyl-Sepharose (SP-) cation exhanger. This is another strong cation exchanger, the functional group is a negatively charged sulphate group that is attached to the matrix through a propyl chain (468). As with the heparin column, all the fractions with TFIIIC activity also contained TFIIIB activity (Fig. 3.2 **A** & **B**). Although recovery of TFIIIB activity is reasonable, very poor recovery of TFIIIC activity was obtained. The poor recovery of TFIIIC activity may simply reflect inactivation during the chromatographic procedure. Another possibility is that the two components of TFIIIC, TFIIIC1 and TFIIIC2, are separated by the gradient, as has previously been reported for a variety of different columns (118, 607). The few fractions that contained TFIIIC activity may represent the overlap between TFIIIC1-containing and TFIIIC2-containing fractions. To test the possibility that TFIIIC1 and TFIIIC2 fractionate differently, I tried combining fractions from different parts of the gradient to see if they would complement each other for TFIIIC activity. As shown in Figure 3.2 **C**, combining fractions from lower in the gradient than the fractions possessing TFIIIC activity, with fractions higher in the gradient, resulted in a synergistic increase in TFIIIC activity. This synergy suggests that TFIIIC is split into at least two essential components during gradient chromatography on SP-Sepharose.

I also considered using the weak cation exchanger carboxylmethyl (CM) for the initial fractionation of HeLa nuclear extract. However, the low binding capacity of this ion exchanger renders it much more suitable for a later stage in the purification scheme.

**Figure 3.1**

**Fractionation of TFIIIB activity and TFIIIC activity on a heparin gradient**

15mg of HeLa nuclear extract was applied to a 1ml heparin-Sepharose column. After washing with 10 column volumes of Hep buffer containing 100mM KCl (Hep100), fractions were eluted with a 100mM-1M linear KCl gradient, generated by appropriate mixing of Hep100 and Hep buffer containing 1M KCl (Hep1000). Eluted fractions were tested for the presence of TFIIIB or TFIIIC by in vitro transcription assays.

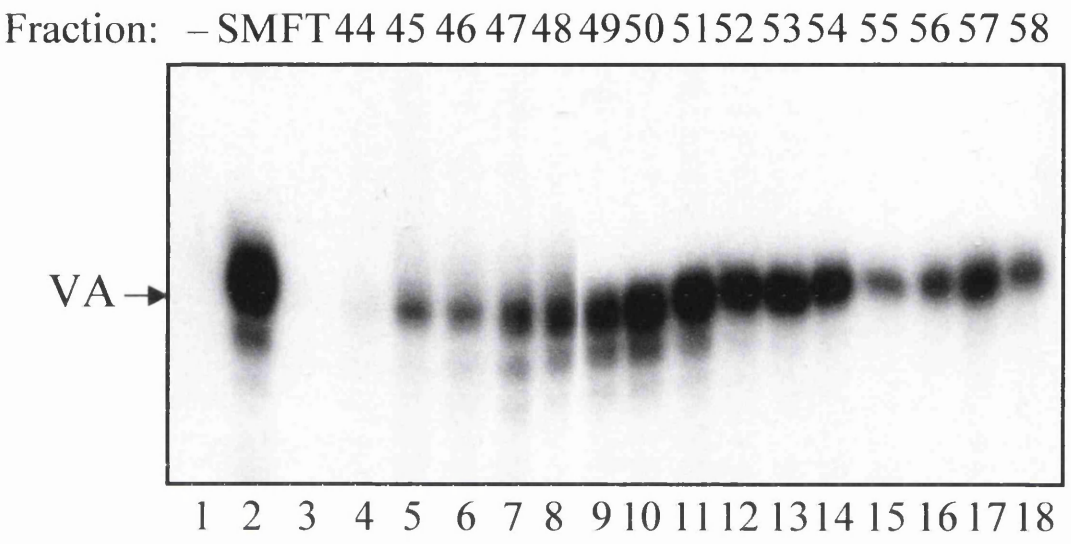
**A)** TFIIIB activity of eluted fractions.

Fraction numbers are indicated. SM, starting material; FT, flowthrough. TFIIIB activity was assayed using 4μl of the indicated fraction, 2μl of PC-C and 250 ng of $pVA_I$; after 15 min incubation at $30^0$C, nucleotides were added to initiate transcription.

**B)** TFIIIC activity of eluted fractions.

Transcription reactions were performed as described in **(A)**,except 2μl of PC-B was substituted for PC-C as the complementing fraction.

**A**


Fraction: – SM FT 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

**B**

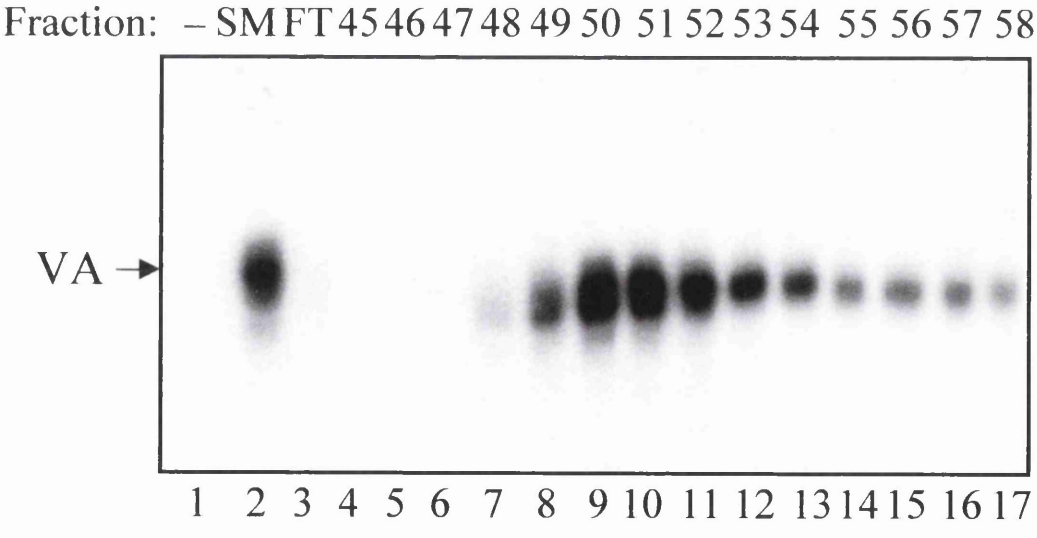

Fraction: – SM FT 45 46 47 48 49 50 51 52 53 54 55 56 57 58
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

**Figure 3.2**

**Fractionation of TFIIIB activity and TFIIIC activity on SP-Sepharose**

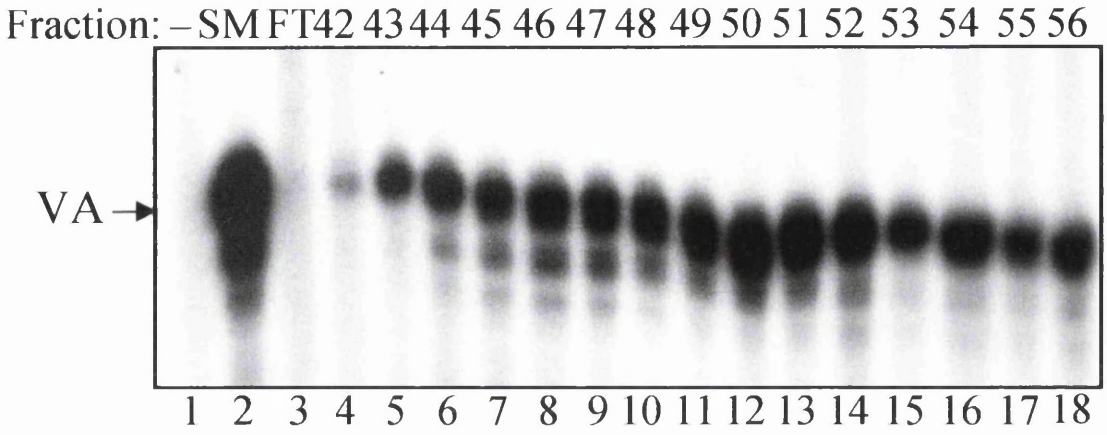
15mg of HeLa nuclear extract was applied to a 1ml SP-Sepharose column. After washing with 10 column volumes of 100mM KCl buffer (Hep 100, as in Fig.3.1), fractions were eluted with 6 column volumes of a 100mM-1M linear KCl gradient. Eluted fractions were tested for the presence of TFIIIB or TFIIIC by in vitro transcription assays.

**A)** TFIIIB activity of eluted fractions.
Fraction numbers are indicated. SM, starting material; FT, flowthrough. Lane 1 of **A**, **B** and **C**; and lane 9 of **C** contain LDB buffer instead of a fraction. TFIIIB activity was assayed using 4μl of the indicated fraction, 2μl of PC-C and 250 ng of $pVA_I$; after 15 min incubation at $30^0$C, nucleotides were added to initiate transcription.

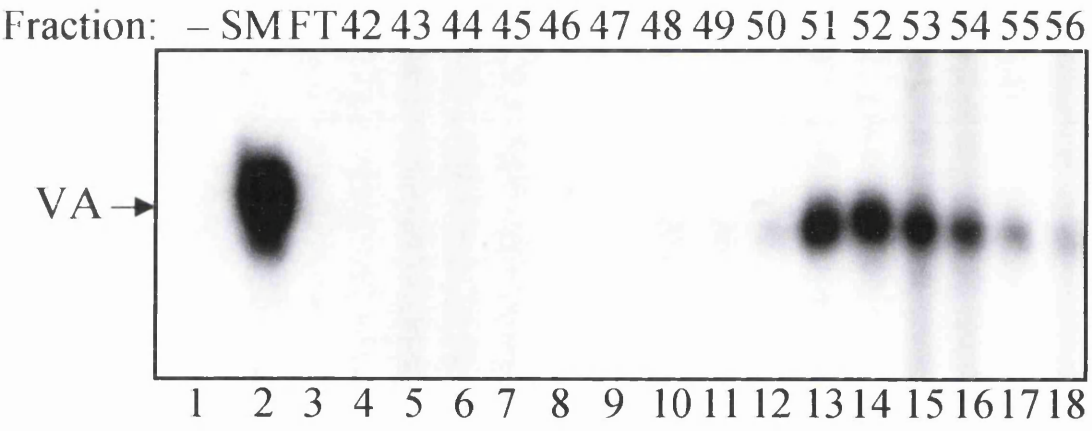
**B)** TFIIIC activity of eluted fractions.
Transcription reactions were performed as described in **(A)**,except 2μl of PC-B was substituted for PC-C as the complementing fraction.

**C)** Synergism of fractions for TFIIIC activity.
Transcription reactions were performed essentially as described in **(A)**. As in **(B)**, a PC-B fraction was substituted for PC-C as the complementing fraction. In lanes 4 and 7, 2μl each of the two indicated fractions was added; in all the other lanes 4μl of the indicated fraction was added.

**A**


Fraction: – SM FT 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

**B**


Fraction: – SM FT 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

**C**

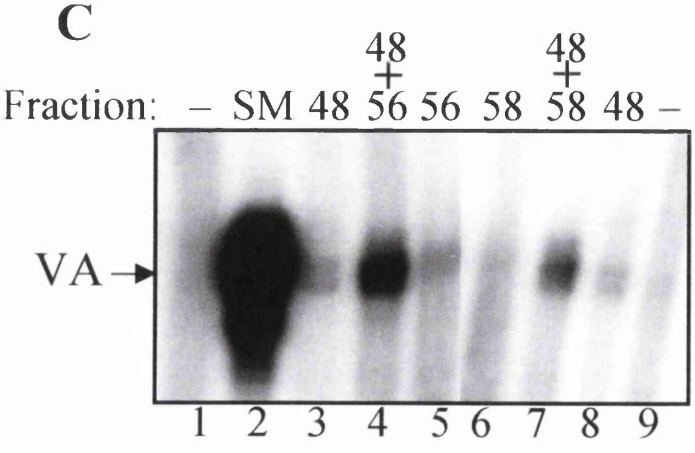

48 + 56
48 + 58
Fraction: – SM 48 56 56 58 58 48 –
VA
1 2 3 4 5 6 7 8 9

Figure 3.3 shows the results of step elution chromatography on phosphocellulose. Step elution could be performed as the elution properties of TFIIIB and TFIIIC had previously been determined. Compared to gradient elution, this potentially offers increased rapidity of purification and concentration of samples, but reduced resolution. The in vitro transcription assays revealed that there is very good recovery of TFIIIB activity and TFIIIC activity. In contrast to the heparin and SP columns, there is complete resolution of TFIIIB activity and TFIIIC activity (Fig.3.3, compare **A** & **B**). The recovery of activity and resolution of TFIIIB and TFIIIC was consistent between different chromatographic runs (data not shown). I therefore concluded that of the cation exchangers tested, for the initial fractionation of HeLa nuclear extract, phosphocellulose is the column of choice, both in terms of resolution and recovery of activity.

### 3.2.2 Further purification of TFIIIB from PC-B

At each step of a purification scheme there are inevitable cumulative losses, both in terms of the amount of the protein of interest that is recovered and its activity. This limits the number of chromatographic steps that can be employed and requires a compromise between resolution and the yield and activity of the protein. The optimisation of each chromatographic step is paramount to obtaining reasonable quantities of active, relatively pure protein.

Although knowledge of the pI and molecular size of the protein of interest can provide certain information as to the behaviour of the protein on a particular column, the effectiveness of particular columns for purification can be determined largely only by trial and error (468).

I tested a variety of different column types for their effectiveness for the purification of TFIIIB from PC-B for potential use in a final purification scheme, and for cofractionation studies. The choice of columns to test was based on the need to combine columns that fractionate by different criteria in order to obtain optimal purification in the minimal number of steps, and information from previously reported attempts at purification of human TFIIIB.

Figure 3.4 **A** shows the results of step elution chromatography of PC-B on Cibacron blue 3G-A-Sepharose. Cibacron blue 3G-A is a blue dye adsorbent with

**Figure 3.3**

**Fractionation of TFIIIB activity and TFIIIC activity on phosphocellulose**

HeLa nuclear extract was applied to a column of activated phosphocellulose at a final concentration of 5mg/ml of resin. The column was extensively washed with PC-A buffer containing 100mM KCl (PC-A(0.1)), until absorbance at 280nm of eluting fractions returned to zero. TFIIIB-containing fractions were eluted with PC-A buffer containing 350mM KCl (PC-A(0.35)). TFIIIC-containing fractions were step eluted using PC-A buffer containing 600mM KCl (PC-A(0.6)). After dialysis of eluted fractions against LDBO buffer, until the salt concentration returned to 100 mM KCl, fractions were tested for the presence of TFIIIB or TFIIIC by in vitro transcription assays.

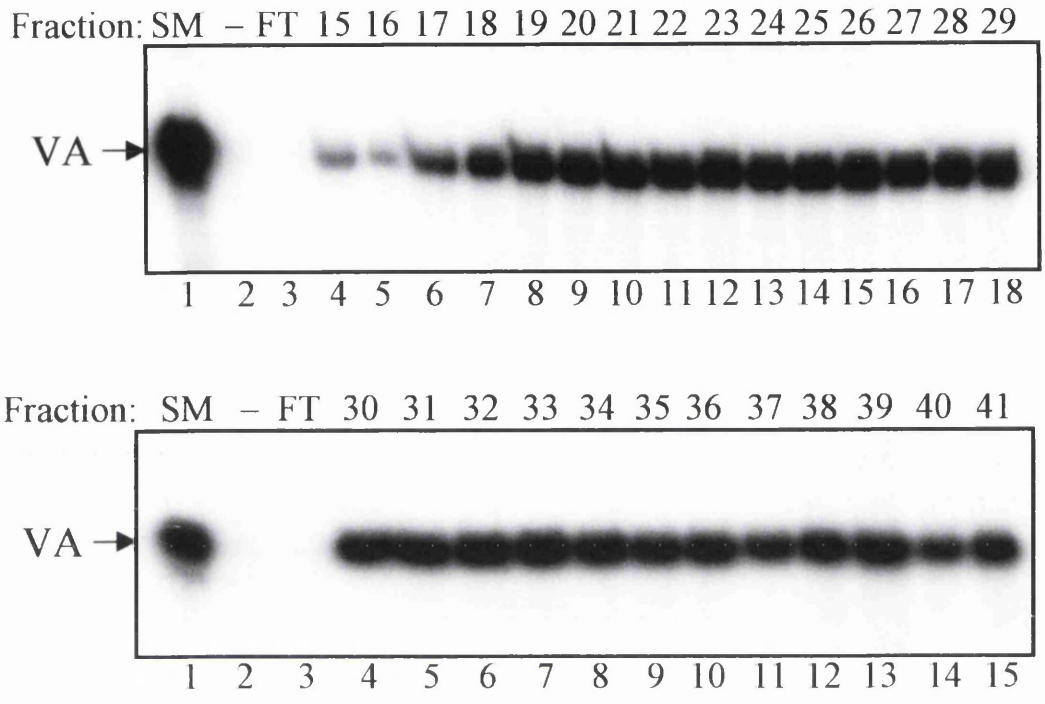
**A)** TFIIIB activity of eluted fractions.

Fraction numbers are indicated. SM, starting material; FT, flowthrough. TFIIIB activity was assayed using 4μl of the indicated fraction, 2μl of PC-C and 250 ng of $pVA_I$; after 15 min incubation at $30^0$C, nucleotides were added to initiate transcription.

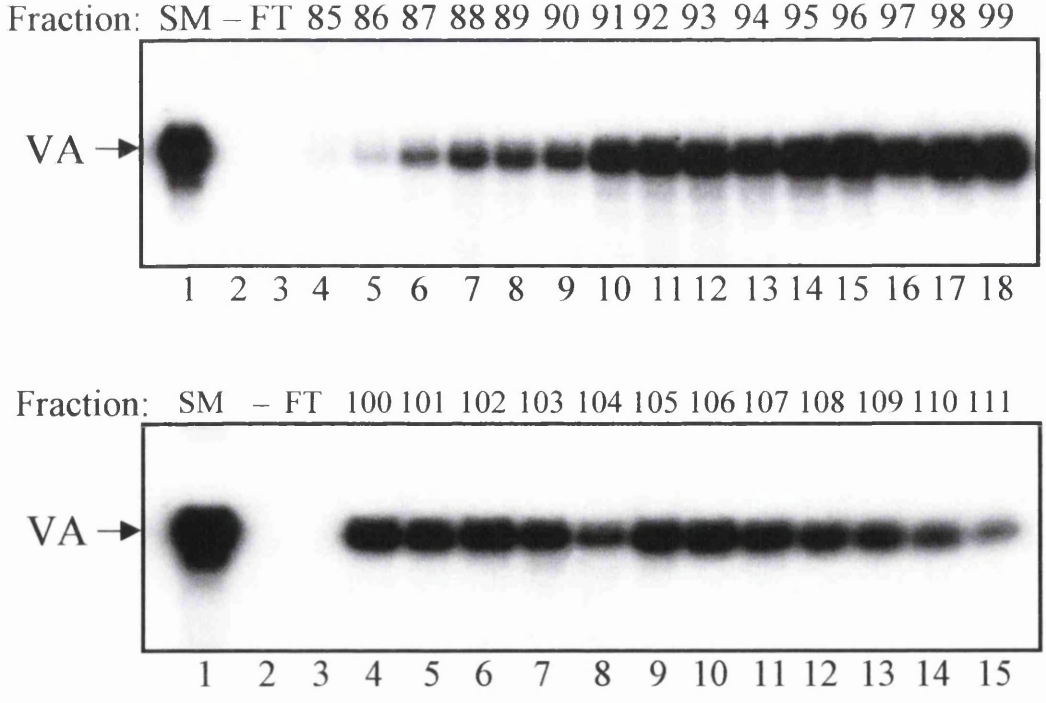
**B)** TFIIIC activity of eluted fractions.

Transcription reactions were performed as described in **(A)**,except 2μl of PC-B was substituted for PC-C as the complementing fraction.

**A**


Fraction: SM – FT 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Fraction: SM – FT 30 31 32 33 34 35 36 37 38 39 40 41
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

**B**


Fraction: SM – FT 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Fraction: SM – FT 100 101 102 103 104 105 106 107 108 109 110 111
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

pseudo-affinity properties. The dye behaves as an analogue of ADP-ribose and has affinity for most proteins that bind a purine nucleotide. The specificity of Cibacron blue is not limited to nucleotide-binding proteins, however. The dye will bind a large number of non nucleotide-binding proteins; interferon and human serum albumin were both purified with the aid of Cibacron blue chromatography. The binding of proteins to Cibacron blue is poorly understood; however, from the structure of the dye, hydrophobic and electrostatic interactions, and hydrogen bonding, are all likely (468).

Eluted fractions from the Cibacron blue column were tested for TFIIIB activity by in vitro transcription assay. Two different templates were separately tested; $VA_I$ and the U6 promoter fused to the β-globin gene (Fig.3.4 **A**). The U6 promoter is a much weaker class III promoter than $VA_I$, hence the lower transcript levels obtained with starting material using the U6 promoter compared to $VA_I$ (compare lane 2 of upper and lower panel, Fig.3.4 **A**). TFIIIB activity capable of supporting U6 transcription eluted in the 2M salt/5M urea fractions, with peak activity approximately half that of the starting material (Fig.3.4 **A**, upper panel, compare lane 2 and lanes 16 &17). A very low level of activity is detectable in the flowthrough and the fractions eluted with 1M salt (upper panel, lanes 3-11).

In contrast to the results obtained with the U6 promoter, no TFIIIB activity capable of supporting $VA_I$ transcription was detectable in any of the eluted fractions (lower panel, lanes 3-18). This suggests that the TFIIIB requirements for $VA_I$ transcription differ from those for U6 transcription. This finding is in general agreement with a number of studies suggesting that the TFIIIB requirement of type III promoters is different from that of types I and II (340, 365, 374, 463, 513, 514, 552, 604).

Teichmann *et al.* also found that the human TFIIIB requirements for U6 and $VA_I$ transcription can be physically separated on Cibacron blue (514). In contrast to my results, Teichmann *et al.* found that TFIIIB activity capable of supporting $VA_I$ transcription was detectable in the 2M salt/5M urea fractions. TFIIIB activity capable of supporting U6 transcription was detected in largest amounts in the 600mM NaCl elution, but significant activity was also detected in the 60mM NaCl flowthrough and 2M NaCl/5M urea elution (514). The presence of TFIIIB activity supporting U6 transcription in all these fractions implies that the column was

overloaded in Teichmann's study. The lack of TFIIIB activity for $VA_I$ transcription in my 2M NaCl/5M urea fractions could be explained by partial disruption of this form of TFIIIB by the 1M NaCl elution I adopted compared with Teichmann's 0.6M NaCl elution. Waldshmidt *et al.* were able to obtain TFIIIB activity capable of reconstituting tRNA transcription in their 2M salt/5M urea fractions after an elution with 1M NaCl, however (545).

Another significant difference between the results I obtained and those of Teichmann *et al.* and Waldshmidt *et al.* is in the fractionation of the bulk of the protein. Teichmann *et al.* and Waldshmidt *et al.* found that the bulk of the protein eluted in the flowthrough and 0.6M elutions, and the 1M elution, respectively (514, 545). The stronger binding of TFIIIB to Cibacron blue than the bulk of the protein and the increase in purity that this offers was the main reason for my considering Cibacron blue chromatography. I found that the bulk of the protein was retained on the column after elution with 1M NaCl and eluted with 2M salt/5M urea (data not shown). This discrepancy may be explained by the source of the Cibacron blue column used. Teichmann *et al.* (Teichmann, M., personal communication) and Waldshmidt *et al.* both used Cibacron blue-agarose columns purchased from Sigma, whereas the Cibacron blue column I used was purchased from Pharmacia Biotech. The Cibacron blue dye used by manufacturers is sometimes not very pure and may contain a variety of minor components or isomers of the main component (191). Variations in purity can have quantitative and qualitative effects on protein binding (468). The degree of substitution of dye can also have a significant effect on protein binding.

Teichmann *et al.* suggest that the TFIIIB required for U6 transcription is a subcomplex of the TFIIIB required for $VA_I$ transcription. By Cibacron blue chromatography, they were able to generate TFIIIB capable of supporting U6 transcription from a TFIIIB fraction previously shown to be capable of supporting $VA_I$ transcription but not that of U6 (514). Another possible explanation for the lack of TFIIIB activity for $VA_I$ transcription that I observed, is the disruption of the complex with the dissociation of the TFIIIB subcomplex sufficient for U6 transcription and the retention on the column of one or more components required for $VA_I$ transcription.

Although I have not attempted harsher elution conditions to investigate further this possibility, I have tested the effect of eluting with 0.6M NaCl rather than

1M NaCl. When I eluted with 0.6M NaCl, I was able to recover low levels of TFIIIB activity for $VA_I$ transcription in the 2M NaCl/5M urea fractions (data not shown). This suggests that washing with 1M NaCl rather than 0.6M NaCl causes some disruption of the TFIIIB complex required for $VA_I$ transcription.

The fractionation of HeLa nuclear extract on heparin-Sepharose yielded reasonable quantities of active TFIIIB. I therefore decided to test whether this was also true using PC-B as the starting material. The subsequent use of the partially purified TFIIIB in regulatory studies makes recovery of reasonably active protein at this stage of purification an important requirement. Following application of the sample and washing to remove unbound protein, bound protein was eluted with a 3 column volume 100mM-1M salt gradient. The TFIIIB activity of eluted fractions is shown in Figure 3.4 **B**. As with the nuclear extract, reasonable TFIIIB activity was recovered. A steeper gradient was applied than previously to keep the samples concentrated and thus less prone to inactivation.

The weak cation exchanger carboxymethyl, which is based on charged carboxylate groups, was also tested. Previous work has shown that, under certain conditions, TFIIIB does not bind the column and can be collected in the flowthrough whereas pol III and any contaminating TFIIIC bind to the column (White, R.J., personal communication). I was consistently able only to recover fractions with extremely low TFIIIB activity, however (data not shown). This may reflect inactivation of the TFIIIB caused by its inevitable dilution resulting from its elution in the flowthrough.

One of the columns Waldshmidt *et al.* used in their purification of human TFIIIB was hydroxyapatite (545). Hydroxyapatite ($(Ca_5(PO_4)_3OH)_2$) is an inorganic adsorbent made of a crystalline form of calcium hydroxide phosphate. The mechanism of adsorption of proteins to hydroxyapatite is not properly understood. It has been postulated that dipole-dipole bonding may be involved; however, the adsorption of buffer ions to the hydroxyapatite complicates matters and interactions may more resemble those of ion exchangers (468).

Figure 3.4 **C** shows the results of gradient chromatography of PC-B on hydroxyapatite. A 10mM-1M potassium phosphate (pH 7.0) gradient was applied over six column volumes to elute bound protein. TFIIIB activity eluted relatively early in the gradient, between 50mM and 250mM potassium phosphate (pH 7.0), reducing time required for purification and dialysis, which meant samples were left

**Figure 3.4 - Screening of resins for the further purification of TFIIIB**

**A) Cibacron blue chromatography of a PC-B fraction**

1mg of PC-B was applied to a 1ml Cibacron blue column. After extensively washing with CB buffer containing 100mM NaCl (CB100), bound protein was step eluted with CB buffer containing 1M NaCl (CB1000), followed by a second step elution with CB buffer containing 2M NaCl and 5M urea (CB2000U). Eluted fractions were assayed for TFIIIB activity by in vitro transcription.

Upper panel - Fraction numbers are indicated. SM, starting material; FT, flowthrough. TFIIIB activity was assayed using 4µl of the indicated fraction, 2µl of PC-C and 500 ng of pU6/Hae/RA.2; after 15 min incubation at $30^0$C, nucleotides were added to initiate transcription.

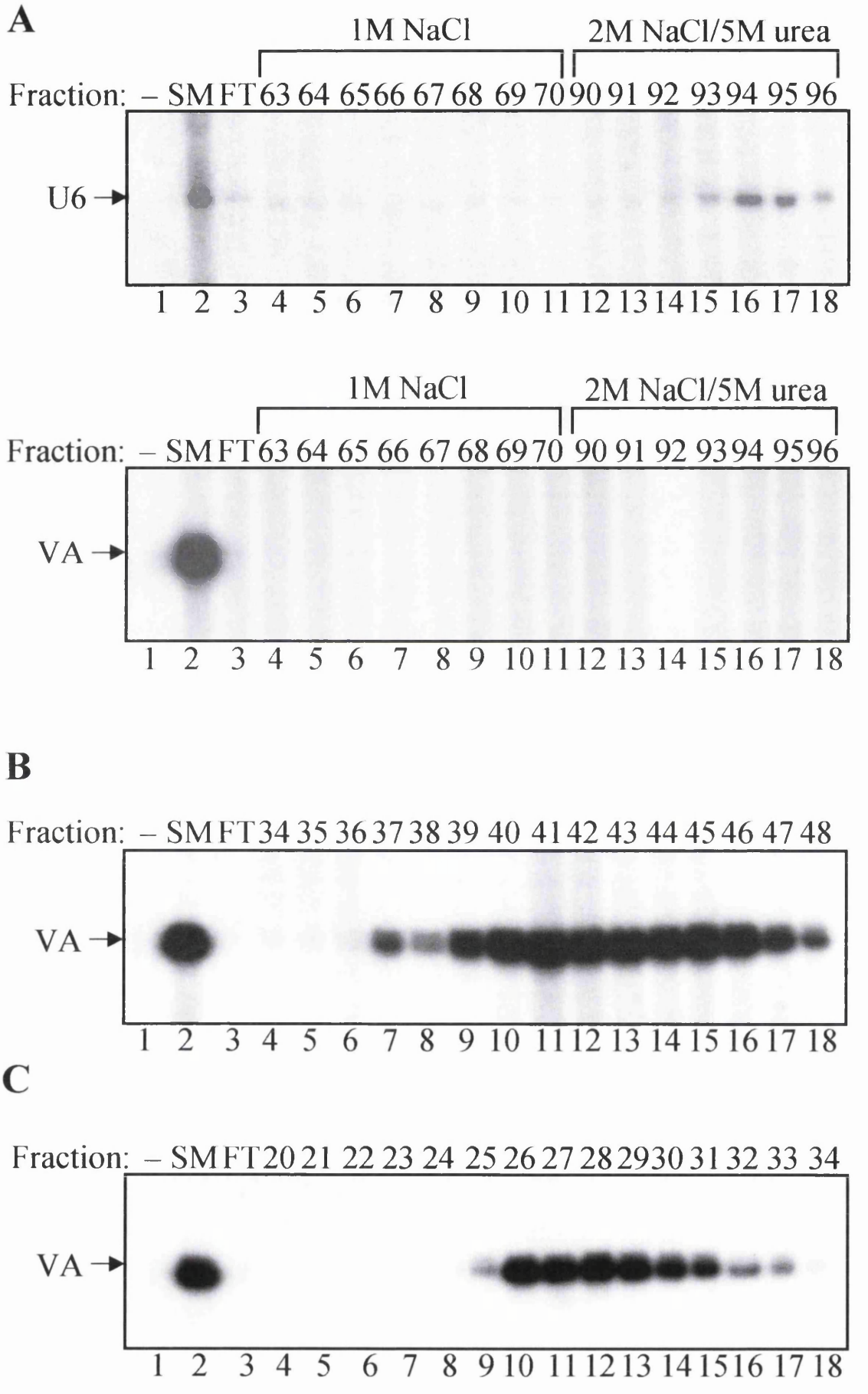
Lower panel - Transcription reactions were performed as described for the upper panel except 250ng of $pVA_I$ replaced pU6/Hae/RA.2 as the pol III template added.

**B) Heparin gradient chromatography of a PC-B fraction**

4 mg of PC-B was applied to a 1ml heparin-Sepharose column. After extensive washing, fractions were eluted with a 100mM-1M linear KCl gradient. Eluted fractions were tested for TFIIIB activity by in vitro transcription assay. Transcription was carried out as described in **(A)** using 250 ng of $pVA_I$ as template.

**C) Hydroxyapatite chromatography of a PC-B fraction**

1mg of PC-B was applied to a 1ml hydroxyapatite column. After extensive washing with HAP buffer containing 10mM potassium phosphate pH7.0 (HAP10), a 10mM-500mM potassium phosphate pH 7.0 gradient was applied. Eluted fractions were tested for TFIIIB activity by in vitro transcription assay as described in **(B)**.


A
1M NaCl
2M NaCl/5M urea
Fraction: – SM FT 63 64 65 66 67 68 69 70 90 91 92 93 94 95 96
U6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
1M NaCl
2M NaCl/5M urea
Fraction: – SM FT 63 64 65 66 67 68 69 70 90 91 92 93 94 95 96
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
Fraction: – SM FT 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
C
Fraction: – SM FT 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

at $4^0$C susceptible to inactivation and degradation for less time. As the in vitro transcription of Figure 3.4 **C** shows, good recovery of TFIIIB activity was obtained, although Bradford's assay of eluted protein revealed that a lot of protein was eluted in similar fractions to TFIIIB, reducing the extent of purification obtained.

### 3.2.3 Verification of antibodies for use in immunoaffinity chromatography

Immunoaffinity chromatography separates the protein of interest from contaminating proteins on the basis of a biospecific interaction between the protein of interest and an antibody that has been immunologically raised against a specific part of that protein. The biospecificity of this technique provides the opportunity to achieve fold purification in a single step that cannot be achieved with four or five steps of conventional chromatography. It is possible to achieve > 90% purity from crude extract in a single immunoaffinity step. The success (or failure) of immunoaffinity chromatography is critically dependent on the quality of the antibodies used. The antibody is the ultimate adsorbent provided that it is highly selective for the protein of interest and that it binds the protein tightly but reversibly. Before embarking on immunoaffinity chromatography, it is crucial to verify that the antibodies to be used do indeed recognise the proteins that they were raised against and that they do not react with a host of other proteins.

The recent identification and cloning of several components of human TFIIIB and human TFIIIC has provided the opportunity to carry out immunoaffinity chromatography as a means of purification, which was previously not possible due to a lack of knowledge as to the composition of these two proteins. It enables the raising of antibodies against specific components of hTFIIIB and hTFIIIC that can be used for the immunoaffinity purification of these two activities.

We have three different BRF antibodies, 128, 482 and 330, each raised against a different C-terminal region of the human protein. 128 was raised against residues 533-547 of human BRF. This sequence has been strongly conserved in evolution and corresponds to part of the yeast homology region III, one of three regions of strong conservation within a diverged C-terminus. Yeast homology region III was identified as a sequence of strong conservation following the sequence comparison of BRF from three distant yeast species (271). The subsequent cloning

of BRF from *C.elegans* and humans revealed that this region is strongly conserved in these organisms also (316). 482 was raised against residues 452-466 of human BRF, which corresponds to part of yeast homology region II. 330 was raised against residues 664-677 of human BRF, which corresponds to the extreme C-terminus of the protein. This part of the protein is not conserved between humans and *C.elegans* and is absent in the yeast BRFs (316). We also have an antibody against the TFIIICβ subunit (TFIIIC110) of TFIIIC2. This antibody, 4286, was raised against residues 897-911 of human TFIIICβ.

All of the aforementioned antibodies are rabbit polyclonals. Antibodies are raised by injecting the antigen into the animal at regular intervals and waiting for an immune reponse before bleeding the animal. It is essential that the antigen injected is as pure as possible as trace amounts of other proteins may be much more antigenic. Furthermore, approximately 90% of the antibodies in a rabbit antiserum are not specific to the antigen injected but represent immunoglobins that happened to be present in the animal at the time. The verification of the antibodies is thus absolutely essential.

The 4286 antiserum specifically recognises a 110 kDa polypeptide, which corresponds to the size of TFIIICβ, both in a crude nuclear extract and in a PC-C fraction, as revealed by Western blotting (Figure 3.5). The presence of a few weaker bands of slightly smaller size in the lane of nuclear extract (lane 1) may represent TFIIICβ that has been partially degraded or modified in some way. Five times as much protein was loaded in lane 1 compared to lane 2, yet the difference in intensity of the 110 kDa band obtained is less than two-fold. The PC-C fraction is clearly enriched for the 110 kDa polypeptide specifically recognised by this antiserum, as would be expected for TFIIICβ. This suggests that the 110 kDa band seen in the lane of nuclear extract is indeed TFIIICβ and not another polypeptide of the same size that the antibody has cross-reacted with.

The TFIIICβ antiserum is also able to specifically immunoprecipitate TFIIIC2 from HeLa nuclear extract, of which TFIIICβ is one of five subunits. Immunoblotting of precipitated material with an antiserum against the TFIIIC220 (TFIIICα) subunit of TFIIIC2, Ab4 (481), revealed that the 4286 antiserum specifically coimmunoprecipitated TFIIIC220, whereas the corresponding

**Figure 3.5**

**The 4286 antiserum specifically recognises TFIIICβ**

75μg of HeLa nuclear extract (lane1) and 15μg of PC-C (lane 2) were resolved on a SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting using the 4286 antiserum.

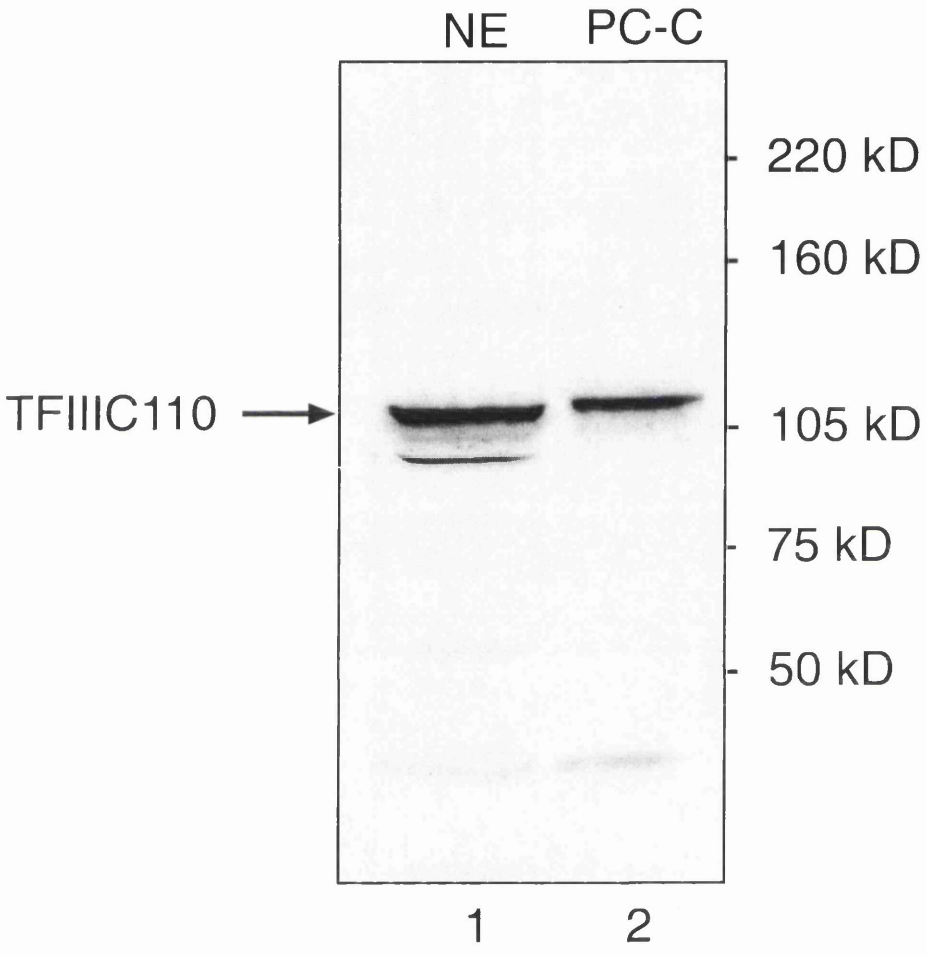
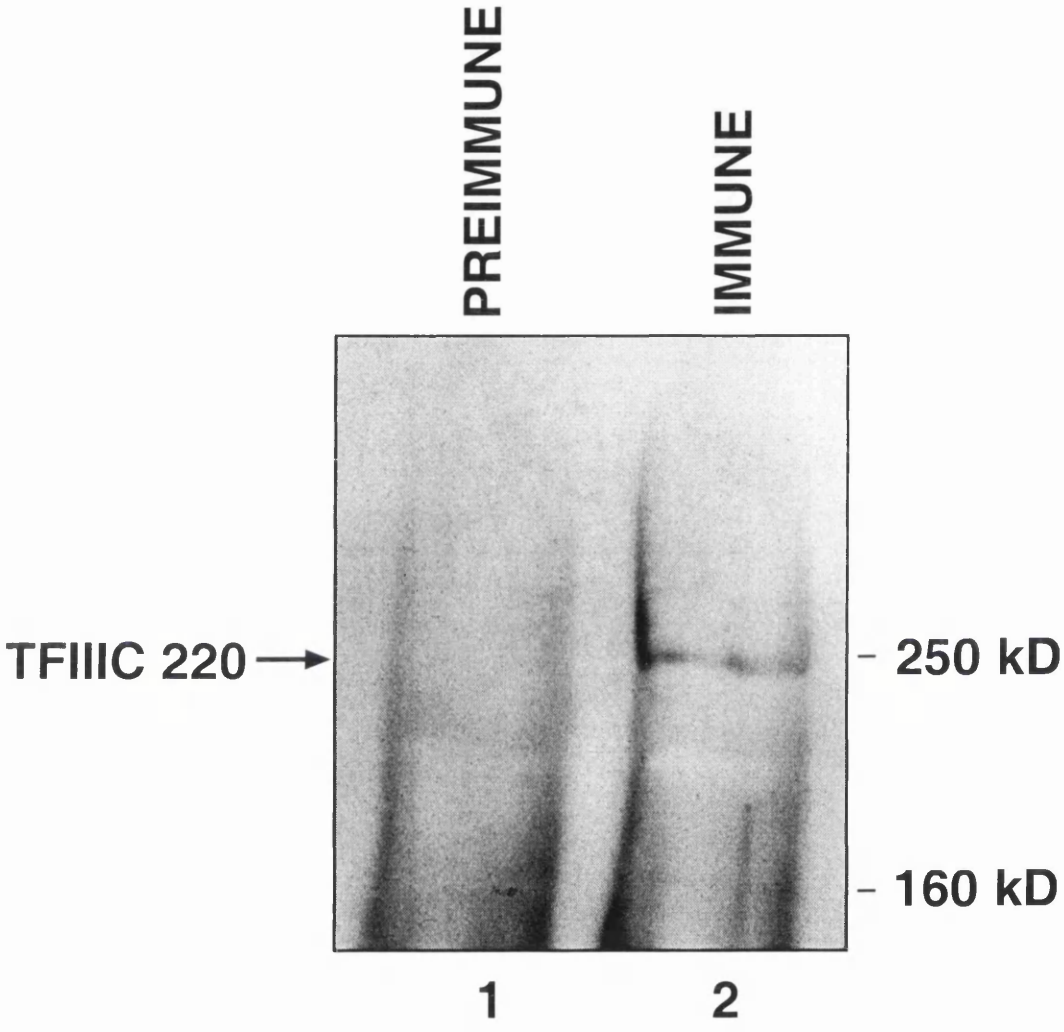

NE
PC-C
220 kD
160 kD
TFIIIC110
105 kD
75 kD
50 kD
1
2

**Figure 3.6**

**The 4286 antiserum specifically immunoprecipitates TFIIIC2 from crude extract**

HeLa nuclear extract (150μg) was immunoprecipitated using the 4286 antiserum (lane 2) or the corresponding preimmune serum (lane 1). After washing five times with 500μl of LDB buffer, immunoprecipitated material was resolved on a SDS-7.8% polyacrylamide gel and then analysed by immunoblotting using the Ab4 antiserum against the TFIIIC220 subunit of TFIIIC2.


PREIMMUNE
IMMUNE
TFIIIC 220
250 kD
160 kD
1
2

preimmune serum did not (Figure 3.6). This suggests that 4286 can immunoprecipitate the native TFIIIC2 complex.

The three BRF antisera specifically recognise a 90 kDa polypeptide corresponding in size to human BRF, both in crude nuclear extract and in PC-B fractions, as revealed by immunoblotting (data not shown). They also specifically immunoprecipitate $^{35}$S-labelled in vitro translated human BRF from rabbit reticulocyte lysate, whereas the corresponding pre-immune sera do not (Figure 3.7 and data not shown).

Figure 3.7 also shows the results of peptide elution experiments for 128 and 482, in which I tested whether synthetic peptide corresponding to the sequence of BRF that these two antibodies were raised against could be used for elution of BRF from the antibody. Antibodies generally bind very tightly to their antigen and elution of the protein of interest from the antibody without requiring harsh conditions that inactivate the protein or destroy the antibody can prove difficult. Peptide elution, which acts by displacement competition between the peptide and the protein of interest for the antigen-binding site on the antibody, offers the best solution to this problem, if it is possible. With peptide elution the protein of interest can be gently eluted in a buffer of one's choice.

As Figure 3.7 shows, the peptide specific for 482 can achieve efficient elution of immunoprecipitated BRF after only 30 min of incubation with the 482-immunoprecipitated material. In contrast, BRF immunoprecipitated with 128 could not be eluted with the synthetic peptide the antibody was raised against, even after 5 hours of incubation together.

The BRF antisera were also tested for their ability to immunoprecipitate endogenous BRF by immunodepletion experiments using HeLa nuclear extract. Immunodepleted extracts were tested for their ability to support in vitro transcription of the $VA_I$ template in the absence of any other added proteins. Given that BRF is essential for $VA_I$ transcription, effective immunodepletion by the antisera should cause a sharp reduction in transcript levels. Extracts immunodepleted using 128 and 330 were able to support only very low levels of $VA_I$ transcription compared to their corresponding pre-immune sera (Figure 3.8, lanes 11-14). In contrast, extract immunodepleted with 482 supported levels of transcription comparable to that of extract immunodepleted with the preimmune sera (Figure 3.8, lanes 9 & 10). This suggested that the 482 antiserum is unable to immunoprecipitate endogenous BRF,

although it clearly is able to immunoprecipitate exogenous in vitro translated BRF (Figure 3.7). It may be that the epitope on BRF that 482 specifically recognises is involved in an endogenous interaction of BRF with other proteins and thus in a cell extract it is buried. In rabbit reticulocyte lysate, however, the in vitro translated human BRF should be relatively free of interactions with other proteins. The compatibility of human BRF and rabbit TFIIIB components is not known; however, regardless of this, the human BRF is likely to be in vast excess of proteins that potentially could interact. The BRF epitope in this environment should be exposed and thus can be specifically recognised by the 482 antiserum.

The possibility that 482 specifically recognises an epitope on BRF that is involved in protein-protein interaction is supported by the strong conservation of the sequence that the antibody was raised against between five disparate species (316). Conservation of sequence is suggestive of its involvement in protein-protein interactions. The sequence that 482 was raised against corresponds to part of the yeast homology domain II. This domain is implicated by a number of studies to be involved in the binding of TBP (11, 102, 265, 271).

As a positive control for the immunodepletion experiments, MTBP6 was used (Fig 3.8, lanes 1-3). This is a mouse monoclonal antibody that specifically recognises a sequence in the non-conserved N-terminal region of TBP. The presence of TBP in pol I- and pol II- specific complexes as well as TFIIIB restricts its usefulness in the immunopurification of TFIIIB compared with a TFIIIB-specific component such as BRF. As Figure 3.8 shows, MTBP6 is very effective at immunodepleting extracts of TFIIIB activity. Transcription can be restored by the addition of PC-B but not PC-C. MTBP6 also specifically coimmunodepletes BRF with the TBP, as revealed by Western blotting of immunodepleted extracts (data not shown).

### 3.2.4 Immunoaffinity purification of human TFIIIB

Having discarded 482 on the basis of its inability to immunoprecipitate endogenous BRF, the next stage was the application of the determined immunospecificity of 128 and 330 to column chromatography. Initially I kept

**Figure 3.7**

**128 and 482 antisera specifically immunoprecipitate BRF**

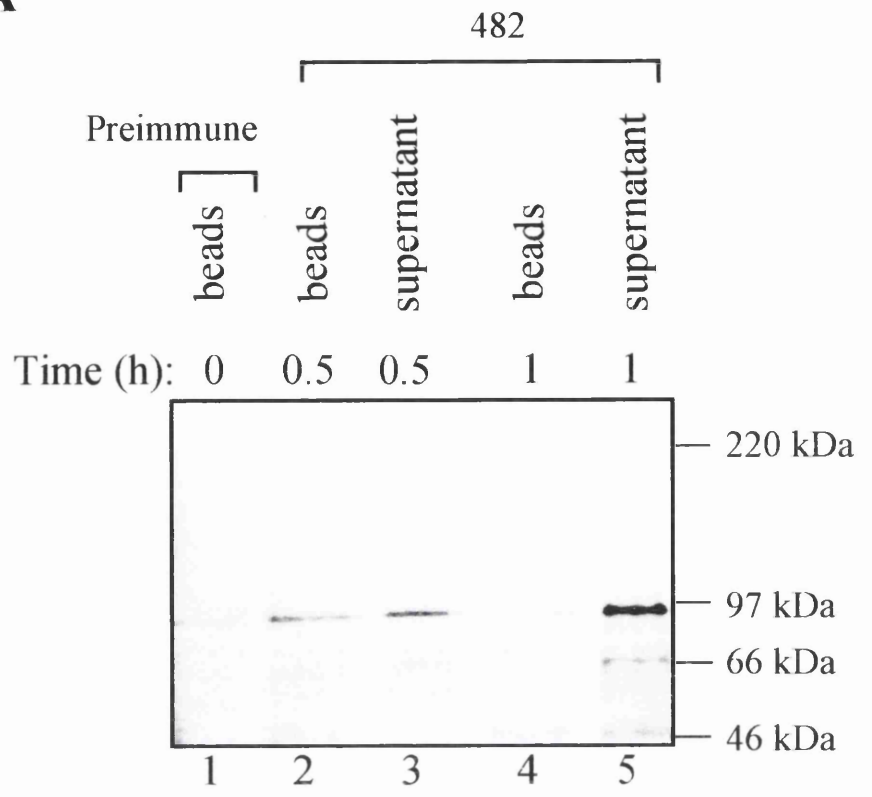
**A) Time course of peptide elution of in vitro translated BRF specifically immunoprecipitated with 482 antiserum**

Reticulocyte lysate (20µl) containing in vitro translated BRF was immunoprecipitated using equal amounts of the anti-BRF antibody 482 (lanes 2-5) or the corresponding preimmune serum (lane 1) prebound to protein A-Sepharose. After washing with LDB buffer to remove unbound protein and unincorporated label, 20µl of 2.5mg/ml BRF-2 peptide (corresponding to the peptide sequence 482 antiserum was raised against) was added. Immunoprecipitated material and BRF-2 peptide were incubated on an orbital shaker for 0 h (lane1), 0.5 h (lanes 2 & 3) or 1h (lanes 4 & 5) at $4^0$C. Supernatants were removed and the protein A-Sepharose beads were further washed five times with LDB buffer. Protein eluted into the supernatant and immunoprecipitated material retained on the protein A-Sepharose was resolved on a 7.8% SDS-polyacrylamide gel and then visualised by autoradiography.

**B) Time course of peptide elution of in vitro translated BRF specifically immunoprecipitated with 128 antiserum**

Immunoprecipitation was carried out essentially as described in **(A)** except 128 antiserum (lanes 3-8 & lanes 10-15) and its corresponding preimmune serum (lanes 2 & 9) were used. Precipitated material was agitated on an orbital shaker with 20µl of 2.5mg/ml BRF-1 peptide (which correponds to the peptide sequence 128 antiserum was raised against) for 0 h (lanes 2 & 9), 1 h (lanes 3 & 4), 2 h (lanes 5 & 6), 3 h (lanes 7,8,10 &11), 4 h (lanes 12 & 13) or 5 h (lanes 14 &15). Supernatant was immediately removed at the specified time point and the protein A-Sepharose beads were further washed five times with LDB buffer. Protein eluted into the supernatant and immunoprecipitated material retained on the protein A-Sepharose was resolved on a 7.8% SDS-polyacrylamide gel and then visualised by autoradiography. Lane 1 shows 30% of the input reticulocyte lysate containing in vitro translated BRF.

**A**


482
Preimmune
beads
beads
supernatant
beads
supernatant
Time (h): 0 0.5 0.5 1 1
220 kDa
97 kDa
66 kDa
46 kDa
1 2 3 4 5

**B**

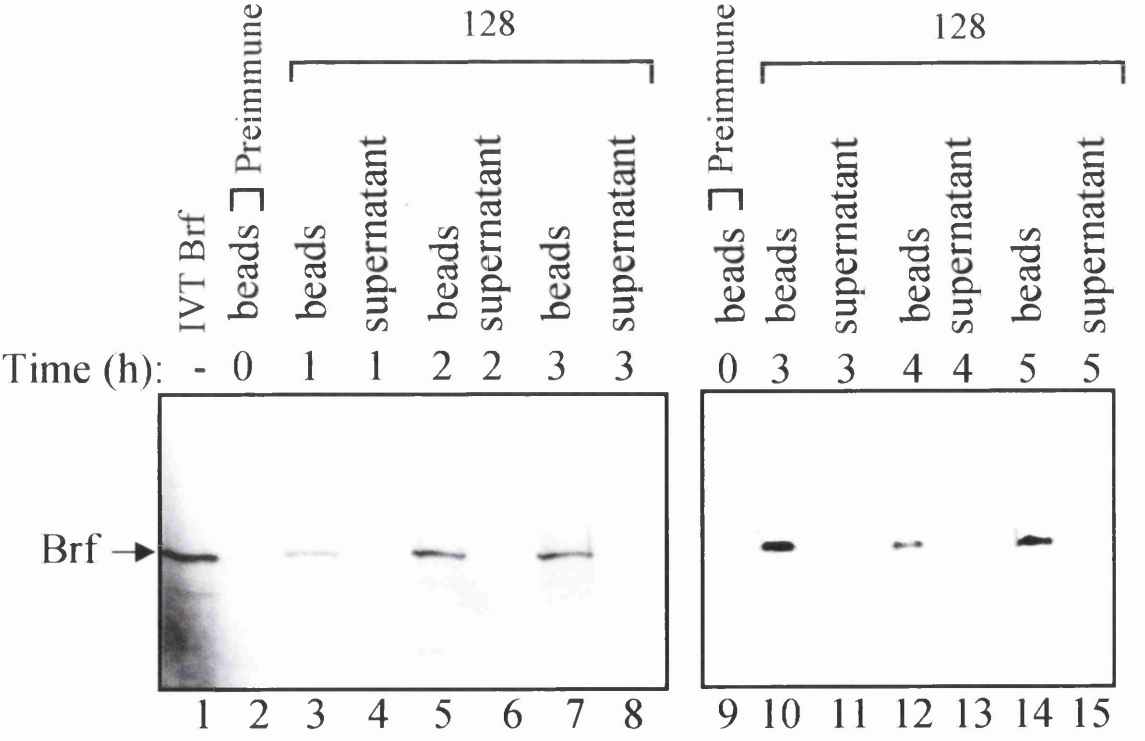
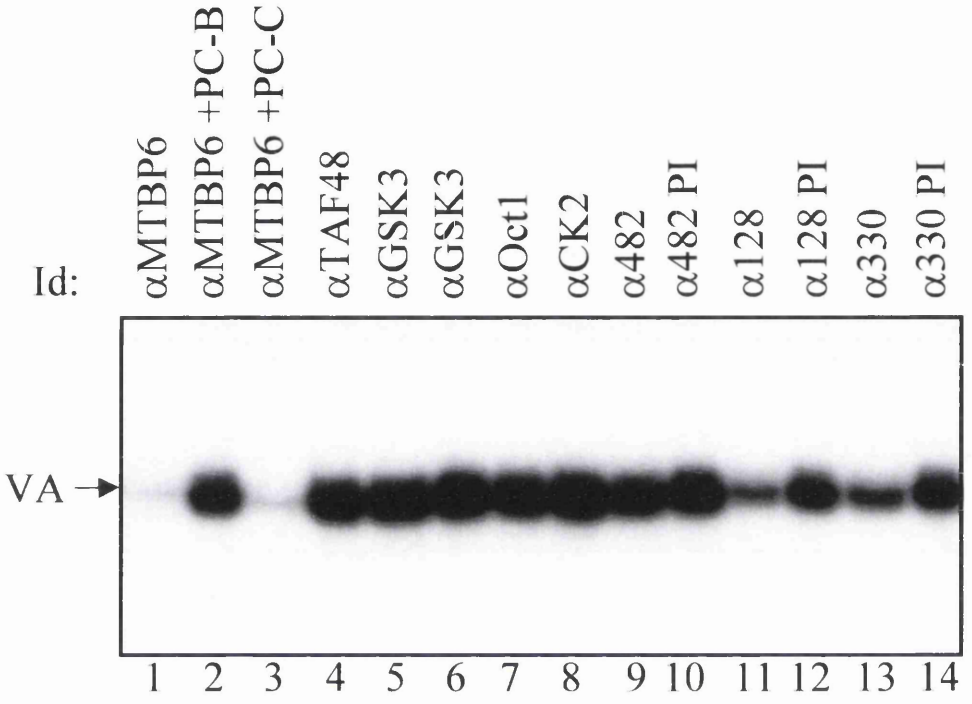

128
128
IVT Brf
Preimmune
beads
beads
supernatant
beads
supernatant
beads
supernatant
Preimmune
beads
beads
supernatant
beads
supernatant
beads
supernatant
Time (h): - 0 1 1 2 2 3 3 0 3 3 4 4 5 5
Brf
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

**Figure 3.8**

**Immunodepletion of RNA polymerase III transcriptional activity from HeLa nuclear extract using the anti-BRF antisera 128 and 330 but not with the 482 antiserum**

Identical amounts of the indicated antisera or preimmune sera prebound to 20μl of protein A-Sepharose beads were incubated with 150μg of HeLa nuclear extract on ice for 3 h. To keep the beads in suspension, the samples were gently mixed by tapping every 10 minutes. After 3 h the beads were pelleted and the supernatants were removed for analysis by in vitro transcription.

RNA polymerase III transcription was assayed using 2μl of the immunodepleted extracts and 250 ng of $pVA_I$; after 15 min incubation at $30^0$C, nucleotides were added to initiate transcription. In lanes 2 and 3, 2μl of PC-B or PC-C, respectively, was added. Lanes 5 and 6 represent the activities from a duplicate immunodepletion using the same antiserum. MTBP6 (lanes 1-3) is a mouse monoclonal antiserum raised against the N-terminal region of TBP and is used as a positive control. The activity of extract immunodepleted with the anti-BRF antisera 482,128 or 330 is shown in lanes 9, 11 and 13, respectively. The activity of extract immunodepleted with the corresponding preimmune serum is shown in lanes 10, 12 and 14, respectively.


Id:
αMTBP6
αMTBP6 +PC-B
αMTBP6 +PC-C
αTAF48
αGSK3
αGSK3
αOct1
αCK2
α482
α482 PI
α128
α128 PI
α330
α330 PI
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14

conditions as similar as possible to those used for the immunodepletions, as these had worked exceptionally well.

As for the immunodepletions, antibody was prebound to protein A-Sepharose by agitation at $4^0$C for 3 h of a 1:1 mixture of antiserum and protein A-Sepharose. The protein A-Sepharose beads were then extensively washed with LDB buffer to remove unwanted material and transferred to a disposable Polyprep® column (Biorad). HeLa nuclear extract was then slowly recirculated through the column for ~ 2 h at $4^0$C. Recirculation was carried out by hand, as opposed to utilising a chromatographic machine, to minimise dilution and possible inactivation of the sample. Gravity flow through the column was slowed by means of an attachable valve. After ~2 h the recirculated material was collected for assaying how effectively the column had depleted the extract of TFIIIB activity and the column was washed with 12 column volumes of CB buffer containing 100mM NaCl, to remove unbound protein. Bound protein was then eluted with CB buffer containing 2M NaCl and 2M urea. Fractions containing protein were then dialysed for 6-10 hours against LDBO buffer to remove the urea and renature the proteins, and to lower the salt.

A major difference between the immunopurification of TFIIIB and the immunodepletions is the need to recover TFIIIB activity. The tightness of antibody –antigen interactions poses the risk of disrupting intramolecular interactions within the TFIIIB complex when attempting to elute. Although peptide elution overcomes this problem, this was not a viable option for 128, as shown in Figure 3.7. The interaction between antigen and antibody is likely to be composed of electrostatic and hydrophobic interactions and H-bonding. This is why elution buffers for conventional chromatography generally work badly; they are targeting only a single type of interaction; for example, a high ionic strength buffer will weaken electrostatic forces, but it also strengthens hydrophobic interactions.

It has been reported that TFIIIB is quite a labile protein (235). This limits how harsh elution conditions can be used to disrupt the antibody-antigen interaction without irreversibly disrupting the protein. Chromatography on Cibacron blue revealed surprisingly that hTFIIIB activity is recoverable from fractions exposed to 2M salt/5 M urea, however (see Figure 3.4 **A**). Elution was therefore attempted with 2M salt/2M urea; the concentration of urea was reduced to cut the time needed for sufficient dialysis.

It was possible that without the covalent coupling of antibody to protein A-Sepharose by chemical crosslinking, such a harsh elution buffer may also remove antibody. Such eluted antibody may be inhibitory in an in vitro transcription assay and would prevent reuse of the column. I therefore tested whether covalent coupling was necessary by screening a range of salt and/or urea concentrations for their ability to remove antibody prebound to protein A-Sepharose. The levels of antibody removed by the buffer and the levels remaining bound to protein A was analysed by Western blotting, probing directly with an anti-rabbit secondary antibody. The majority of antibody appeared to remain bound to the protein A-Sepharose under harsh elution conditions. Although some antibody could be detected in the 2M salt/2M urea supernatants, this amount was not more than two fold greater than that detected with other buffers, including LDB.

Some TFIIIB activity was recovered in the 2M salt/2M urea fractions. The immunodepletion of TFIIIB activity from the starting material was very poor, however. This was surprising, considering that such good immunodepletion was obtained by immunoprecipitation. Although I only recirculated the nuclear extract for ~ 2 h compared with 3 h agitation in an immunoprecipitation, a previous time course of binding of BRF by 128 revealed little difference between 2 and 3 h, with low levels bound after only 30 min. However, the length of time needed for interaction is not necessarily transferable between the batch-like adsorption of an immunoprecipitation and column adsorption. It is also noteworthy that in an immunoprecipitation all of the starting material is in contact with protein A-Sepharose for the entire incubation length, which is clearly not possible with a recirculated starting material.

The tight binding of an antibody to its antigen is thought to involve more than one step and is slow compared with most interactions of enzymes with their substrates (468). For immunoaffinity chromatography, there are two interactions that concern us; the interaction of antibody with protein A and the interaction of antibody with its specific antigen. Of these two interactions, the interaction of antibody and antigen is thought to be significantly slower kinetically. To try and improve immunodepletion, I decided to preincubate the antibody and antigen before applying to the column. In addition to allowing more time for this interaction to occur, this method enables all of the antigen to be exposed to the antibody for the entire time. Preincubation is also advantageous because where antibody is bound to protein A-

Sepharose first, a proportion (estimated to be nine-tenths) of the antibody is effectively lost as the antibody has bound to the Sepharose beads in such an orientation that it is unable to bind antigen.

Preincubation of HeLa nuclear extract with the antiserum for 1 h on ice prior to recirculating the mixture slowly for ~ 2 h did improve immunodepletion and recovery of activity, but only to a small extent (data not shown). This is despite reducing the ratio of antibody to extract 6.7 fold in order to try and conserve antiserum and reduce the effect of any proteases that may be present in the antiserum. However, the amount of antibody did not seem to be the limiting factor for immunodepletion, as increasing the amount of antibody relative to the amount of extract three-fold had no significant effect.

It seems plausible that the poor immunodepletion may be due in part to the vast quantity of other proteins in the nuclear extract that may lead to steric hindrance, perhaps weakly interacting non-specifically with the antigen-binding site of the antibody or with the antigen itself. Furthermore, in a nuclear extract the endogenous interactions of the protein of interest are likely to be intact; one or more of these specific interactions may be mediated by the antigenic site or be very close to it, rendering it poorly accessible. The TFIIIB activity that is immunodepleted may reflect TFIIIB in which the endogenous interactions have temporarily been disrupted. In an immunoprecipitation the frequent agitation of the sample is more likely to mechanically disturb any weak non-specific or specific interactions that prevent the antigen and antibody interacting. The epitope that 128 was raised against is highly conserved, so it is quite probable that it is involved in a specific interaction with another component of the transcriptional machinery.

I therefore decided to repeat the chromatographic procedure but using a more purifed source of TFIIIB as starting material, a PC-B fraction rather than crude nuclear extract. PC-B lacks TFIIIC activity, so at least one TFIIIB-interacting protein that could obscure the epitope has probably been displaced. Pol III activity is found in PC-B fractions, so one or more specific interactions involving pol III subunits and BRF or other TFIIIB components could still be intact. I decided to keep the amount of antiserum constant, which meant an increase in the proportion of antibody relative to total protein, although PC-B is clearly enriched for TFIIIB.

Analysis of fractions revealed a complete lack of transcriptional activity in the recirculated starting material, suggesting very effective immunodepletion;

however, recovery of activity was not improved from previous attempts. Another possible explanation for this remarkable transformation in transcriptional activity of the recirculated starting material was that it was getting inactivated in some way, such as by protein degradation by proteases in the serum.

To investigate this possibility further, I carried out an in vitro transcription assay for TFIIIB activity using PC-B preincubated in the presence or absence of 128 or 330 antiserum or the corresponding preimmune sera. The reactions in which PC-B was preincubated with 128 or 330 produced no transcript. This could be a specific inhibitory effect caused by the antibodies; however, the reactions preincubated with preimmune sera also lacked transcriptional activity, implying protein degradation or something potently inhibitory common to all the sera. Although a cocktail of protease inhibitors was added to the PC-B fraction prior to adding the antiserum, it is possible that insufficient was added. To test this, I titrated increasing amounts of the cocktail of inhibitors into in vitro transcription reactions containing PC-B and antiserum. This had no discernable effect on the level of transcript. Although the cocktail contained inhibitors of the major types of proteases, it is likely that some proteases present would have remained active. I therefore decided to purify the antisera on protein A-Sepharose by the low salt method of Harlow and Lane (195), prior to preincubation with PC-B, in order to remove any contaminating proteases or inhibitory activities present.

Purifying the antisera appeared to solve this problem. Preincubation of purified antiserum with PC-B in an in vitro transcription assay did not adversely affect transcript levels. It also revealed that using PC-B as starting material did improve immunodepletion, but the bulk of activity remained in the starting material. Recovery of activity concurrently improved and this was improved further by a reduction of the size of the column relative to the amount of purified antibody and PC-B. This was to try to concentrate samples on elution, in order to preserve their activity.

To increase the purity of eluted fractions I attempted washing the column more stringently before elution. This proved unsuccessful; increasing the concentration of salt in the wash buffer from 100mM to 200mM resulted in a complete loss of recoverable activity. There are two obvious possible interpretations of this result; one is that one or more essential components of TFIIIB are very loosely associated with the complex and are dissociated when the salt concentration

is increased. This association may also have been weakened by the interaction of the antibody with TFIIIB. It has been reported previously that TFIIIB is not a stable molecular entity (235), lending support to this interpretation. The other interpretation is that the specific interaction formed between the antibody and TFIIIB is very weak and disrupted by the increase in salt concentration. The elution of activity in the flowthrough could easily escape detection due to excessive dilution. The first interpretation seems more likely; as discussed earlier, antibodies generally interact very tightly with their specific antigen. The formation of a tight antigen-antibody complex is a slow process, however, and it is possible that insufficient time was allowed for this to form; perhaps only weak interactions easily disrupted by salt existed between the antigen and the antibody.

In light of the improved immunodepletion and recovery of activity obtained using PC-B rather than nuclear extract as my starting material, I carried out an immunopurification using a PC-B fraction that had been purified further on another column. A fraction enriched for TFIIIB from a heparin gradient of a PC-B fraction was used (BHep). The in vitro transcription assay for TFIIIB activity of eluted fractions is shown in Figure 3.9 (lanes 10-18). Another column using PC-B as the starting material was run in parallel; the activity of eluted fractions from this column were assayed at the same time and are also shown in Figure 3.9 (lanes 1-9). For both columns, the first fraction eluted with 2M salt/2M urea contained the bulk of the eluted TFIIIB activity. A low level of TFIIIB activity was also detectable in the peak FT fraction from each column; this may represent weakly bound TFIIIB that interacted non-specifically with the columns.

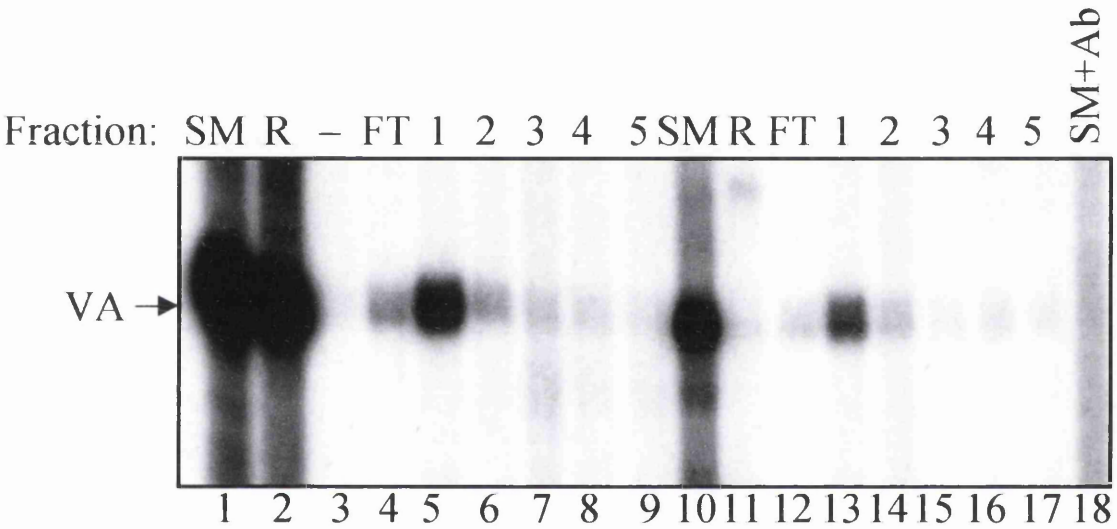
Immunodepletion of TFIIIB activity was improved drastically using BHep as starting material rather than PC-B. Using PC-B, immunodepletion is ~30% (compare lanes 1 & 2), whereas with BHep it is over 90% efficient (compare lanes 10 & 11). This is probably a reflection of the epitope recognised by the BRF antibody being more exposed in the more purified fraction. It is also noteworthy that the purified antiserum preincubated with the BHep fraction inhibits transcription (lane 18), although it does not when incubated with PC-B (data not shown). This suggests that the antiserum may specifically inhibit TFIIIB, but this requires further investigation. The effect of purified preimmune serum on BHep has not been tested, so it is plausible that the effect is non-specific. Assuming that it is a specific effect,

**Figure 3.9**

**Immunoaffinity purification of human TFIIIB**

Human TFIIIB was immunopurified in parallel from two different sources of TFIIIB, PC-B (lanes 1-9) and the more purified BHep (lanes 10-18). Following preincubation of the starting material with purified 330 antiserum on ice for 1 h, this mixture was slowly recirculated through a column of protein A-Sepharose for ~ 2 h. The column was then washed with 20 column volumes of CB buffer containing 100mM NaCl (CB100). Protein was eluted with CB buffer containing 2M salt/5M urea (CB2000U). After dialysis to remove the urea and lower the salt concentration to 100mM KCl, fractions were assayed for TFIIIB activity by in vitro transcription.

Fraction numbers are indicated. SM, starting material; R, recirculated starting material; FT, flowthrough; SM+Ab, preincubated starting material and antiserum mixture. Lane 3 contains LDB buffer. TFIIIB activity was assayed using 12μl of the indicated fraction, 2μl of PC-C and 250 ng of $pVA_I$; after 15 min incubation at $30^0$C, nucleotides were added to initiate transcription.


Fraction: SM R – FT 1 2 3 4 5 SM R FT 1 2 3 4 5 SM+Ab
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

the lack of inhibition observed with PC-B may reflect the masking of the inhibitory effect due to an excess of TFIIIB that is not interacting with the antibody.

The immunoaffinity purified BHep fraction, whose TFIIIB activity is shown in Figure 3.9 (lane 13), represents my most purified TFIIIB fraction. Considering that it has been subjected to three different chromatographic steps, three extensive dialyses, exposure to the denaturant urea and at least three freeze-thaw cycles the activity of this fraction is remarkably good. Silver staining of the fraction revealed that it represents a complex set of polypeptides (data not shown). The TFIIIB is estimated to have been purified in the range of 100-400 fold from HeLa nuclear extract. This partially purified TFIIIB fraction represents a significant improvement in the purity of TFIIIB compared to most sources of TFIIIB that are commonly used in regulatory studies of the pol III transcriptional apparatus.

### 3.2.5 Immunoaffinity purification of human TFIIIC

In parallel to developing an immunoaffinity step for the purification of human TFIIIB, the same was done for human TFIIIC. The procedure that gave the best immunopurification of hTFIIIC was essentially the same as that for hTFIIIB.

In contrast to the relatively poor immunodepletion observed using 330 or 128 and PC-B, preincubation of PC-C with purified 4286 antiserum resulted in >90% depletion of TFIIIC activity. It may be that the epitope on TFIIICβ specifically recognised by 4286 is much more accessible than the epitopes on BRF recognised by 128 and 330, respectively. There are other possible explanations, however, such as the kinetics of the interaction. The TFIIIC contained in PC-C also represents a purer source of TFIIIC than PC-B is of TFIIIB.

Although excellent immunodepletion was consistently obtained using the 4286 antiserum, recovery of TFIIIC activity was much more variable between different chromatographic runs. This seems to be a feature of this protein; it has also been found to be the case for TFIIIC eluted from phosphocellulose (312), suggesting it is relatively easily inactivated.

For an immunoaffinity step to achieve any degree of purification the antibody used must interact specifically with the protein of interest. The antibody specifically

selects the protein of interest for adsorption and not other proteins, although inevitably there is some non-specific binding both to the antibody and the protein A-Sepharose. It is theoretically possible that the immunodepletion of hTFIIIC from PC-C observed using 4286 antiserum and the immunodepletion of hTFIIIB from PC-B observed using 128 or 330 is simply due to non-specific binding to protein A-Sepharose or the antibody. I have tested this possibility for all three antisera by carrying out mock immunopurifications using the appropriate preimmune serum, which is preincubated either with PC-B or, for the 4286 preimmune serum, PC-C. As a positive control, an immunopurification using the appropriate antiserum was carried out in parallel.

Figure 3.10 **A** shows the results obtained using the 4286 antiserum and the corresponding preimmune serum. No TFIIIC activity was eluted in any of the fractions from the mock immunopurification (Fig.3.10 **A**, lanes 12 to 17). For the 4286 column run in parallel, some TFIIIC activity was eluted (see lane 5), although recovery of TFIIIC activity from this particular chromatographic run was very poor compared to that often obtained (see, for example, Figure 3.10 **B**). More indicative of the specificity of the immunoaffinity step is the extent of immunodepletion of TFIIIC activity using 4286 compared to the preimmune serum. With 4286, there was almost complete immunodepletion of TFIIIC activity from PC-C ( Fig.3.10 **A**, compare lanes 1 and 3), whereas using preimmune serum resulted in no or very little immunodepletion (compare lane 11 with lanes 1 & 3). Although the activity of the recirculated PC-C from the preimmune serum is slightly lower than that of the starting material, this is not entirely unexpected given that it was recirculated at $4^0$C for ~2h and exposed to purified preimmune serum which is slightly inhibitory (compare lanes 1, 10 and 11). The recirculated material is also likely to have been diluted slightly compared to the starting material. Immunopurification using the 4286 antiserum thus appears to specifically immunodeplete PC-C of TFIIIC activity. A similar result was obtained for 330 and 128; they specifically immunodepleted PC-B of TFIIIB activity, whereas mock immunopurifications with the preimmune sera did not deplete PC-B of TFIIIB activity (data not shown).

Although there is a very slight inhibitory effect of the preimmune serum on the PC-C, this pales into insignificance when compared with the inhibitory effect of the purified 4286 antiserum (compare lanes 10 &2). Such a strong inhibitory effect of the antiserum compared to the preimmune serum suggests that this inhibition is a

**Figure 3.10**

**Immunoaffinity purification of human TFIIIC**

TFIIIC was immunopurified from a PC-C fraction using anti-TFIIICβ antibody 4286. 2mls of PC-C was preincubated with 300μl of purified 4286 antiserum on ice for ~ 1h. This solution was then recirculated through a 350μl column of protein A-Sepharose at $4^0$C for ~ 2h. Recirculated material was collected and the column was washed with 25 column volumes of CB buffer containing 100mM NaCl (CB100). Immunopurified TFIIIC was eluted with CB buffer containing 2M salt, 2M urea (CB2000U). Eluted fractions were dialysed against LDBO buffer for 8-12 h until the salt concentration was ~ 100mM, and then were further dialysed against LDB buffer for 2-4 h to ensure removal of any residual urea. Eluted fractions were then tested for TFIIIC activity by in vitro transcription assay.

Transcription was reconstituted using 12μl of the indicated fraction, 2μl of PC-B and 250ng of $pVA_L$. Fraction numbers are indicated. SM, starting material; R, recirculated starting material; FT, flowthrough; SM+Ab/PI, preincubated starting material and antiserum or preimmune serum mixture.

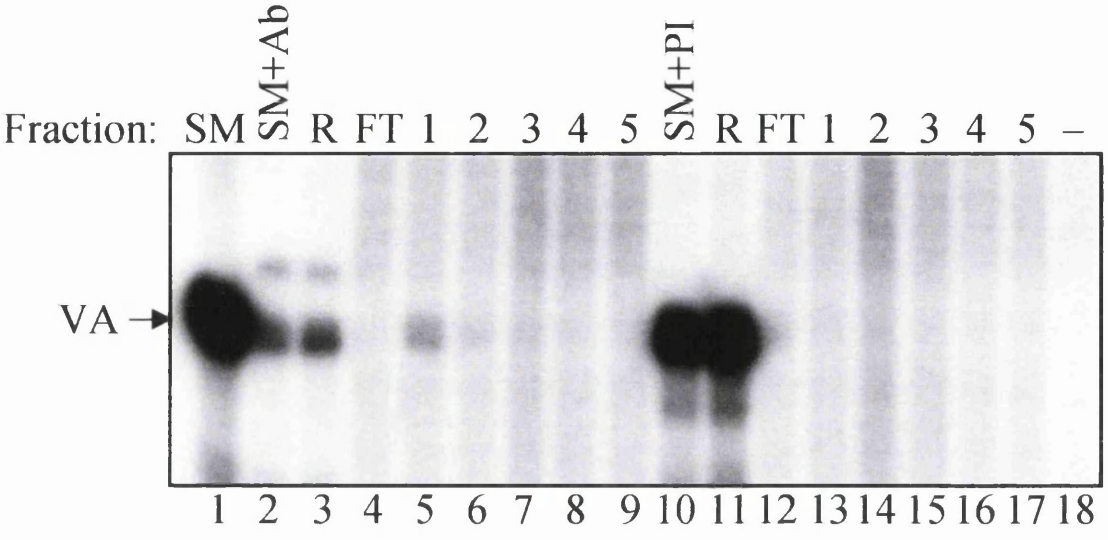
**A) 4286 specifically immunopurifies hTFIIIC**

Lanes 1-9 show the TFIIIC activity of fractions eluted from the 4286 immunoaffinity column. Lanes 10-18 show the TFIIIC activity of fractions from a mock immunopurification conducted at the same time using the corresponding preimmune serum.

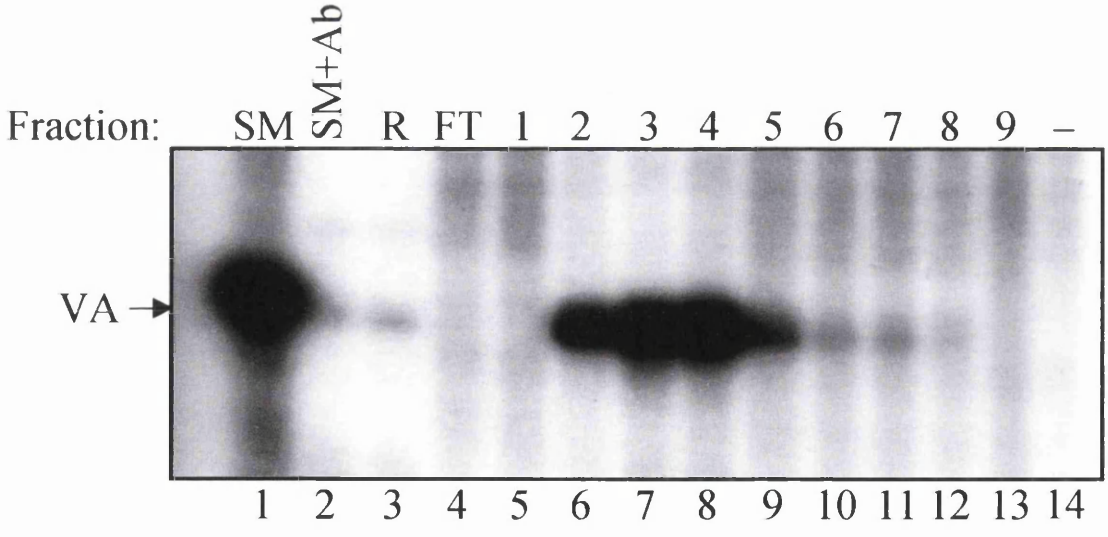
**B) Immunopurification of hTFIIIC with good recovery of activity**

Transcription reactions were carried out as described above. Lanes 5-13 show the TFIIIC transcriptional activity of eluted fractions.

**A**


Fraction: SM SM+Ab R FT 1 2 3 4 5 SM+PI R FT 1 2 3 4 5 –
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

**B**


Fraction: SM SM+Ab R FT 1 2 3 4 5 6 7 8 9 –
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14

specific effect. The reason for the inhibition requires further investigation; it may be that the region of TFIIICβ that 4286 was raised against is involved in the interaction with another component of the transcriptional apparatus. A less interesting but equally plausible explanation is that antibody bound to TFIIICβ may simply sterically restrict TFIIICβ's interactions, mediated through other sites of the protein, with other pol III components, thus preventing the formation of a functional pre-initiation complex.

Figure 3.10 **B** shows the results of another immunopurification using 4286, for which there was very good recovery of TFIIIC activity. Two of the eluted fractions had activity almost comparable to that of the starting material. No TFIIIC activity was detected in the flowthrough. Estimation of the concentration of the eluted fractions by comparison with known amounts of protein on a silver stained gel suggests that the fractions are purified 10-20 fold compared to PC-C. This represents a 100-200 fold purification from HeLa nuclear extract. This is a significant improvement in the purity of TFIIIC compared to that often used in regulatory studies involving human TFIIIC.

Attempts to try and improve upon this level of purification by washing the immunoaffinity column more stringently before elution resulted in a complete loss of activity, as was found to be the case for the TFIIIB immunoaffinity columns.

### 3.2.6 DNA-affinity purification of TFIIIC

TFIIIC binds specifically and with high affinity to the B-block region of the split internal promoter of tRNA and $VA_I$ genes (44, 73, 107, 154, 553, 607). This unique property has previously been exploited for the partial purification of TFIIIC, both in yeast and in humans (71, 156, 408, 505). In contrast to TFIIIC, human TFIIIB and pol III alone are completely devoid of sequence-specific DNA-binding activity and are only able to interact with DNA non-specifically and with low affinity.

TFIIIC was purified using a B-block oligonucleotide, containing the B-block region of the adenoviral $VA_I$ gene, coupled to Sepharose beads (246). I purified

TFIIIC essentially as described by Dean and Berk (118), but with a few modifications from their method (see **2.18.8** and below).

Figure 3.11 show the results of purification on this resin of a PC-C fraction. After washing the resin with 20 column volumes of LDB buffer, bound protein was eluted in two steps. As the in vitro transcription assay shows, TFIIIC activity was recovered both in the 0.35 M KCl elution (Fig.3.11, lanes 5-10) and in the 2M KCl/0.1% IGEPAL elution (Fig.3.11, lanes 11-15).

This contrasted with the results of Dean and Berk, who were unable to detect any TFIIIC transcriptional activity either in their 0.25M KCl elution or in their 2M KCl/0.1% NP40 elution (118). However, combining 0.25M KCl eluate with that from the 2M KCl/0.1% NP40 elution was able to restore transcriptional activity, suggesting that TFIIIC was split into two components by fractionation on the B-block resin, as previously observed on Mono Q (118).

The presence of TFIIIC transcriptional activity in both the 0.35M KCl elution and in the 2M KCl/0.1% IGEPAL elution that I found suggested that TFIIIC fractionates heterogeneously on this column but is not resolved into separate components. However, when I combined the peak TFIIIC fraction from the 0.35M KCl elution with that of the 2M KCl/0.1% IGEPAL elution there was a synergistic increase in transcription levels (compare lanes 6 and 11 with lane 16). This suggests that the two fractions do represent different complementing TFIIIC species; that transcriptional activity could be obtained by the fractions alone may reflect cross-contamination of the two activities. It is also possible that the complementing PC-B fraction is contaminated with one of the two activities; TFIIIC1 elutes from phosphocellulose between 280 and 390 KCl and thus may be found to some extent in the PC-B fraction.

In contrast to the PC-B fraction that I used, the PC-B fraction used by Dean and Berk had been rechromatographed on phosphocellulose and gradient eluted and subsequently tested by in vitro transcription assays reconstituted with Mono Q-purified TFIIIC1 and TFIIIC2, for the presence of these two activities (118).

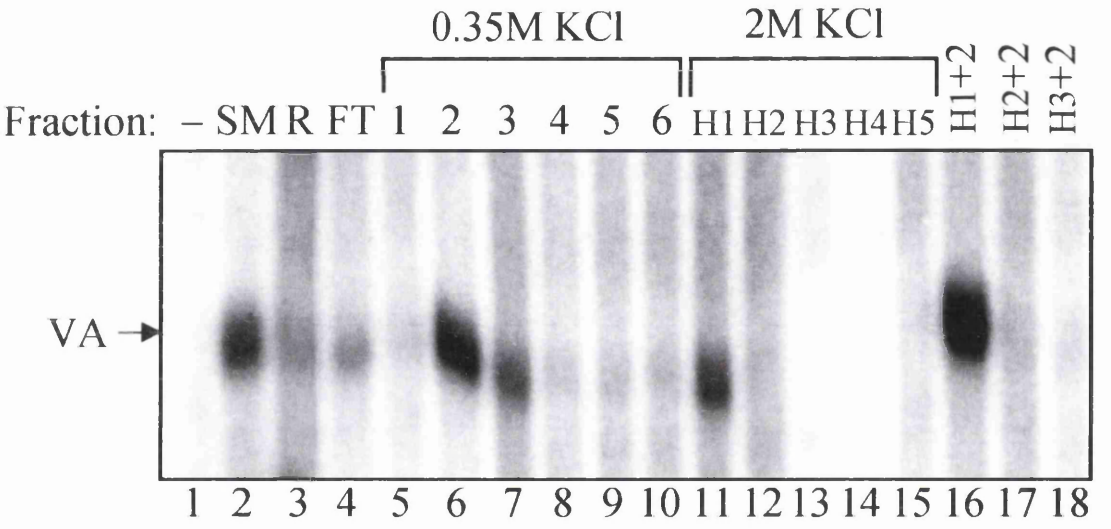
In order to try and obtain better synergy, I reconstituted the in vitro transcription reaction using a more purified source of TFIIIB from a heparin gradient that should lack TFIIIC1 or TFIIIIC2. Unfortunately, however, little synergy was obtained; a reconstitution using PC-B done in parallel suggested that the aliquots of the high salt fractions used were not active. Dean and Berk reported that the activity

**Figure 3.11**

**DNA-affinity purification of human TFIIIC**

Human TFIIIC was DNA-affinity purified from PC-C on a B-block oligonucleotide resin. After extensive washing with LDB buffer, bound protein was eluted in two steps of PC-A buffer containing 0.35M KCl (PC-A(0.35)) and PC-A buffer containing 2M KCl but lacking EDTA ((PC-A(-EDTA)(2.0)). Eluted fractions were assayed for TFIIIC activity by in vitro transcription.

Transcription was reconstituted using 12μl of the indicated fraction, except in lanes 16-18 in which 6μl of both fractions was used. As the source of TFIIIB and pol III, 2μl of PC-B was added to each reaction. $pVA_I$ (250ng) was used as the specific pol III template. Fractions were preincubated for 15 min at $30^0$C with PC-B and template before transcription was initiated by nucleotide addition.
SM, starting material; R, recirculated starting material; FT, flowthrough. Lanes 5-10 show the TFIIIC activity of PC-A(0.35)-eluted fractions. Lanes 11-15 show the TFIIIC activity of PC-A(-EDTA)(2.0)-eluted fractions.


0.35M KCl
2M KCl
Fraction: – SM R FT 1 2 3 4 5 6 H1 H2 H3 H4 H5 H1+2 H2+2 H3+2
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

of the high salt eluate was not stable to multiple rounds of freeze/thaw and that the inclusion of BSA (0.1mg/ml) in the elution buffer was critical for the recovery and stability of activity (118). Although I did not use BSA I did add insulin, which is sometimes used as an alternative to BSA, to a final concentration of 0.2mg/ml. To my knowledge BSA is not any better at stabilising dilute proteins than insulin. However, my fractions seemed extremely susceptible to inactivation.

It was important to distinguish whether the low salt eluate and the high salt eluate of mine contain overlapping TFIIIC activities and to what extent their ability to reconstitute transcription alone is the consequence of a contaminated PC-B fraction. To address this, I carried out band shift analyses of the fractions using a B-block oligonucleotide as my specific probe. Since TFIIIC1 cannot bind to the B-block, the amount of probe bound should be a direct reflection of the level of TFIIIC2 activity of the fractions, although in the context of the whole promoter TFIIIC1 levels affect stability of binding. The band shift assays suggested that there is considerable overlap of activities, at least in terms of TFIIIC2 DNA-binding activity. This result may reflect the higher concentration of salt that I used for the low salt elution compared to that used by Dean and Berk (0.35M KCl versus 0.25M KCl) (118). The 100mM increase in salt concentration may significantly destabilise the interaction of TFIIIC2 with the B-block, although this requires further investigation.

Silver staining of the fractions revealed that a large number of bands seem to be common to both the low salt and the high salt fractions. Although some of these bands may be contaminants, the silver stain is supportive of some overlap of two distinct TFIIIC activities in the low and high salt fractions, as suggested by band shift and in vitro transcription analyses. The silver stain also clearly showed strong enrichment of particular polypeptides in the high salt fraction compared to the low salt fraction and vice-versa, which is supportive of the existence and partial separation of two distinct TFIIIC activities.

TFIIIC (combination of the peak 0.35M KCl fraction and the peak 2M KCl fraction) has been purified ~ 8-12 fold from PC-C. This represents an ~80-120 fold purification of TFIIIC from HeLa nuclear extract.

Potentially, TFIIIC could be purified up to 10-20 fold more by immunoaffinity chromatography (as described in **3.2.5**) of this sequence-specific DNA-affinity purified TFIIIC to give a final purification in the range of 800-2400

fold. However, the extreme susceptibility of the DNA-affinity purified TFIIIC to inactivation makes it unlikely that activity would be recoverable following its application on another column. This was not necessarily the case for the immunoaffinity purified TFIIIC, however, which seemed more resistant to inactivation. The opportunity thus existed to combine two different affinity steps for the purification of human TFIIIC.

In order to have sufficient immunoaffinity-purified TFIIIC for further chromatography on the B-block oligonucleotide resin, I carried out several large scale immunopurifications of TFIIIC. In vitro transcription assays and silver staining revealed a similar level of purification and recovery of activity to that obtained on a small scale. The immunopurified TFIIIC was applied to the B-block column and DNA-affinity chromatography was carried out in an identical manner to that for PC-C. Unfortunately, however, neither TFIIIC transcriptional activity nor B-block binding activity could be recovered.

### 3.2.7 Investigation of the reported HAT activity of human TFIIIC

The packaging of DNA into chromatin has a general repressive effect on nuclear transcription. The structure of chromatin is dynamic, however, and the modification of local chromatin structure is an additional level by which the transcription of specific genes is controlled (588). There are two groups of factors that can affect the chromatin state; nucleosome remodelling factors and proteins that affect the acetylation state of histones. Histone acetyltransferases (HATs) acetylate histones at N-terminal lysine residues reducing the basicity of the histone (500). This has been correlated with a less compact chromatin state and the stimulation of transcription (534).

A number of in vitro and in vivo studies have demonstrated that pol III transcription is subject to chromatin-mediated repression, although different class III templates seem to vary widely in their susceptibility to repression (411). Kundu et al. have shown that human TFIIIC2 can partially relieve chromatin-mediated repression of a tRNA gene in vitro at a higher concentration than is required for transcription of the DNA template (298). Work from the same laboratory suggests that human TFIIIC possesses weak intrinsic histone acetyltransferase activity (230,

298). Pretreatment of TFIIIC with *p*-hydroxymercuribenzoic acid (PMA) resulted in a partial loss of both TFIIIC HAT activity and TFIIIC-dependent chromatin transcription, but with no effect on DNA transcription (298). This suggests that the relief of chromatin repression by TFIIIC may be mediated, at least in part, by its intrinsic HAT activity. For the yeast U6 gene, TFIIIC has a dominant role in relieving chromatin repression; disruption of chromatin structure only enhances transcription when TFIIIC interactions with the B-box are impaired (357, 384). It is interesting to note, however, that yeast TFIIIC lacks any detectable HAT activity.

Kundu et al. immunopurified hTFIIIC on M2 agarose from a cell line expressing a FLAG-tagged TFIIICβ subunit (298). Suprisingly, an in-gel HAT assay using this purified TFIIIC revealed weak HAT activity associated with three polypeptides of ~220kDa, ~110kDa and ~90kDa, corresponding in size to the TFIIICα, TFIIICβ and TFIIICδ subunits of TFIIIC2 (298). This result has been confirmed using recombinant proteins for TFIIICβ (298) and TFIIICδ (230).

In light of the weakness of the HAT activity observed, I attempted to verify that hTFIIIC does indeed possess HAT activity using my partially purified TFIIIC fractions. Figure 3.12 **A** shows the results of a comparison of the HAT activity of my immunopurified TFIIIC with a partially purified TFIIIB fraction, using core histones as the specific substrate for acetylation. This clearly shows that the immunopurified TFIIIC fraction has significantly higher levels of HAT activity than the partially purified TFIIIB fraction assayed in parallel (compare lanes 2 & 3). Consistent with the results from Roeder's laboratory, the HAT activity of the partially purified TFIIIC fraction was very low compared with that of p300 (compare lanes 1 & 2). As found by Roeder and coworkers, histones H3 and H4 were specifically acetylated, with the preferential acetylation of histone H4. There was no detectable autoacetylation of hTFIIIC.

In contrast to Kundu et al., no sodium butyrate was included in the HAT reaction mixture (298). Sodium butyrate is a deacetylase inhibitor but was omitted on the basis that it can have non-specific inhibitory effects and this could reflect the low HAT activity of the hTFIIIC observed by Kundu et al. (298). The HAT activities observed with the various fractions shown in Figure 3.12 **A** thus represent the level of acetylation relative to deacetylation. It is thus plausible that the difference in histone acetylation observed between the immunopurified TFIIIC and

the partially purified TFIIIB fraction simply reflects higher levels of deacetylase activity in the TFIIIB fraction. TFIIIB specifically cofractionates and associates with the retinoblastoma protein, pRb (314). As discussed in Chapter 4, pRb has recently been shown to associate with the histone deacetylase HDAC1 (53, 349), lending support to this possibility.

It is important to realise that the TFIIIB and TFIIIC fractions assayed are partially purified fractions. Given the very weak HAT activity of the immunopurified TFIIIC fraction, this activity observed could easily be caused by a small level of contamination of the fraction with a potent HAT such as p300.

To investigate the reported HAT activity of hTFIIIC further, I decided to carry out HAT assays on immunoprecipitated TFIIIC, TFIIIB and mock immunoprecipitated material obtained using the appropriate preimmune sera. This revealed no significant differences in the HAT activities of immunoprecipitated TFIIIC and TFIIIB compared to the mock immunoprecipitations (data not shown). It may be that the amount of TFIIIC immunoprecipitated is too low for its weak HAT activity to be detectable above background levels, or its HAT activity may be partially inactivated by the vigorous agitation required during immunoprecipitation.

Figure 3.12 **B** (lanes 1-6) shows the HAT activity of TFIIIC-containing fractions from the DNA-affinity column of Fig. 3.11. This reveals a slight depletion of HAT activity from the PC-C used as starting material (compare lanes 1 & 2). A very low level of HAT activity is detectable in the FT fraction (lane 3). In contrast, there is a very significant enrichment of HAT activity in the peak fraction from the 2M KCl elution and the peak fraction from the 0.35M KCl elution (lanes 5 & 6 respectively), both of which are enriched in TFIIIC activity. This result is supportive of hTFIIIC possessing intrinsic HAT activity.

The TFIIIC DNA-affinity fractions are purified on the basis of their specific B-block binding activity. Previously it has been shown that TFIIICβ and an N-terminal 83 kDa fragment of TFIIICα are sufficient to reconstitute the B-block binding activity of hTFIIIC (481). The strong enrichment in HAT activity in the B-block affinity-purified TFIIIC fractions thus correlates well with the finding by Kundu et al. (298), that TFIIICα and TFIIICβ contain their own intrinsic HAT activity, since the fractions should be particularly enriched in these two subunits of TFIIIC2.

Lanes 7 & 8 of Fig. 3.12 **B** show the HAT activity of an immunopurified TFIIIC fraction and a mock-immunopurified TFIIIC fraction, respectively. There is very little difference in HAT activity of the two fractions, which is not supportive of hTFIIIC possessing intrinsic HAT activity. Analysis of the HAT activity of other TFIIIC immunopurified fractions revealed a similar result.

Together my results are unable to verify the finding by the Roeder laboratory that human TFIIIC possesses weak intrinsic HAT activity (230, 298) although some results in support of this were obtained.

**Figure 3.12**

**HAT activity of partially purified TFIIIC fractions**

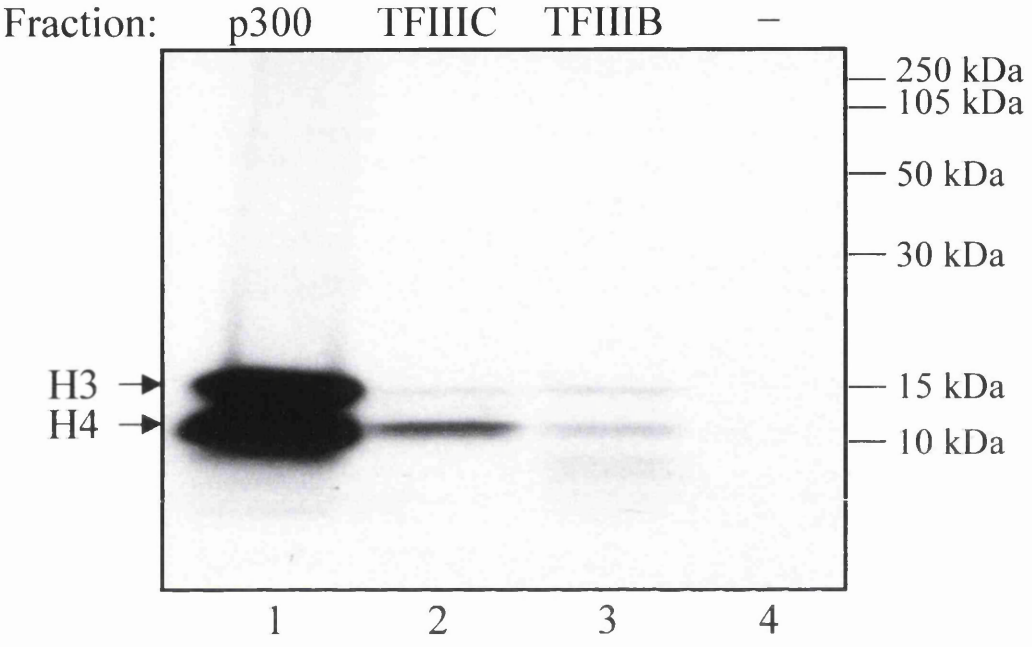
**A) Comparison of HAT activity of an immunopurified TFIIIC fraction with a partially purified TFIIIB fraction and p300**

Equal amounts of immunopurified TFIIIC (lane 2) and a partially purified TFIIIB fraction (BHep) (lane 3) were assayed for HAT activity. A small quantity of recombinant p300 (lane 1) was also assayed as a positive control. Lane 4 lacks any additional source of protein apart from the core histones added as the specific substrate for assaying acetylation. The fractions were incubated for 30 min at $30^0$C with 1 μg of chicken core histones and 0.25μCi of $^{14}$C acetyl CoA in a final reaction mixture of 50mM Tris pH 8.0, 5% glycerol, 0.1mM EDTA, 50mM KCl, 1mM DTT and 1mM PMSF. Samples were resolved on a 12% polyacrylamide-SDS gel. After fixing the gel, it was incubated in Amplify™ (Amersham) for 15-30 min to reduce the exposure time of the gel required. Acetylated products were visualised by autoradiography, after exposure to BIOMAX X-ray film (Kodak) for 3 days at $-80^0$C.

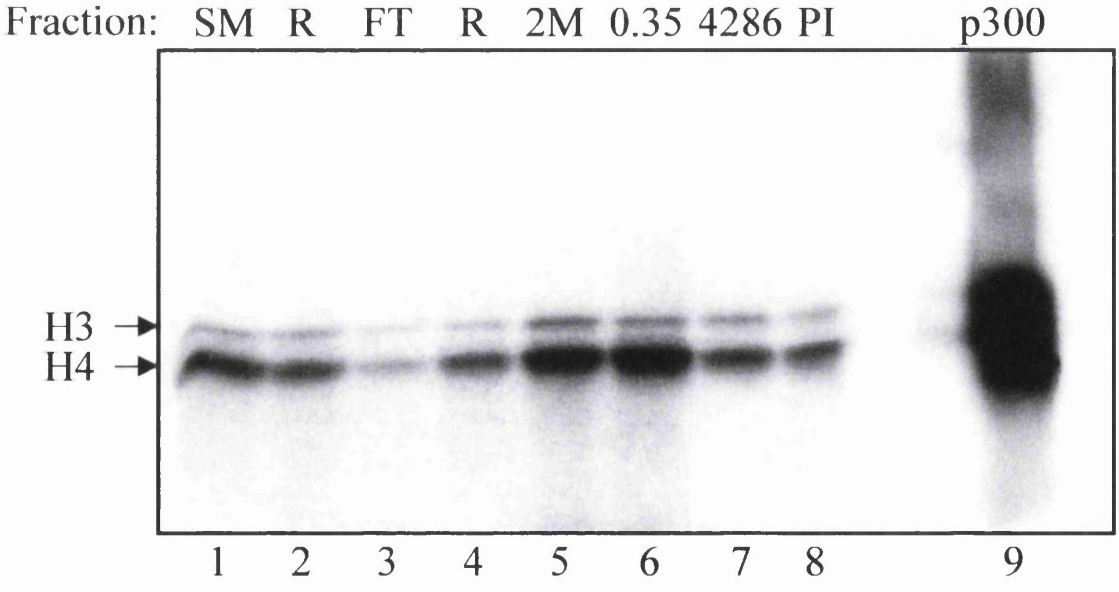
**B) B-block affinity-purified TFIIIC fractions are enriched in HAT activity**

Fractions were assayed for HAT activity as described in **(A)**. 1μl of p300 was assayed (lane 9) and 15μl of all other fractions (lanes 1-8). Lanes 1-6 represent peak fractions from the DNA-affinity column of Fig.3.11. SM, starting material; R, recirculated starting material, FT; flowthrough; 2M, peak fraction from 2M KCl elution; 0.35, peak fraction from the 0.35M elution. Although equal volumes of fractions were assayed, silver staining revealed that the 2M KCl and the 0.35M KCl fractions assayed were approximately four times less concentrated than SM and R. Lanes 7 & 8 represent the first eluted fraction of the 4286 immunoaffinity column and the mock immunoaffinity column respectively of Fig.3.10A (lanes 5 & 13). Silver staining revealed that these two fractions are of comparable concentration.

**A**


Fraction: p300 TFIIIC TFIIIB –
250 kDa
105 kDa
50 kDa
30 kDa
15 kDa
10 kDa
H3
H4
1 2 3 4

**B**


Fraction: SM R FT R 2M 0.35 4286 PI p300
H3
H4
1 2 3 4 5 6 7 8 9

## 3.3 Discussion

### 3.3.1 Partial purification of mammalian TFIIIB

Despite considerable effort in the past fifteen years by a number of different laboratories, substantial purification of intact and active mammalian TFIIIB has remained rather elusive. A great many studies have implicated TFIIIB as a target for regulation, but these have tended to rely on rather impure sources of hTFIIIB obtained through conventional chromatography. The cloning and identification of TBP and hBRF as essential components of hTFIIIB provided the opportunity to use a novel approach, namely that of immunoaffinity chromatography, as a means of purification. It is now well documented that this technique can potentially achieve levels of purification in a single step that conventional chromatographers may struggle to even approach over a whole purification scheme. A significant proportion of my time and effort was therefore devoted to developing an effective immunoaffinity step for purifying TFIIIB, as well as screening a number of different conventional column types for potential use in a final fractionation scheme. Attempts have previously been made to immunopurify hTFIIIB in our laboratory using anti-TBP antibodies; however, this approach resulted only in the isolation of disrupted hTFIIIB complexes inactive for transcription (570). An immunoaffinity step was therefore developed using antibodies against BRF. There was the additional advantage of BRF being specific to TFIIIB, whereas TBP is part of pol II- and pol I-specific complexes as well as TFIIIB.

Three different BRF antibodies, each raised against a different region of the BRF protein, were tested for their potential use in the immunopurification of TFIIIB. However, only two of these, 128 and 330, were capable of immunodepleting endogenous BRF. There was very little to choose between the 128 and 330 antisera in terms of the yield of active TFIIIB typically obtained or the extent of purification. In the final purification scheme, nuclear extract was first purified on phosphocellulose and a heparin gradient prior to the immunoaffinity step, resulting in a 100-400 fold purification of active hTFIIIB from HeLa nuclear extract. This represents a significant improvement in the purity of TFIIIB compared to that normally used for regulatory studies in our laboratory.

During gradient chromatography on Mono Q, hTFIIIB fractionates into two components, suggesting that one or more subunits may be loosely associated. In support of this, washing the immunoaffinity column with buffer containing 200mM NaCl rather than 100mM NaCl prior to the high salt elution of bound hTFIIIB complex was consistently found to result in a complete loss of TFIIIB activity. Whatever the reason, the inability to wash the immunopurified complex very stringently without the loss of transcriptional activity meant that only a modest level of purification (~10 fold) was obtained by the immunoaffinity step.

The cause of this loss of activity is an important issue to be resolved as it may provide valuable information as to how the immunoaffinity step could be improved. It may be that the hTFIIIB complex is intrinsically unstable, as suggested by Huet et al. for the yeast TFIIIB complex. Alternatively, it may be that the particular BRF antibodies used weaken the association of one or more subunits of TFIIIB. If the latter possibility were to be true, other BRF antibodies raised against different regions of the BRF protein may offer the opportunity to wash more stringently without incurring a loss of activity. Another possible explanation is that washing with buffer containing 200mM NaCl disrupts the interaction between the antibody and TFIIIB. The establishment of a tight antigen-antibody complex is thought to occur in more than one step and is rather slow kinetically. Perhaps insufficient time was allowed for progression from the initial weak interaction of antigen with antibody to the formation of a stronger interaction that is relatively resistant to salt. This could easily be tested simply by preincubating the antigen and antibody for longer periods of time, for example overnight at $4^0$C, and seeing whether the column can be washed any more stringently without the loss of activity.

During the preparation of this manuscript, Schramm et al. reported the cloning of human B″ (463). They also show that antibodies raised against hB″ specifically deplete extracts of pol III transcriptional activity. Significantly, the addition of recombinant hB″ alone to depleted extracts was able to rescue transcription, suggesting that hB″ is not tightly associated with other TFIIIB components. Conversely, for extracts depleted of pol III transcriptional activity using anti-BRF or anti-TBP antibodies, a combination of recombinant TBP and BRF were found to be sufficient to restore transcription. Together, these results suggest that the TFIIIB I immunopurified using anti-BRF antibodies may be partially

deficient in B″. The sensitivity of the TFIIIB complex to washing with 200mM NaCl may reflect the complete loss of weakly associated hB″. Thus, the addition of recombinant hB″ to eluted fractions from the immunoaffinity column washed with 200mM NaCl may be able to restore pol III transcriptional activity. It would also be of interest to see if recombinant B″ can stimulate transcription synergistically when mixed with active immunopurified TFIIIB that was obtained.

The recent work of Schramm et al. suggests that hB″, like its yeast counterpart, is only loosely associated with the TFIIIB complex. A better approach to achieving highly purified active TFIIIB may therefore be to separately immunopurify hB″ and TBP-BRF and to reconstitute the active complex by mixing the separately purified components. The tendency of a protein complex to dissociate easily makes the substantial purification of intact complex extremely difficult to achieve.

### 3.3.2 Partial purification of mammalian TFIIIC

As with hTFIIIB, washing the immunopurified TFIIIC complex more stringently before eluting from the antibody column resulted in a significant reduction in the level of eluted TFIIIC activity. The cause of this loss of activity has not been investigated; however, hTFIIIC is relatively easily dissociated into two components, TFIIIC1 and TFIIIC2 (118, 607). The TFIIIC1 component remains almost completely undefined, possibly because it is not a very stable entity itself. The sensitivity to washing at increased salt concentrations might therefore be caused by the disruption of the interaction between TFIIIC1 and TFIIIC2, or perhaps, weak interactions within the TFIIIC1 subcomplex.

Immunopurified TFIIIC is estimated to have been purified ~100-200 fold from HeLa nuclear extract while DNA-affinity purified TFIIIC is estimated to have been purified ~80-120 fold. Unfortunately, the single attempt to combine the DNA-affinity and immunoaffinity purification steps failed to yield active TFIIIC. It might be an insufficient amount of immunoaffinity-purified TFIIIC was applied to the B-block oligonucleotide column. The attempt to combine the two purification steps certainly deserves repeating. Having obtained two different sources of partially

purified TFIIIC that have been purified by different criteria, this enabled the significance of results obtained with one source of TFIIIC to be verified using the other source of purified protein.

For future work there is a lot of scope for improving upon the level of purification obtained, if required. All five subunits of TFIIIC2 have been cloned and each of these is a potential target in an immunoaffinity step depending on the extent of their surface exposure. The TFIIIC could also be purified further by conventional chromatography prior to an immunoaffinity step. The behaviour of TFIIIC on a variety of conventional column types was not investigated as part of this work. It would also be interesting to test the behaviour of TFIIIC on an oligonucleotide resin encompassing both the A- and B-block, to see if this stabilises TFIIIC1 and the interaction between TFIIIC1 and TFIIIC2, thereby enabling their co-elution as a single complex.

### 3.3.3 Human TFIIIC as a histone acetyltransferase

The DNA-affinity purified TFIIIC is significantly enriched in HAT activity compared with PC-C, supporting the findings of the Roeder laboratory that hTFIIIC has weak intrinsic HAT activity (230, 298). However, the immunoaffinity purified TFIIIC was not enriched in HAT activity compared with a mock immunopurified TFIIIC fraction of comparable concentration. Other proteins present in these partially purified fractions can account for both of these results. The enrichment in HAT activity in the DNA-affinity purified TFIIIC could easily result from the specific or non-specific co-fractionation of small amounts of a potent HAT, such as p300. Similarly, the lack of enrichment observed in the immunopurified TFIIIC could easily result from background levels of acetylation and deacetylation that have a masking effect on the HAT activity of the TFIIIC. These results suggest that if hTFIIIC does have intrinsic HAT activity, it is very weak. The enrichment in HAT activity in partially purified TFIIIC fractions can only be regarded as weak supporting evidence for the role of hTFIIIC as a HAT.

In the last five years a number of transcriptional coactivators for pol II transcription have been found to possess HAT activity; these include GCN5 (60), PCAF (601), p300 (398), CBP (398), $TAF_{II}250$ (378) and the nuclear hormone

receptor coactivators ACTR (85) and SRC1 (496). The intrinsic HAT activities of GCN5 and CBP have recently been demonstrated to be essential for their coactivator function (303, 359). It is now widely accepted that histone acetylation of chromatinised templates facilitates their transcription. Histone acetylation of a chromatinised 5S rRNA gene has been shown to facilitate binding of the 5S gene-specific factor TFIIIA and increase transcription of this gene (229, 321, 529, 534). Both in yeast and in humans, TFIIIC has been shown to be able to relieve chromatin-mediated repression of pol III transcription (66, 298). Thus, recent reports that hTFIIIC has intrinsic HAT activity are not too surprising. The treatment of hTFIIIC with the HAT inhibitor PMA results in a partial loss of HAT activity and inhibits TFIIIC-dependent chromatin transcription without affecting transcription of naked DNA (298). Although this is suggestive of a link between hTFIIIC relief of chromatin repression and its HAT activity, the inhibitory effect of PMA on TFIIIC-dependent chromatin transcription may be coincidental.

Human TFIIIC has diverged significantly from its yeast counterpart and none of the reported HAT-containing subunits of human TFIIIC have significant sequence homology to any of the yeast subunits. In accordance with the lack of homology, no HAT activity has been found to be associated with yeast TFIIIC (83). Thus, relief of chromatin-mediated repression by yeast TFIIIC seems to be by a HAT-independent mechanism and the same may be true for hTFIIIC.

In recent years it has become clear that some of the HATs efficiently acetylate non-histone proteins both in vitro and in vivo. Among the targets for acetylation of non-histone proteins are transcription factors. The acetylation of transcription factors can have both a stimulatory and an inhibitory effect on transcription, depending on the particular transcription factor and the site of acetylation. For example, acetylation of p53 at a site directly adjacent to its sequence-specific DNA-binding domain increases the sequence-specific DNA-binding activity of p53 and transcription of p53-activated genes (178). In contrast, the acetylation of HMGI(Y) within its DNA-binding domain disrupts its binding to DNA and reduces transcription of HMGI(Y)-responsive genes (386). The HAT activity of hTFIIIC is extremely weak compared to that of many other HATs, which raises the issue as to whether such a weak activity is physiologically significant. It is possible, however, that the activity is so weak because histones are a poor substrate for hTFIIIC and the in vivo target of hTFIIIC HAT activity is non-histone proteins

that remain to be identified. To date this possibility has not been investigated, although it would appear that hTFIIIC does not possess autoacetylation activity.

Several lines of evidence suggest that the enzymatic activity of histone acetyltransferases may be regulated. The HAT activity of CBP increases sharply at the G1-S transition of the cell cycle (4). This coincides with the hyperphosphorylation of CBP and a peak of cdk2/cyclin E activity, implicating the involvement of a phosphorylation event (4). The HAT activity of hGCN5 is also regulated by phosphorylation; phosphorylation by DNA-PK inhibits its HAT activity (20). There is also some evidence that suggests that the HAT activities of p300 and PCAF can be regulated by the viral oncoprotein E1a (289). The weak HAT activity of hTFIIIC tested may be due to the lack of an activating post-translational modification, such as phosphorylation. All five subunits of TFIIIC2 are metabolically labelled with $^{32}$Pi in vivo (481). It may be that one of these phosphorylation events stimulates the HAT activity of hTFIIIC. At present, however, the finding by the Roeder laboratory that hTFIIIC is indeed a HAT still awaits independent verification.

# Chapter 4.

# Repression of TFIIIB by the pocket proteins

## 4.1 Introduction

The retinoblastoma protein, RB, is an important tumour suppressor that is frequently inactivated in a variety of different human malignancies (173, 214, 557, 580). Indeed, it has been suggested that the regulatory pathway involving RB may be disrupted in all human cancers (557). RB is involved in constraining the growth and proliferation of the cell (173, 214, 557, 580). In the absence of RB function the cell is desensitised to environmental conditions and normal regulatory signals and is prone to uncontrolled growth and proliferation. Introduction of the wild-type *Rb* gene into a number of different human cancer cell lines that lack functional RB was found to suppress growth and proliferation, soft agar formation and tumorigenicity in nude mice (42, 233, 427). Definite proof for the essential role of RB as a tumour suppressor is provided by gene knockout experiments in mice. Homozygous inactivation of the *Rb* gene is lethal, mouse embryos die in midgestion with neuronal and haematopoietic defects implicating a role for RB in differentiation. However, heterozygotes survive and are strongly predisposed to cancer (232, 245, 322, 347, 396, 581).

A diverse array of cellular proteins have been found to associate with RB and novel targets are still being discovered (214, 512, 566). The relative contributions of the various targets to the physiological effects of RB is still the subject of intense study. Probably the best characterised target of RB is the E2F family of transcription factors (3, 132, 310, 556). E2F is a heterodimeric transcription factor composed of an E2F polypeptide, of which there are six types, and a DP polypeptide, of which there are three types (3). RB inhibits E2F by binding to and masking its transactivation domain, thus preventing E2F activating gene expression from promoters that contain E2F-binding sites (131, 560). E2F-RB complexes can bind to E2F-binding sites in promoters and repress basal transcription from these promoters

(131, 560). Thus, RB converts E2F from a transcriptional activator to a transcriptional repressor (560). This dominant repressive effect on transcription is dependent, at least at some E2F-regulated promoters, on histone deacetylase activity (53, 346, 349). The histone deacetylase HDAC1 can associate with RB and is specifically recruited to E2F-regulated promoters via this association (53, 346, 349).

The E2F transcription factors regulate the expression of a battery of genes many of which encode proteins essential for DNA replication or which promote cell cycle progression (131, 566). Overexpression of E2F-1 can drive quiescent cells through G1 and into S phase of the cell cycle (428). On the basis of the identity of the genes that are regulated by E2F, one would predict that inhibition of E2F by RB would be a potent block upon cell proliferation (131, 566). Overexpression of RB in many cell types causes a G1 arrest; however, without inactivating all the genes encoding E2F and DP proteins one cannot be certain the arrest is solely due to inactivation of E2F (131).

The activity of RB is tightly regulated and depends on its phosphorylation status (239, 418, 482, 557). In a hypophosphorylated state RB is active, whereas in a hyperphosphorylated state RB is inactive. The phosphorylation of RB is controlled by cyclin-dependent kinases (CDKs) (239, 418, 482, 557). The CDKs integrate negative and positive growth signals from the environment. For example, the synthesis of the D-type cyclins is rapidly induced on mitogenic stimulation; on growth factor withdrawal, cyclin D synthesis ceases immediately (418). Cyclin D may act as a growth factor sensor (418). The activity of CDKs depends on the levels of the regulatory cyclin subunits (239, 418, 482). The CDKs enable RB to indirectly sense the environment and to coordinate gene expression required for cellular growth and proliferation with growth factor availability (214, 239, 418, 482).

One of the major checkpoints of the cell cycle is the restriction point, R, in late G1 (404). Passage through R constitutes a transition from a serum-dependent to a serum-independent state and cells are committed to a single round of DNA replication and cell division (404). RB is a major determinant of whether cells can pass through R (482, 557).

The restraining influence of RB on cell proliferation, that is, an increase in cell number, can be largely explained by its inhibitory effect on E2F (566). However, it is difficult to reconcile the ability of RB to suppress cell growth, that is, an increase in cell mass, with its ability to inhibit E2F (566). With the exception of

c-myc, none of the known E2F targets provide any obvious links to the control of cell growth (566). One might imagine that controlling cell proliferation is sufficient to achieve indirect control of cell growth. However, inhibition of DNA replication has little immediate effect on the growth of a variety of different mammalian cell types (566). Although cell proliferation and growth are undoubtedly intimately linked, available evidence suggests that any dependency is the other way round. In bacteria and yeast it is necessary for the cell to reach a critical size threshold before DNA replication and cell division can proceed (394). This has also been found to be true for murine fibroblasts (273).

A significant imbalance between growth and proliferation will cause a cell to undergo apoptosis (428, 566). Although the overexpression of E2F drives quiescent cells into S phase, they then apoptose (428). This strongly suggests that the control of E2F is insufficient to account for the growth inhibitory effects of RB. RB is also in vast molar excess of E2F in the cell (214, 557).

Recently it has been shown that RB represses pol III transcription in vitro and in vivo (579). There is also some evidence suggesting that pol I transcription may be a physiological target of RB (78, 539). Repression of pols I and III provide a potential mechanism by which RB can achieve growth control (567, 568). The rate of growth of cells is directly proportional to the rate of protein accumulation, which is largely dependent on the rate of protein synthesis (27). rRNA and tRNA are important determinants of the biosynthetic capacity of the cell . The levels of rRNA and tRNA in the cell are unlikely to be in large excess for reasons of cell economy and it is possible that suppression of their synthesis by RB results in their becoming limiting for translation, thus restricting cellular growth.

Knockout mice provided definite proof that pol III transcription is a physiological target of RB. Primary embryonic fibroblasts from $Rb^{-/-}$ mice synthesised tRNA and 5S rRNA at a rate approximately 5-fold higher than equivalent cells from $Rb^{+/+}$ mice (579). In contrast, the overall level of pol II transcription remained unchanged.

RB specifically represses TFIIIB (93, 314). In a reconstituted system repression of $VA_I$ transcription by recombinant RB can be overcome by addition of partially purified TFIIIB whereas addition of pol III or TFIIIC fractions had little or no effect (314). In this reconstituted system in the absence of added RB, TFIIIC was found to be the limiting factor whereas TFIIIB was in relative excess (314). This

demonstrates the potency of the repressive effect of RB on TFIIIB; RB severely reduces the level of pol III transcription yet its target, TFIIIB, is not even initially limiting for transcription in this system. Furthermore, pull-down assays and immunoprecipitations revealed that RB and TFIIIB stably interact (314). This is supported by the cofractionation of a population of endogenous RB molecules with endogenous TFIIIB over a variety of different chromatographic columns (314). TFIIIB activity was also found to be specifically elevated in primary embryonic fibroblasts from $Rb^{-/-}$ mice (314). This large body of evidence clearly demonstrates that TFIIIB is a specific target for repression by RB. This is also consistent with previous data which showed that TFIIIB is the limiting factor for pol III transcription during the G1 phase of the cell cycle, a time when RB is active; and that it is no longer limiting in S phase, a time when RB is inactivated by hyperphosphorylation (571).

The retinoblastoma protein RB is structurally and functionally related to two other cellular proteins called p107 and p130 (173, 385, 580). Collectively, the three proteins are often referred to as the pocket proteins because they share most extensive sequence homology in a bipartite region called the pocket domain. The pocket domain of RB is both necessary and sufficient for its proliferation- and growth-suppressive functions (427). Like RB, the ectotopic expression of p107 and p130 in tumour cells can inhibit cellular growth and proliferation (97, 427, 593, 615). The pocket domains of the three pocket proteins bind a number of common target proteins such as E2F and the oncoproteins of several DNA tumour viruses, suggesting that there may be some redundancy between the pocket proteins (131, 173, 214, 385). p107 and p130 are much more closely related to each other (~50% amino acid identity) than to RB (30-35% identity). Gene knockout experiments revealed that there is significant redundancy between p107 and p130. p107- or p130-deficient mice develop normally, however, $p107^{-/-}$ $p130^{-/-}$ double knockout mice die shortly after birth (99). In contrast, mice lacking RB die in midgestion, suggesting that some distinct functions of RB cannot be performed by p107 or p130 (95, 245, 322).

Recently, Sutcliffe et al. showed that p107 and p130 are also able to repress pol III transcription; thus, this is a property shared by all three pocket proteins (504). In this chapter evidence is presented which suggests that endogenous p107 and p130 stably interact with a specific component of the pol III transcriptional apparatus.

Although it has been established that RB specifically represses TFIIIB, the mechanism by which RB achieves this has, until recently, not been investigated. In this chapter I also describe some attempts to elucidate the mechanism by which RB specifically inhibits TFIIIB.

## 4.2 Results

### 4.2.1 Repression of pol III transcription by recombinant RB

To investigate the mechanism(s) by which RB specifically represses TFIIIB, it was necessary to have a purified source of RB for use in assays and for this RB to be active in its ability to inhibit pol III transcription. Since the *Rb* gene has been cloned, the production of recombinant protein was an attractive alternative to purifying the protein from natural sources. Although RB is quite an abundant nuclear protein and it is ubiquitously expressed, it was judged that purification of sufficient quantities from natural sources would probably require vast amounts of nuclear extract. It was also predicted that sufficient purification of RB from its many targets and other cellular proteins could be very difficult and time consuming.

For quantitative reasons and ease of purification, it was thus decided to express RB heterologously using Escherichia coli as host. Suitable RB expression vectors were already available for the expression of RB as a glutathione-S-transferase (GST) fusion protein or as a $His_6$ fusion protein. Both of these RB fusion proteins were expressed for use in assays enabling the verification of results obtained with one RB fusion protein by testing the effect of the other. Where results differ, this suggests the effects seen may not be due to RB but may be a consequence of the specific tag or contaminants that have co-purified. The "tagging" of RB enabled its rapid and efficient purification in a single step on an affinity column specific for the tag. Rather than express full-length protein, RB containing residues 379-928 (RB(379-928)) was expressed, as this has been found to give higher levels of expression. White et al. previously demonstrated that this region of RB is sufficient to inhibit pol III transcription (579).

Having expressed and purified RB, it was essential to test that the protein is active in its ability to repress pol III transcription. When expressing eukaryotic proteins in a prokaryotic system obtaining biologically active protein can sometimes be a big problem (468). This may be caused by the lack of an essential post-translational modification or incorrect folding resulting from the environment in which it was expressed and the lack of appropriate molecular chaperones (468). The protein may also be inadvertently inactivated by the procedure used to extract and purify it. The procedures used for disrupting cells to obtain the protein of interest are

often quite harsh and the balance between disrupting too gently and obtaining very poor yields and disrupting too harshly and inactivating the protein seems quite fine.

I have been able to purify both GST-RB(379-928) and $His_6$-RB(379-928) that are of very high activity in their ability to repress in vitro pol III transcription, as shown in Figure 4.1 for GST-RB(379-928), for example. However, consistent with the findings of others, there seemed to be considerable variation in the activity of the purified RB between different preparations (314). Attempts to pinpoint the cause of the variation in activity of RB between different batches have been unsuccessful to date. It was thus essential that each preparation of GST-RB(379-928) or $His_6$-RB(379-928) was individually tested for its ability to repress pol III transcription before use in an assay.

As shown in Figure 4.1, addition of nanogram amounts of GST-RB(379-928) to an in vitro pol III transcription assay can potently inhibit transcription of the specific pol III template. This is not a non-specific effect of the buffer composition of the recombinant RB, as an equivalent amount of the same buffer was added to the reaction shown in lane 1 which contains no recombinant protein (compare lanes 1 and 3& 4). As an additional control, GST that was made and purified in parallel to the RB was added to one of the reactions. Addition of the same amount of GST as the largest amount of RB added clearly demonstrated that the inhibition of pol III transcription by the GST-RB(379-928) is specific (compare lane 2 with lanes 3 & 4). Addition of GST did not inhibit transcription at all; rather perhaps it very slightly stimulated transcription compared with no addition of recombinant protein (compare lanes 1 & 2). This may be a non-specific stabilising effect on the pol III transcription components caused by the addition of protein.

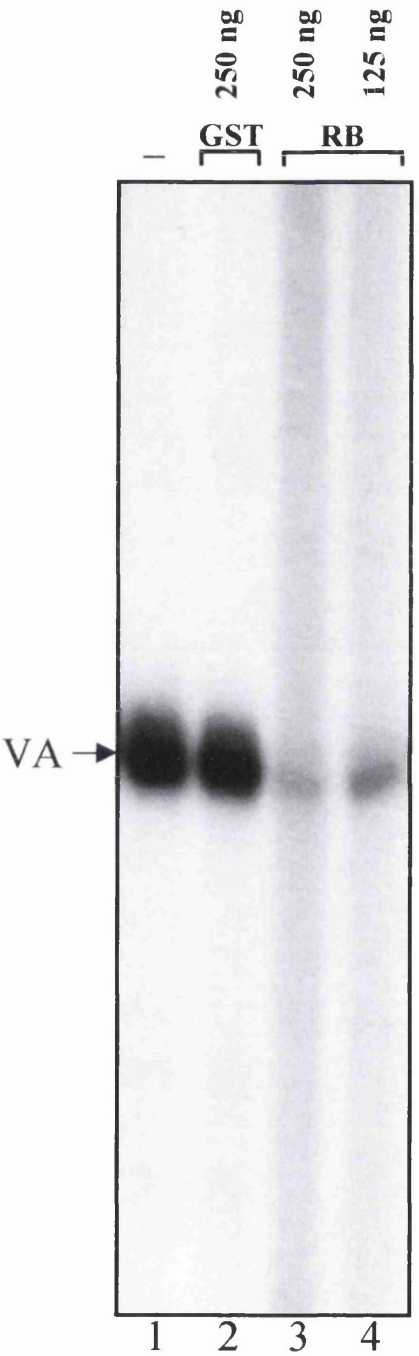
### 4.2.2 Recombinant RB potently represses pol III transcription in the presence of histone deacetylase inhibitor

Transcriptional repression by RB of E2F-regulated promoters involves at least two distinct mechanisms. RB binds to E2F within its transactivation domain, preventing it from interacting with the basal pol II transcription factor TFIID (448). However, the ability of RB to inhibit transactivation by E2F does not account for its ability to reduce transcription to below the basal levels observed when the E2F

**Figure 4.1**

**Repression of pol III transcription by recombinant RB**

250 ng of $pVA_I$ was preincubated for 15 min at $30^0$C with HeLa-PC-B (2μl) and HeLa-PC-C (2μl) (all lanes) in a reaction volume of 15μl containing GST-RB elution buffer (lane 1), 250 ng of GST (lane 2) or with 250 ng or 125ng of GST-RB(379-928) (lanes 3 and 4, respectively). Transcription was initiated by the addition of a mixture of rATP, rCTP, rGTP and [$\alpha^{32}$P]-UTP and was allowed to proceed for 1 h. Ethanol-precipitated transcription products were separated on a 7M urea 7% polyacrylamide sequencing gel and were visualised by autoradiography.


250 ng
250 ng
125 ng
–
GST
RB
VA
1
2
3
4

binding sites are unoccupied. Additionally, several RB mutants have been isolated that bind E2F but fail to significantly repress transcription of E2F-regulated promoters (52).

As discussed in the previous chapter, the acetylation of chromatin is an important mechanism by which transcription may be regulated. A number of transcriptional activators have been found to possess intrinsic histone acetyltransferase activity which for several coactivators has been shown to be essential for their activating function (303, 359, 500). Hence, the discovery that several transcriptional repressors, such as Mad and NCoR, associate with histone deacetylases and that this activity is essential for their repressive function (5, 308) came as little or no surprise. The possibility of an involvement of histone deacetylases in transcriptional repression by RB was loosely suggested by the finding that two RB binding proteins, RbAp46 and RbAp48, are part of the mammalian Sin3 deacetylase complex (52).

Recently, three different groups have independently shown that RB specifically interacts with the histone deacetylase HDAC1 both in vitro and in vivo (53, 346, 349). Furthermore, E2F1, RB and HDAC1 can form a trimeric complex in vitro (53, 349). However, an E2F mutant that is unable to bind RB also failed to bind HDAC1 (53). This suggested that RB might act as a bridging factor between HDAC1 and E2F to enable the recruitment of deacetylase activity to E2F-regulated promoters. The significance of the association between RB and histone deacetylase was tested by Luo et al. using the chromatin immunoprecipitation method (346). They found that the acetylation status of histones at a chromatinised promoter changed upon binding of RB (346). The deacetylation of histones is predicted to result in a more compact chromatin structure that is less accessible to transcription factors and thus inhibitory to transcription (177). Treatment of cells with the potent histone deacetylase inhibitor trichostatin A (TSA) relieved RB-mediated repression of some of the E2F-regulated promoters tested. Although this demonstrated that deacetylase activity is required for the full repressive effect of RB at some E2F-regulated promoters, other promoters seemed insensitive to treatment with TSA, indicating that the involvement of associated deacetylase activity in RB-mediated repression is selective.

RNA polymerase III transcription is also likely to be affected by acetylation, as exemplified by recent reports suggesting that TFIIIC2 possesses histone

acetyltransferase activity. It is plausible that the histone deacetylase activity associated with RB antagonises the HAT activity of TFIIIC2. To test whether the repression of pol III transcription by RB requires histone deacetylase activity, the effect of increasing amounts of TSA on transcription carried out in the presence or absence of exogenous RB was tested (503). Figure 4.2 shows the effects of TSA on in vitro transcription from the $VA_I$ template (503). The GST-RB (379-928) potently repressed transcription of the $VA_I$ gene (compare lanes 1 & 2). Repression was maintained in the presence of increasing amounts of TSA (even-numbered lanes). Transcription carried out in the presence of added GST was not affected by the presence of TSA (odd-numbered lanes), indicating that the lack of derepression in the presence of RB is not due to the TSA having a non-specific toxic effect on transcription.

It is plausible that RB utilises different mechanisms to repress transcription at different pol III templates. For example, some pol III templates may be more susceptible to acetylation than others. I therefore decided to test the effect of TSA on a tRNA gene, which has a weaker promoter than $VA_I$, and thus perhaps is more susceptible to acetylation. As Figure 4.3 shows, similar results were obtained to those with the $VA_I$ gene (503). Transcription carried out with nuclear extract in the absence of recombinant protein was largely unaffected by the inclusion of TSA at a concentration of ~500 nM in the transcription reaction mixture (lanes 1 & 2). Similarly, transcription carried out in the presence of 250 ng of GST and increasing amounts of TSA remained at a fairly constant level (lanes 3-6). Together, these results suggest that the TSA is not inhibitory to transcription and that in vitro transcription with endogenous factors is not affected by acetylation/deacetylation. As with the $VA_I$ template, inclusion of increasing amounts of TSA did not relieve repression by RB (lanes 7-10). TSA is a specific and irreversible inhibitor of histone deacetylases and a concentration of 100 nM is sufficient to block HDAC function. Repression by RB of tRNA transcription was maintained in the presence of ~500nM TSA. Clearly, recombinant RB is able to repress pol III transcription in vitro by a mechanism that does not require histone deacetylase activity.

**Figure 4.2**

**Repression of $VA_I$ transcription by RB is maintained in the presence of the histone deacetylase inhibitor Trichostatin A**

$pVA_I$ (250 ng) was transcribed using 10μg of HeLa nuclear extract that had been preincubated for 15 min at $30^0$C with 250 ng of GST (odd-numbered lanes) or GST-RB(379-928) (even-numbered lanes). Reactions 3 and 4 also contained 165 nM TSA and reactions 5 and 6 contained 330 nM TSA. Transcription was initiated by the addition of nucleotides and allowed to proceed for 1 h.

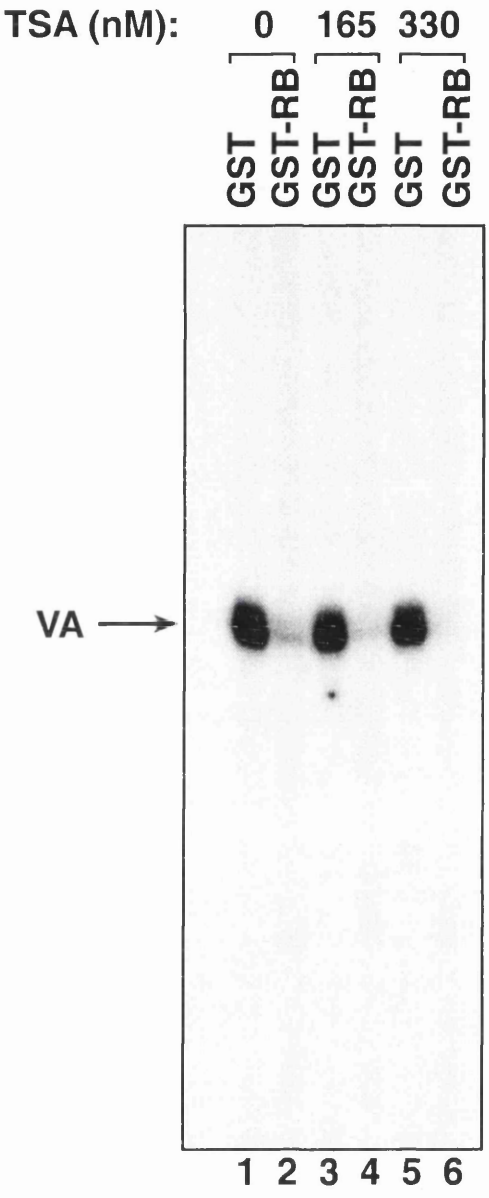
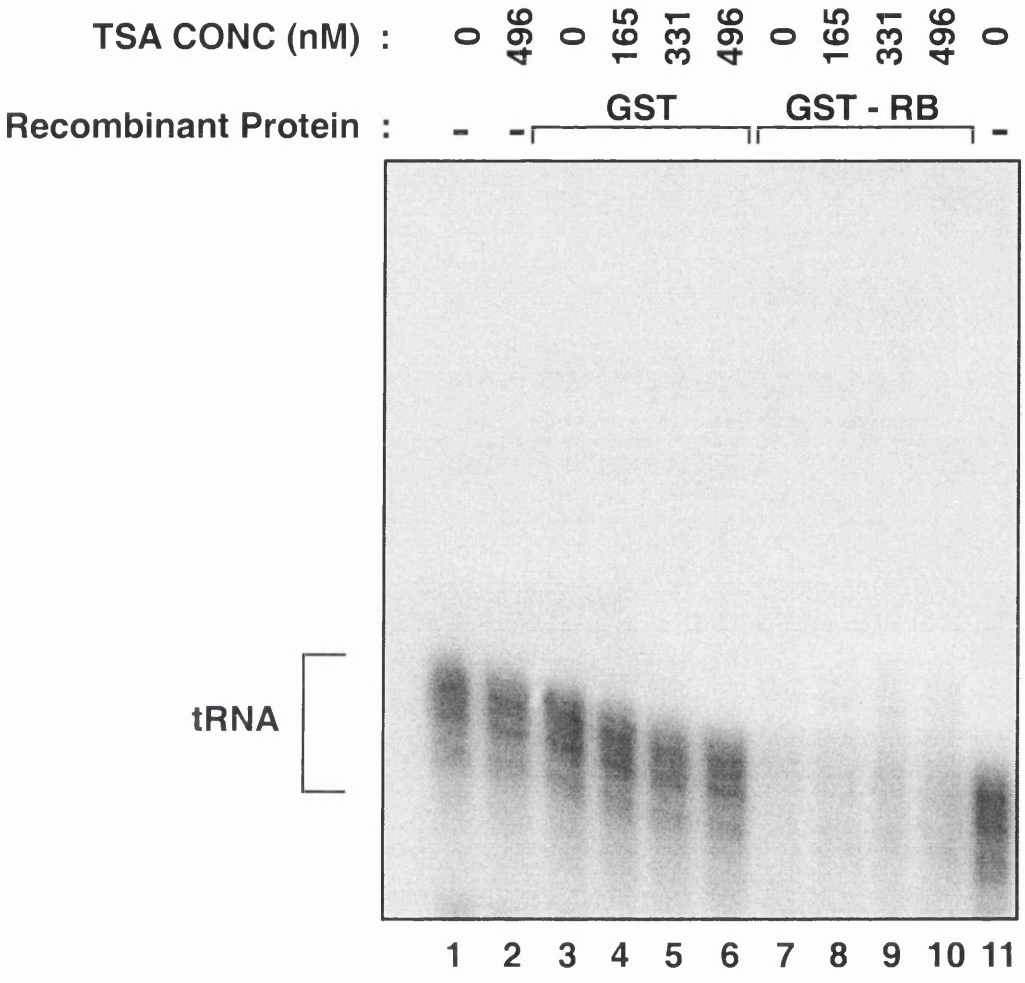

TSA (nM):
0
165
330
GST
GST-RB
GST
GST-RB
GST
GST-RB
VA
1 2 3 4 5 6

**Figure 4.3**

**Repression of tRNA synthesis by recombinant RB is unaffected by the presence of increasing concentrations of TSA**

250 ng of pLeu was transcribed using 10μg of HeLa nuclear extract that had been preincubated for 15 min at $30^0$C without addition (lanes 1,2 and 11), with 250 ng of GST (lanes 3-6) or with 250 ng of GST-RB(379-928) (lanes 7-10). TSA was included at a concentration of 165nM (lanes 4 and 8), 331nM (lanes 5 and 9) or 496nM (lanes 2, 6 and 10). Transcription was initiated by the addition of a mixture of rATP, rCTP, rGTP and [$\alpha^{32}$P]-UTP and was allowed to proceed for 1 h at $30^0$C. Ethanol-precipitated transcription products were separated on a 7M urea 7% polyacrylamide sequencing gel and were visualised by autoradiography.


TSA CONC (nM) :
0
496
0
165
331
496
0
165
331
496
0
Recombinant Protein :
-
-
GST
GST - RB
-
tRNA
1
2
3
4
5
6
7
8
9
10
11

### 4.2.3 Fractionation of HDAC1 and TFIIIB

It is surprising that TSA had no effect on pol III transcription in light of recent reports suggesting that TFIIIC, which is essential for transcription from both of the promoters investigated, has intrinsic HAT activity. However, the transcription assays were conducted using pol III templates carried in plasmid DNAs. It is very unlikely that the pol III templates would be assembled into their native chromatin conformation in this in vitro system, although the assembly of some histones onto the plasmid DNAs is plausible. The possibility therefore remained that acetylation or deacetylation does have an important role in the regulation of pol III templates in their natural chromatin environment. Furthermore, the possibility also existed that repression by RB of pol III transcription in its natural chromatin context involves histone deacetylase activity.

Although it has been demonstrated that endogenous RB and endogenous HDAC1 specifically interact with each other, it is not known what proportion of RB in vivo is associated with HDAC1 (53, 346, 349). This raises the question as to whether the endogenous RB that associates with TFIIIB is also associated with HDAC1.

HDAC1 preferentially binds to the active, hypophosphorylated form of RB, suggesting that its association is important for RB function (346). HDAC1 interacts with RB via an LXCXE-like motif (IACEE) (349); deletion of this motif severely compromises their coimmunoprecipitation (349). HDAC1 binds to the A/B pocket domain of RB (residues 379-792). This same domain of RB is required for its proliferation- and growth-suppressive functions and for its ability to inhibit pol III transcription (568). The viral oncoproteins of several DNA tumour viruses bind the same region of RB through an LXCXE motif. This disrupts the interaction between RB and HDAC1 and also the interaction between RB and E2F (53, 84). This suggests that the binding sites of HDAC1 and E2F on RB may overlap. However, E2F, RB and HDAC1 can form a trimeric complex in vitro (53, 349). In vivo, RB and HDAC1 cooperate to repress certain E2F-regulated promoters; in a $RB^{-/-}$ background the same promoters are insensitive to TSA, presumably because HDAC1 cannot be recruited (346).

It is well established that TFIIIB and RB specifically interact with each other in vitro and in vivo and at physiological ratios (314). Substitutions in the A/B pocket

domain of RB that disrupt this interaction prevent RB from repressing pol III transcription (93). Repression of pol III transcription by RB can also be overcome by adenoviral E1A and SV40 large T antigen (579). It is predicted that the binding of these viral oncoproteins to the pocket domain of RB disrupts the interaction between TFIIIB and RB (314). This suggests that TFIIIB and HDAC1 probably bind to the same region of RB, since the interaction of HDAC1 with RB is disrupted by viral oncoproteins. However, it is not known whether the binding sites of TFIIIB and HDAC1 on RB overlap or are distinct, or whether the binding of one precludes the binding of the other. E2F, RB and HDAC1 can form a trimeric complex in vitro despite viral oncoproteins being able to disrupt the binding both of HDAC1 and E2F to RB.

If the binding of HDAC1 and TFIIIB to RB is mutually exclusive it is unlikely, unless another deacetylase is involved, that the repression of pol III transcription by RB in vivo involves deacetylase activity. Immunoprecipitations were performed to see whether endogenous TFIIIB and HDAC1 specifically interact and, if they do, whether this occurs in a RB-dependent or a RB-independent manner. The HDAC1 polyclonal antiserum used was unable to coimmunoprecipitate BRF, an essential subunit of TFIIIB, from HeLa nuclear extracts (data not shown). The antiserum was also unable to coimmunoprecipitate RB from the same extracts (data not shown). However, this antiserum does immunoprecipitate and has previously been reported to coimmunoprecipitate RB from MCF7 extracts (388). This apparent discrepancy in the ability of the antiserum to coimmunoprecipitate RB is probably a reflection of the different extracts used. HeLa cells are HPV-transformed and contain high levels of the viral oncoprotein E7. HPV E7 protein has previously been shown to prevent RB from associating with deacetylase activity (53). Thus, the absence of RB in the immunoprecipitated material from HeLa nuclear extracts may be because none of the HDAC1 in these extracts is associated with RB. HeLa cells may also contain less HDAC1 than MCF7 cells which could result in less HDAC1 being immunoprecipitated and the levels of coimmunoprecipitated proteins could be reduced to below the levels of detection. The lack of a positive control for the immunoprecipitation precludes any interpretation regarding the absence of BRF in the immunoprecipitated material. Unfortunately, the similarity in size of HDAC1 and the heavy chain of immunoglobulins prevented a clear result from the converse

experiment of immunoprecipitating with a BRF antiserum or a RB antiserum and seeing if HDAC1 specifically coimmunoprecipitates (data not shown).

It was also tested whether endogenous HDAC1 and TFIIIB cofractionate during column chromatography. The consistent cofractionation of two proteins over a variety of different chromatographic columns is suggestive of a stable association. Western blotting of fractions from heparin gradient chromatography of a PC-B fraction revealed that HDAC1 cofractionates closely with TFIIIB activity through this column (data not shown). However, the bulk of the protein appears to have fractionated in similar fractions, suggesting that the cofractionation of TFIIIB and HDAC1 on this column may be fortuitous (data not shown).

Fractions from Mono Q gradient chromatography of a PC-B fraction were also tested. As Figure 4.4 shows, HDAC1 fractionates extremely heterogenously with some HDAC1 detectable in many of the fractions assayed and some adjacent fractions containing quite variable amounts of HDAC1. The column is clearly not overloaded, which could contribute to the heterogeneity, as HDAC1 is notably absent from the flowthrough (lane 2) indicating its specific retention on the column.

Fractionation of TFIIIB on a Mono Q gradient splits TFIIIB into two essential components called 0.38M-TFIIIB and 0.48M-TFIIIB, respectively (91, 340, 371). 0.38M-TFIIIB consists minimally of TBP and BRF. 0.48M-TFIIIB has not been characterised, but is thought to consist minimally of a human homologue of yeast B″ (340). 0.38M-TFIIIB activity was found to peak in fractions 54, 55 and 56 whereas 0.48M-TFIIIB activity peaked in fractions 57, 58 and 59 (White, R.J., unpublished observations). The 0.48M-TFIIIB peak fractions contain only very low levels of HDAC1 and clearly show a depletion in the levels of HDAC1 compared to the starting material, PC-B (compares lanes 10-12 with lane 1). It is likely that these fractions are significantly less concentrated than the starting material, however.

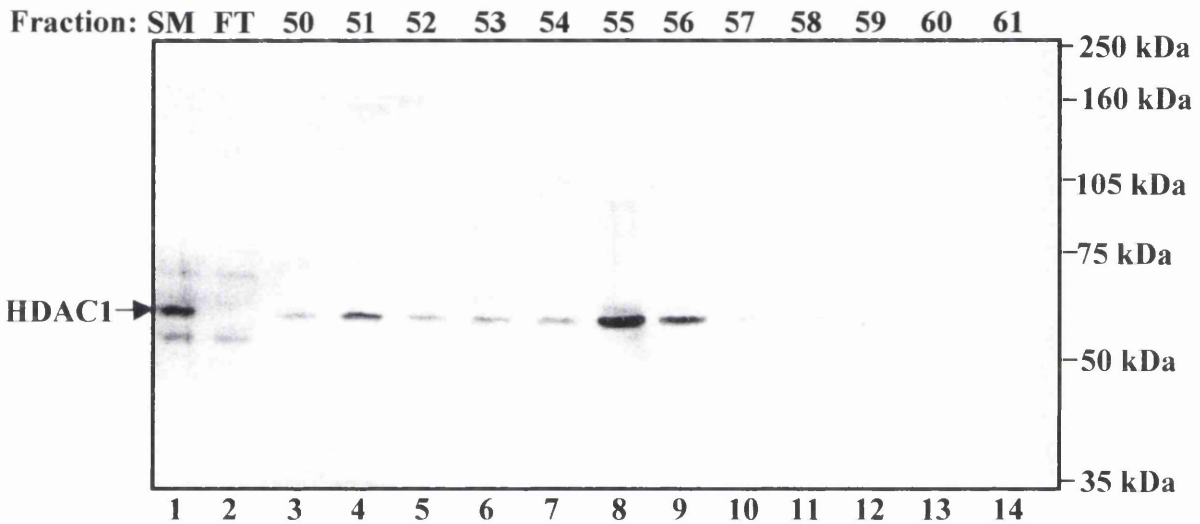
In contrast, two of the 0.38M-TFIIIB peak fractions contain very high levels of HDAC1 compared to the other eluted fractions. Fraction 55 is clearly enriched in HDAC1 compared to the starting material, PC-B, and while fraction 56 contains a similar amount of HDAC1 to the starting material the fraction is probably significantly more dilute than the PC-B. Fractions 55 and 56 contain much more HDAC1 than any other eluted fraction assayed with the sole exception of fraction 51

**Figure 4.4**

**Fractionation of HDAC1 during gradient chromatography of PC-B on Mono Q**

PC-B was applied to an FPLC Mono Q column. After washing extensively with buffer Q containing 100mM KCl, fractions were eluted with a linear 100-500mM KCl gradient. Fractions were then extensively dialysed into LDB, aliquoted, snap frozen and stored at $-80^0$C. Equal volumes (15µl) of the various fractions* were resolved on a SDS-7.8% polyacrylamide gel and then analysed by Western blotting with an antiserum specific for HDAC1 (C19, Santa Cruz).

* Mono Q fractions were kindly provided by R. J. White


Fraction: SM FT 50 51 52 53 54 55 56 57 58 59 60 61
250 kDa
160 kDa
105 kDa
75 kDa
50 kDa
35 kDa
HDAC1
1 2 3 4 5 6 7 8 9 10 11 12 13 14

which contains approximately two-thirds as much HDAC1 as fraction 56 (compare lanes 8 & 9 with lanes 3-7 and 10-14). Although fractions 55 and 56 are peak 0.38M-TFIIIB fractions, suggesting that HDAC1 and TFIIIB may associate, fraction 54 also contains high levels of 0.38M-TFIIIB yet contains low levels of HDAC1 comparable to other fractions with very little TFIIIB activity. The most straightforward interpretation of this is that the high levels of HDAC1 in fractions 55 and 56 are not due to a stable association with TFIIIB. However, without knowing how TFIIIB itself has fractionated interpretation is near impossible.

Although the TFIIIB activity of individual fractions is probably a good indication of the amounts of TFIIIB protein in the fractions, it is not a substitute and may not accurately reflect the distribution of the protein. For example, fractions 52 and 53 have much lower 0.38M-TFIIIB activity than fractions 54-56 but it is plausible that they actually contain higher amounts of TFIIIB. These two fractions perhaps contain high levels of a specific repressor of TFIIIB that has cofractionated but which is not associated with the TFIIIB in fractions 54-56. Indeed RB, which has previously been demonstrated to specifically and stably interact with and repress TFIIIB, peaks in fractions 52 and 53 with much lower levels in fractions 54-56 (data not shown). Unfortunately, the BRF antisera cross-reacted badly with other proteins of a similar size to BRF in the fractions, which meant the BRF content of individual fractions was uncertain (data not shown). The amounts of 0.48M-TFIIIB protein present in individual fractions was prevented from being determined by a lack of knowledge as to its molecular composition and a lack of antibodies against any of its components.

The functional significance of any interaction between TFIIIB and HDAC1 is also important; for example, if HDAC1 inactivates TFIIIB higher levels of HDAC1 may be expected in fractions of lower TFIIIB activity depending on the protein levels. Collectively, the available evidence on the fractionation of HDAC1 and TFIIIB on a Mono Q gradient is not supportive of a close cofractionation of the two proteins. However, the results are open to differential interpretation due to uncertainty as to how reliable an indicator TFIIIB activity is of the levels of TFIIIB protein in the fractions.

Recently, it has been shown that RB does not require histone deacetylase activity for repression of pol III transcription in vivo (503). This is consistent with the lack of requirement observed in vitro (see Figs 4.2 & 4.3). As well as using

transiently transfected pol III templates, which as in the in vitro system may not be properly assembled into chromatin, the effects of inhibiting deacetylase on transcription of an endogenous gene in its natural chromatin environment was also tested (503). Untransfected $Rb^{-/-}$ and $Rb^{+/+}$ mouse embryonic fibroblasts were cultured in the presence or absence of TSA and total RNA was then extracted for analysis of the expression of the B2 gene by northern blotting (503). The B2 gene family was chosen for the northern analysis as previously it has been shown that these genes are highly susceptible to chromatin-mediated repression. Interestingly, TSA elicited an increase in B2 expression levels in both types of fibroblasts (503). This effect is specific, since the levels of a pol II transcript encoding acidic ribosomal phosphoprotein P0 (ARPP P0) remained relatively constant. This represents the first in vivo evidence that acetylation can influence pol III transcription. It may be that deacetylases function in antagonising the reported HAT activity of TFIIIC2. The relative increase in B2 RNA levels in response to TSA was no greater for the $Rb^{+/+}$ cells than for the $Rb^{-/-}$ cells and the higher levels of expression in the $Rb^{-/-}$ cells was maintained (503). Thus, the effect of inhibiting histone deacetylases on pol III transcription in vivo is RB-independent. It may be that an endogenous histone deacetylase can interact directly with a component of the pol III transcriptional apparatus or is recruited via a protein other than RB. The western blots of the Mono Q fractions clearly showed that there is a lot of endogenous HDAC1 that is not associated with RB (data not shown). Alternatively, the effect of HDAC function on pol III transcription may be indirect.

TSA treatment strongly stimulated transcription of a chromosomally integrated class III gene but had no effect in vitro and only a very slight stimulatory effect on transcription of a transiently transfected template (503). This suggests that histone deacetylases and the reported HAT activity of TFIIIC2 probably influence pol III transcription through the acetylation or deacetylation of nucleosomal histone proteins and the modulation of local chromatin structure. Although it remains possible that the physiological target of histone deacetylases and TFIIIC2 HAT activity are non-histone proteins, it is less likely that the effect of acetylation or deacetylation of these proteins would be restricted to pol III transcription carried out in a chromatin environment. In contrast to the TSA effect on pol III transcription that appears to be restricted to pol III templates that are fully assembled into chromatin, TSA sensitivity of E2F-regulated promoters was also observed using

transiently transfected templates (53, 349) which are only partially assembled into chromatin. This suggests that transcription of E2F-regulated promoters may be regulated by the acetylation or deacetylation of non-histone proteins or it may be that these genes are more susceptible to chromatin-mediated repression.

### 4.2.4 Immunopurification of a pol III holoenzyme

A variety of different mechanisms have been proposed for how RB specifically inactivates TFIIIB. One possibility is that RB disrupts the TFIIIB complex in some way, perhaps displacing one or more components. Another nuclear phosphoprotein called Dr1 utilises this mechanism to repress pol III transcription. Dr1 can specifically displace BRF from its interaction with TBP (576). Mutational analysis suggests that BRF and Dr1 compete directly for overlapping binding sites on TBP (576). The binding of BRF to TBP is maintained in the presence of RB, consistent with the ability of anti-RB antibodies to coimmunoprecipitate both of these proteins (314, 503). However, RB may displace an unidentified component of TFIIIB from the complex. Alternatively, intramolecular interactions within TFIIIB may be disrupted that inactivate the complex without any component actually being displaced from it.

RB contains regions of homology to both BRF and TBP, suggesting there may be some structural similarity (314). Larminie et al. proposed a model in which RB mimics TBP and BRF enabling it to disrupt interactions of these two proteins with other components of the complex (314). Subsequently, it has been found that the regions of homology of RB and TBP bear no structural resemblance. It remains to be determined whether the regions of homology of RB and BRF are structurally alike (275).

RB can inhibit pol I transcription by preventing UBF from binding to the rDNA promoter (539). Similarly, RB may inhibit TFIIIB by preventing contacts with promoter DNA. For most pol III templates, TFIIIB is recruited to the promoter through protein–protein interactions with TFIIIC. In yeast, once TFIIIB has been recruited TFIIIC can be stripped away and TFIIIB alone will remain bound to promoter DNA capable of supporting multiple rounds of transcription initiation (263). In contrast, in vertebrates any interactions between TFIIIB and DNA are very

weak and studies to date have been unable to detect a TFIIIB footprint (568). There is circumstantial evidence, however, that DNA contacts are made and that their disruption will impair transcription (568).

RB may inhibit TFIIIB by disrupting any of its intermolecular interactions that are required for transcription. The interactions of TFIIIB with TFIIIC, pol III or promoter DNA are all potential targets for disruption. Below I describe the immunoisolation of a pol III complex containing TFIIIB, TFIIIC and RNA polymerase III. This provided the opportunity to test whether RB represses pol III transcription by specifically disrupting the interactions between TFIIIB and TFIIIC or TFIIIB and pol III.

Using isolated components, the formation of a functional preinitiation complex on pol III templates has been demonstrated to occur in a precisely ordered and stepwise fashion (318). However, alternative assembly pathways may exist in vivo. For RNA polymerase II transcription, a similar stepwise assembly pathway has been defined in vitro. However, in vivo several large complexes that contain pol II, some or all of the general initiation factors and various cofactors have been discovered (187, 286). These so-called holoenzymes can exist in the absence of DNA and suggest an alternative assembly pathway of the pol II preinitiation complex in vivo whereby many or all the essential initiation components are simultaneously recruited to the promoter as part of this preassembled complex (187, 286). In yeast, evidence from a combined biochemical and genetic approach by Young and co-workers strongly suggests that the physiologically relevant form of pol II that is recruited to most pol II promoters in vivo is a pol II holoenzyme (38, 521).

Such holoenzymes are seemingly not restricted to the pol II system. Functional pol III complexes containing TFIIIB, TFIIIC and pol III, but which are not associated with DNA, have been isolated by gel filtration or density gradient centrifugation both from Drosophila and human cell extracts (65, 585). The stability of these complexes to 1M KCl suggests that they are unlikely to result from the fortuitous aggregation of constituent components (585). Further support for the possible existence of a pol III holoenzyme is provided by the recent immunopurification from HeLa cell extracts of a complex that contains all the essential components for tRNA, 5S RNA and $VA_I$ transcription (551). Using immobilised anti-FLAG monoclonal antibody, this complex was purified from a HeLa cell line that constitutively expresses a FLAG epitope-tagged subunit of pol III.

After washing with low salt so as not disturb any weak interactions, the complex was eluted with FLAG peptide. In addition to the various pol III subunits, western blot analysis revealed that low levels of BRF, TBP, TFIIICα and TFIIICβ had co-purified. Footprinting analyses revealed that TFIIIC1 was also present in the eluate. In contrast, none of these proteins were detectable in the eluate from a control immunopurification using nuclear extract from untransfected HeLa cells.

An RNA polymerase holoenzyme as originally defined in prokaryotes is an entity composed of RNA polymerase and other subunits that can initiate specific transcription autonomously. Many of the reported pol II "holoenzymes" required additional factors in order to carry out transcription and thus strictly do not really represent holoenzymes. In contrast, the immunopurified pol III complex described above was capable of pol III transcription of tRNA, 5S RNA and $VA_I$ genes alone and thus may be regarded as a true holoenzyme (551).

Unlike the pol III complexes isolated by gel filtration and density gradient centrifugation, this immunopurified complex was disrupted by a moderate increase in salt concentration; however, it was stable to dilution, suggesting that it was unlikely to have resulted from a concentration-dependent non-specific aggregation of components (551). Preincubation of the nuclear extract prior to immunopurification, with ethidium bromide or Chromomycin A3 which are reagents commonly used to distinguish between DNA-dependent and DNA-independent protein associations, had no effect on the levels of holoenzyme immunopurified (551). Therefore, DNA was unlikely to be responsible for the association of TFIIIB, TFIIIC and pol III in this complex.

The immunopurification of a complex containing TFIIIB, TFIIIC and pol III by Wang et al.(551) raised the possibility that the same may be achievable using one of the various antisera that we have against specific components of the basal pol III transcriptional apparatus. This was tested by carrying out immunopurifications, essentially by the same method as described in the previous chapter, with the different antisera and assaying for the coimmunopurification of TFIIB, TFIIIC and pol III. Unfractionated HeLa nuclear extract was used as the starting material as phosphocellulose chromatography disrupts the holoenzyme complex (551), which may partly explain why the existence of such complexes has largely eluded

detection. The other advantage of using an unfractionated extract is that TFIIIB, TFIIIC and pol III will be present at physiological ratios.

After recirculating a mixture of preincubated antiserum and nuclear extract through a column of protein A-Sepharose for ~1h at $4^0$C, any unbound material was removed by washing with 20 cvs of a low salt buffer (CB100). Bound material was then eluted with a buffer containing 2M salt and 2M urea, as previously. Eluted material was tested for the presence of TFIIIB and TFIIIC by in vitro transcription assays. The presence of RNA polymerases in the eluted material was assayed by virtue of their ability to transcribe poly(dA.dT) in a transcription factor-independent manner.

The mouse monoclonal antibody MTBP6 (424), which specifically recognises an epitope in the N-terminal region of TBP, was found to consistently coimmunopurify low levels of TFIIIC and polymerase with TFIIIB immunopurified from HeLa nuclear extract (Figure 4.5 and data not shown). The level of immunodepletion of TFIIIB from the nuclear extract was rather poor (Fig. 4.5 **A**, compare lane 1 with lanes 2 & 11), as previously observed with the BRF antisera 128 and 330, when using such crude extract. Despite this, reasonable amounts of TFIIIB activity were detected in the eluted fractions from the two MTBP6 columns (Fig 4.5 **A**, lanes 4-10 & 13-18), whereas no activity was detected in the FT fractions (lanes 3 & 12), which is consistent with the specific immunopurification of TFIIIB by MTBP6. A control immunopurification was carried out in parallel using a rabbbit polyclonal antibody called SI-1 raised against another N-terminal region of TBP. In contrast to MTBP6, the SI-1 antibody consistently failed to deplete TFIIIB activity from extracts when used in immunoprecipitations (data not shown). In support of the specificity of the immunopurification of TFIIIB by MTBP6, there was no TFIIIB activity either in the FT or any of the other eluates from the SI-1 column, nor did any appear to have been depleted from the starting material (data not shown).

As Figure 4.5 **B** shows, very low levels of TFIIIC were also immunodepleted from the starting material (compare lanes 1 and 2). As for TFIIIB, no TFIIIC was found in the flowthrough (Fig. 4.5 **B**, lane 3). The depleted TFIIIC activity eluted entirely in fractions containing TFIIIB activity. For both of the columns, the peak fraction of TFIIIC activity was also the peak fraction of TFIIIB activity (compare Fig.4.5 **A** & **B**, lanes 6 and lanes 14). Clearly, a very small amount of TFIIIC in the nuclear extract copurified with the TFIIIB specifically immunopurified by the

MTBP6 antibody. Some polymerase was also retained on the column and was found to elute in the same peak TFIIIB fractions as TFIIIC (Fig. 4.5 **C**).

The retention of small amounts of TFIIIC and polymerase on the column with TFIIIB and their elution in the same fractions suggested that some TFIIIB, TFIIIC and pol might be specifically associated together in a complex. However, it was also possible that the copurification of small amounts of TFIIIC and polymerase with TFIIIB was entirely fortuitous due to non-specific interactions with the column or with each other and TFIIIB. The former possibility was tested by assaying the fractions from the control SI-1 column to see if any TFIIIC or polymerase binds independently of TFIIIB. No TFIIIC or polymerase was found to bind to the SI-1 column, which suggests that their retention on the MTBP6 column was not due to adventitious association with protein A-Sepharose.

The BRF antisera 128 and 330 which were shown in the previous chapter to specifically immunopurify TFIIIB from nuclear extract, were also tested to see if any TFIIIC or polymerase is copurified. Significantly, the two antisera, which immunopurify similar amounts of TFIIIB, behaved very differently in their ability to copurify TFIIIC and polymerase. The 330 antiserum, like MTBP6, consistently copurified TFIIIC and polymerase with TFIIIB (data not shown). In contrast, the 128 antiserum consistently failed to copurify TFIIIC or polymerase with TFIIIB (data not shown). The inability of 128 to copurify TFIIIC or pol and the consistency of these results renders it extremely unlikely that non-specific interactions are responsible for the coimmunopurification of TFIIIB, TFIIIC and pol observed using MTBP6 or 330. The epitope on BRF that 128 recognises may be involved in the specific interactions of BRF with TFIIIC and pol III or may be spatially close to these regions, thus preventing the antibody from precipitating any TFIIIB in the extracts that is specifically associated with TFIIIC and polymerase.

Although the results indicate that the coimmunopurification of TFIIIB, TFIIIC and pol III is caused by specific interactions between the components, the data provide no information as to how this is achieved. The most obvious mechanism is that the three components form a trimeric complex. However, TFIIIB can interact directly both with TFIIIC and pol III, raising the alternative possibility that partial complexes such as TFIIIB-TFIIIC and TFIIIB-pol III are responsible for the coimmunopurification of the three components.

**Figure 4.5 - Copurification of low levels of TFIIIC and polymerase activity with TFIIIB immunopurified from HeLa nuclear extract using a monoclonal antiserum specific for TBP**

The results are shown from two separate immunoaffinity columns performed in parallel in which different volumes of the MTBP6 antibody contained in tissue culture supernatant was used. Lanes 2-10 represent fractions from the column in which 500µl of MTBP6 was preincubated with 1ml of nuclear extract prior to recirculation through protein A-Sepharose. Lanes 12-18 represent fractions from the column in which 1ml of MTBP6 was used. For both columns, numbered fractions (eg, 1,2,3...) correspond to fractions sequentially eluted with CB buffer containing 2M KCl and 2M urea, obtained after washing bound material with 20cvs of low salt buffer (CB100). R: recirculated starting material. FT: flowthrough. The starting material used (SM) was the same HeLa nuclear extract for both columns.

**A) TFIIIB activity of eluted fractions**

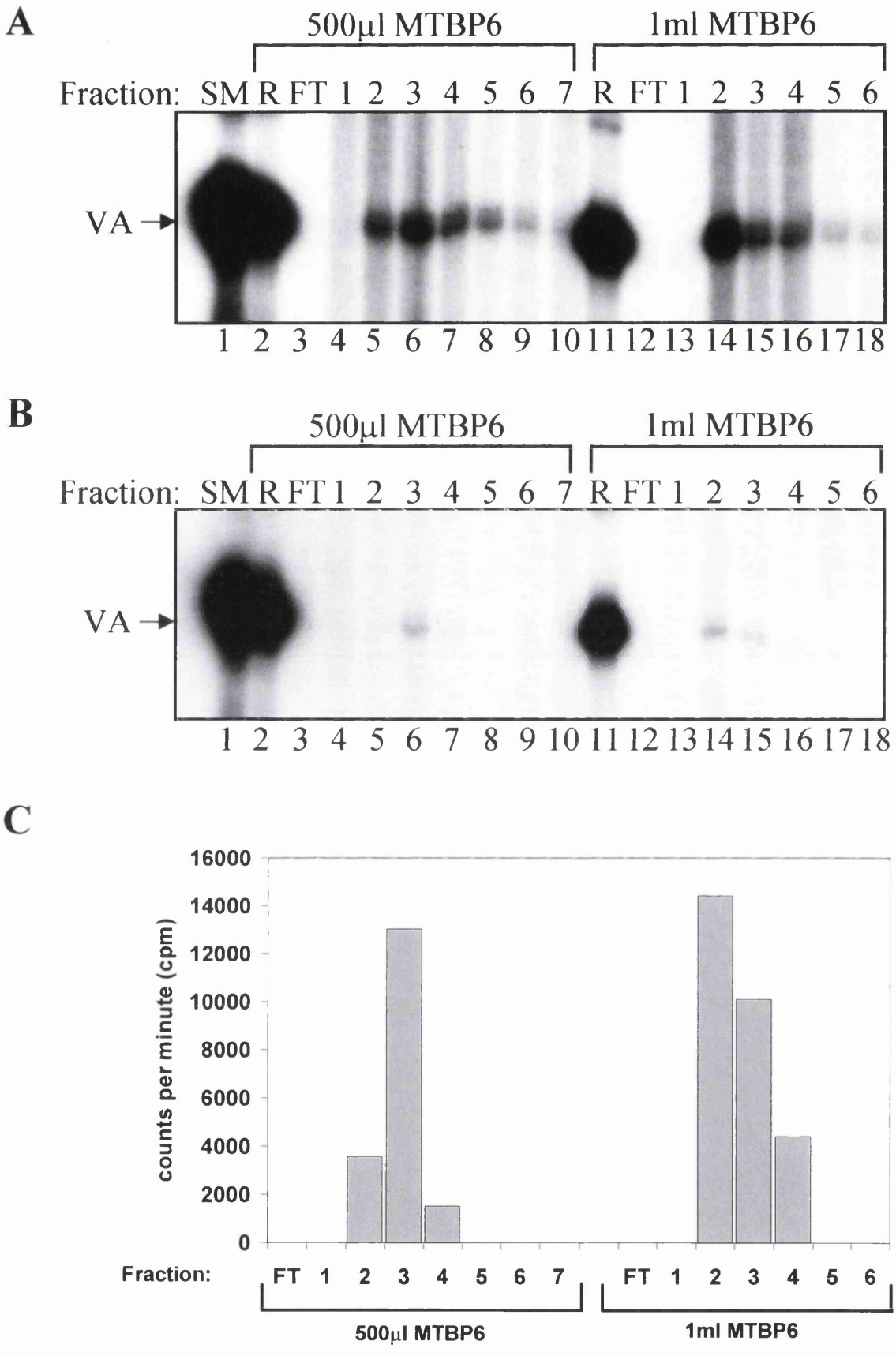
To assay for TFIIIB activity, in vitro pol III transcription was reconstituted using 2µl of PC-C as a source of TFIIIC and pol III, 250 ng of $pVA_I$ as the specific pol III template and 12µl of the individual fraction as the sole source of any TFIIIB. Note only 2µl of SM and R were assayed rather than 12µl.

**B) TFIIIC activity of eluted fractions**

Fractions were assayed for TFIIIC by reconstituted pol III transcription also, essentially as described in (**A**), except a PC-B fraction was used rather than PC-C, the fractions themselves providing the sole source of any TFIIIC.

**C) Polymerase activity of eluted fractions**

Equal volumes of fractions were assayed for polymerase content by their ability to transcribe poly(dA.dT). Levels of polymerisation were determined by the amounts of $[\alpha^{32}P]$-UTP incorporated into transcripts. At the end of the reaction, samples were spotted onto phosphocellulose discs, to which transcripts can bind. These discs were washed extensively to remove any unincorporated label. The levels of polymerisation were then quantitated by scintillation counting of the discs. Reactions were performed in the presence of 1µg/ml α-amanitin.


A
500µl MTBP6
1ml MTBP6
Fraction: SM R FT 1 2 3 4 5 6 7 R FT 1 2 3 4 5 6
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
500µl MTBP6
1ml MTBP6
Fraction: SM R FT 1 2 3 4 5 6 7 R FT 1 2 3 4 5 6
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
C
counts per minute (cpm)
16000
14000
12000
10000
8000
6000
4000
2000
0
Fraction: FT 1 2 3 4 5 6 7 FT 1 2 3 4 5 6
500µl MTBP6
1ml MTBP6

The TFIIICβ antiserum, 4286, was also found to consistently coimmunopurify TFIIIB, TFIIIC and pol III, providing further support for the specific association of these three components (data not shown). Although TFIIIC can interact directly with TFIIIB and there is recent evidence for a comparable interaction of TFIIIC with pol III (129), this latter interaction may be very weak since TFIIIB is essential for the recruitment of pol III to TFIIIC-bound promoters. One of the principal functions of TFIIIB within the transcription complex is thought to be for it to act as a bridging factor between TFIIIC and pol III (568). Thus, the coimmunopurification of TFIIIB, TFIIIC and pol by 4286 is more consistent with the purification of a trimeric complex than partial complexes.

Regardless of which antiserum was used, much lower levels of the two copurifying components were depleted than the component to which the antiserum was raised. This is consistent with the results of Wang et al. (551) and may reflect a low abundance of pol III "holoenzymes" in the cell relative to the abundance of the "free" forms of the different components. It is also quite probable that the epitopes recognised by the antisera are more accessible on the "free" forms, which will bias the immunoprecipitation in their favour.

The consistent copurification of TFIIIB, TFIIIC and polymerase using three different antisera against three different components of the pol III machinery is supportive of the existence of pol III complexes akin to the pol III holoenzyme of Wang et al.(551). However, stringent controls to eliminate the possible involvement of DNA have to date not been carried out. Furthermore, none of the fractions assayed were able to support in vitro pol III transcription in the absence of any other factors; thus these complexes do not represent true pol III holoenzymes that can autonomously initiate transcription (data not shown). It may be an unidentified essential component is missing. Alternatively, the copurification may have resulted from partial complexes, which are unable to assemble into a functional preinitiation complex. Another possibility is quenching of the holoenzyme activity; for example, excess "free" TFIIIC in the same fraction as the TFIIICβ-immunopurified complex is likely to compete directly with the holoenzyme for binding pol III promoters, thus preventing transcription. Regardless, these complexes enabled the effect of RB on the intermolecular interactions of TFIIIB with pol III and TFIIIC to be tested.

### 4.2.5 RB disrupts the interaction between TFIIIB and TFIIIC

To assay for any effects of RB, immunopurifications were carried out as described above, except that after washing the immobilised pol III "holoenzyme" with low salt buffer, the immunopurified complex was exposed to a solution of recombinant RB. This solution was then collected to assay for the presence of any component displaced by RB. The column was washed again briefly before elution of any protein that remained bound. This eluate was assayed for the specific disappearance of any component that is normally eluted here.

Since RB potently represses pol III transcription, any TFIIIB or TFIIIC eluted in the solution containing recombinant RB may not be detected by in vitro transcription assays. Instead, the presence of TFIIIC in any of the fractions was assayed on the basis of its specific DNA-binding activity, rather than its transcriptional activity. TFIIIC binds specifically to the B-block region of type II promoters. Figure 4.6 **A** shows the results of an electrophoretic mobility shift assay (EMSA) using a radiolabelled B-block oligonucleotide to assay specifically for TFIIIC DNA-binding activity in the various fractions from two MTBP6 immunoaffinity columns. For one of the MTBP6 columns, the immunoimmmobilised pol III "holoenzyme" was exposed to a solution of GST-RB(379-928) (Fig. 4.6 **A**, lanes 8-16). For the sister MTBP6 column on which purification was performed in parallel, the immunoimmobilised pol III "holoenzyme" was exposed to a solution of recombinant GST (Fig. 4.6 **A**, lanes 1-7).

As expected, for the column exposed to GST, strong TFIIIC DNA-binding activity was detected in the high salt eluates (Fig. 4.6 **A**, lanes 4-7), whereas TFIIIC DNA-binding activity was absent from the solution containing GST, which was slowly recirculated through the column for ~ 0.5 h (Fig. 4.6 **A**, lanes 1-3). A strikingly different result was obtained for the column exposed to GST-RB(379-928). Although, some TFIIIC DNA-binding activity was detected in the high salt eluates, this was much reduced compared with that of the corresponding eluates from the GST-exposed column (Fig 4.6 **A**, compare lanes 13-16 with lanes 4-7), suggesting that RB might have displaced some of the TFIIIC specifically bound to TFIIIB. This was confirmed by the appearance of significant amounts of TFIIIC DNA-binding activity in the fractions of the recirculated solution of recombinant RB (Fig. 4.6 **A**, lanes 8-12).

**Figure 4.6**

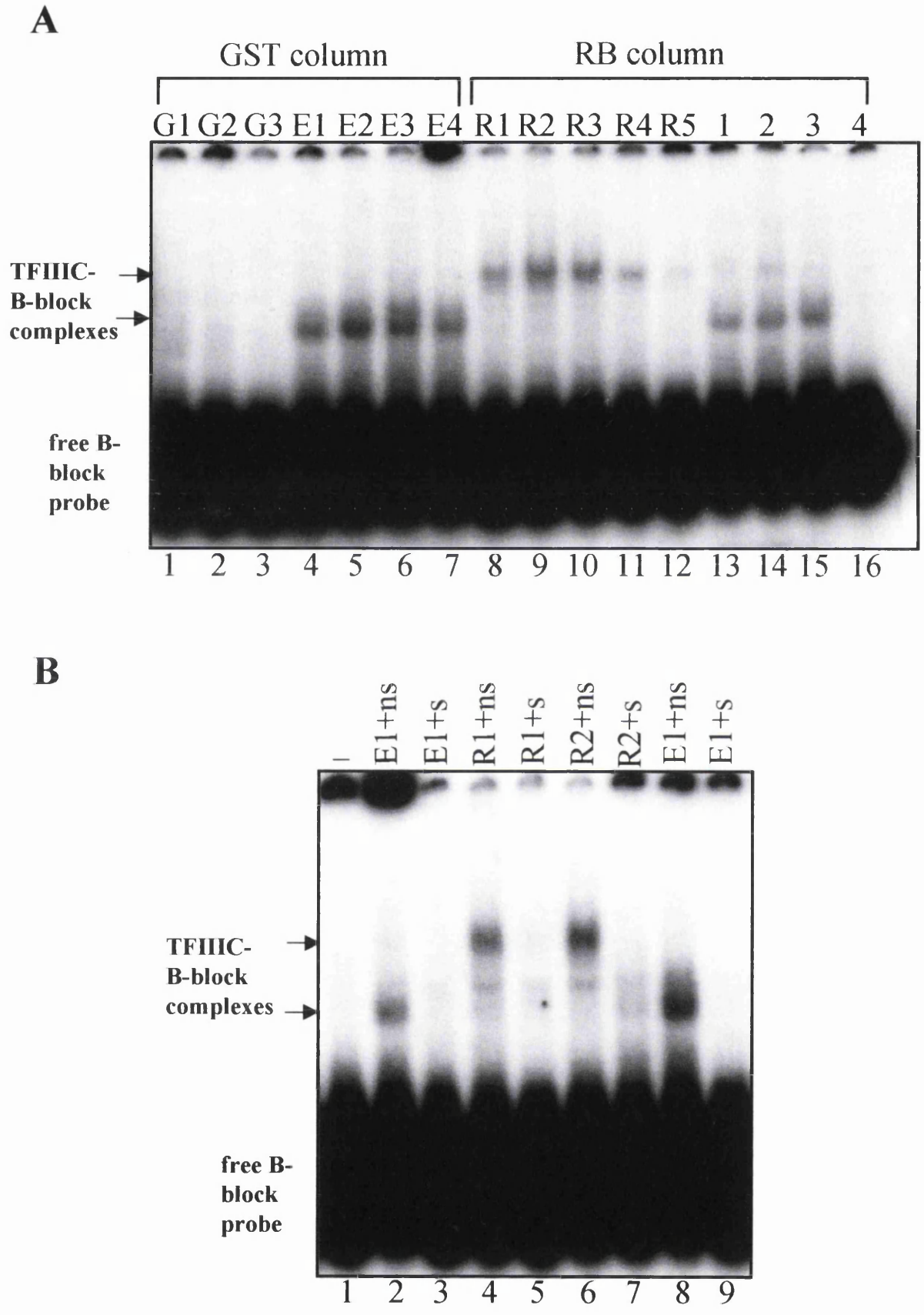
**RB displaces TFIIIC from immunopurified complexes containing TFIIIB, TFIIIC and polymerase**

**A)** EMSA using the B-block oligonucleotide as probe to assay for TFIIIC DNA-binding activity in the various fractions from two MTBP6 immunoaffinity columns to which an immunopurified complex of TFIIIB, TFIIIC and polymerase was bound. One of the columns was exposed to a solution of GST prior to elution with buffer containing 2M salt and 2M urea, whereas the other column was exposed to RB. The TFIIIC DNA-binding activity of fractions from the GST-exposed column are shown in lanes 1-7 and that of fractions from the RB-exposed column are shown in lanes 8-16. Reactions 1-3 contained fractions of the recirculated solution of GST and reactions 4-7 contained fractions eluted with high salt and urea from the same column. Reactions 8-12 contained sequentially collected fractions of the recirculated solution of GST-RB(379-928). Reactions 13-16 contained the 2M salt/2M urea fractions from the RB-exposed column.

4μl of each fraction was tested. To prevent non-specific binding to the probe the fractions were preincubated for 15 min at $30^0$C with 1μg of polydIdC and 100ng of non-specific oligonucleotide prior to addition of 0.5ng of [$\gamma^{32}$P]-ATP labelled B-block oligonucleotide. Formation of DNA-protein complexes was analysed by electrophoresis on a native 4% polyacrylamide gel.

**B)** EMSA of fractions from two 330 immunoaffinity columns to which an immunopurified complex of TFIIIB, TFIIIC and polymerase was bound. Reactions 2 and 3 contained a high salt fraction from a GST-exposed column. Reactions 4-9 contained fractions from a RB-exposed column. Reactions 4-7 contained fractions from the recirculated solution of RB, whereas reactions 8 & 9 contained a high salt fraction. Lane 1 contained probe only. Reactions of odd-numbered lanes contained 100ng of unlabelled B-block oligonucleotide as a specific competitor. Even-numbered lanes contained non-specific competitor.

ns: non-specific competitor; s: specific competitor


A
GST column
RB column
G1 G2 G3 E1 E2 E3 E4 R1 R2 R3 R4 R5 1 2 3 4
TFIIIC-
B-block
complexes
free B-
block
probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B
–
E1+ns
E1+s
R1+ns
R1+s
R2+ns
R2+s
E1+ns
E1+s
TFIIIC-
B-block
complexes
free B-
block
probe
1 2 3 4 5 6 7 8 9

The various RB or GST eluates and high salt eluates were also assayed for the presence of TFIIIB and polymerase. In vitro transcription assays revealed that TFIIIB was retained on the column after exposure to RB and eluted in the high salt eluates, the same was true for polymerase (data not shown). Thus, the appearance of TFIIIC in the RB eluate does not result from the displacement of the whole "holoenzyme" or a TFIIIB-TFIIIC subcomplex in which the integrity of the interaction between TFIIIB and TFIIIC may be maintained. It is only TFIIIC that is displaced strongly, suggesting that RB disrupts the intermolecular interaction between TFIIIB and TFIIIC.

The lack of TFIIIC DNA-binding activity in the GST fractions of the control column clearly demonstrates that the displacement of TFIIIC is a specific effect of RB rather than a non-specific effect due to the addition of excess amounts of recombinant protein. However, the reduced mobility of the TFIIIC-B-block DNA complex in the RB eluates compared to the mobility of the complex in the high salt eluates is curious. This is not unusual for TFIIIC; TFIIIC-B-block DNA complexes of varying mobilities are often found when TFIIIC-containing fractions are assayed. Generally, more dilute fractions tend to result in complexes of higher mobility. The molecular composition of the different shifts commonly produced is unknown. TFIIIC1, which is not required for the B-block binding activity of TFIIIC, may not be part of some of the complexes. The interaction of other proteins with TFIIIC is also likely to affect mobility. It is possible that the reduced mobility of the TFIIIC-B-block DNA complex in the RB eluates is caused by the binding of recombinant RB to TFIIIC. Indeed, an interaction between TFIIIC2 and overexpressed RB has been previously reported (93). However, the anti-RB antibodies tested were unable to supershift the complex and the addition of recombinant RB to the high salt eluates had no effect on mobility of the shift obtained with these fractions (data not shown).

The effect of GST and GST-RB(379-928) on a pol III "holoenzyme" purified using the BRF antiserum 330 was also tested. As for the "holoenzyme" purified using the anti-TBP antibody MTBP6, RB specifically displaced TFIIIC from the complex. As Figure 4.6 **B** shows, the RB eluates from this column also produced a different TFIIIC shift to the high salt eluates. It was essential to be sure that the different shifts obtained were all caused by the specific binding of TFIIIC to the B-block oligonucleotide, as opposed to the non-specific binding of some other protein in the fractions. To test this the fractions were incubated with excess unlabelled

competitor DNA prior to addition of labelled B-block oligonucleotide. Two types of competitor DNA were used; non-specific competitor which differs in sequence to the B-block oligonucleotide and specific competitor which is identical to the B-block oligonucleotide probe but unlabelled. Thus, the intensity of a shift caused by the non-specific binding of protein will be significantly reduced by addition of either competitor DNA, as both will be able to compete for binding of the protein. In contrast, the intensity of a shift caused by specific binding to the labelled probe will be unaffected by non-specific competitor DNA, but greatly reduced by specific competitor DNA. As shown in Figure 4.6 **B**, the preincubation of the fractions with specific competitor DNA dramatically reduced the intensity of the shifts compared to the observed effects when the same fractions were preincubated with non-specific competitor DNA. Despite the difference in the mobility of the shift produced by the RB eluates and the high salt eluates, it is clear that both shifts are caused by sequence-specific binding to the B-block oligonucleotide.

$His_6$-RB was also found to displace TFIIIC from the immunoimmobilised pol III "holoenzymes", thus verifying the results obtained with GST-RB(379-928) (data not shown). These results strongly indicate that RB specifically disrupts the interaction between TFIIIB and TFIIIC. Subsequently, extensive immunoprecipitations have confirmed these preliminary findings that RB may disrupt the specific interaction between TFIIIB and TFIIIC (503).

Together, these experiments and the immunoprecipitations by Sutcliffe et al. (503) clearly demonstrate that RB disrupts the interaction between TFIIIB and TFIIIC2. However, it is unclear if TFIIIC1 is also displaced as the B-block oligonucleotide binding activity of TFIIIC is a property of TFIIIC2 and TFIIIC1 is not required for this function (481). Pol III templates with type III promoters such as 7SK and vertebrate U6 snRNA genes are TFIIIC2-independent but they do require TFIIIIC1 for accurate transcription (397, 604). RB has been found to repress the transcription of every pol III template tested including vertebrate U6 genes (579). Therefore, if RB does not displace TFIIIC1 there must be additional mechanisms by which RB represses transcription. It may be RB represses transcription by different mechanisms at different pol III promoters and by more than one mechanism at some promoters. 7SK and U6 transcription requires a specialised complex called PTF or SNAPc, which is thought to interact with TFIIIB assisting its recruitment to the promoter (568, 604). Recent evidence suggests that RB disrupts this interaction

(215). Although there may be multiple other mechanisms by which RB specifically inhibits TFIIIB, disruption of the interaction between TFIIIB and TFIIIC2 will inhibit the transcription of most class III genes.

### 4.2.6 p107 and p130 stably interact with TFIIIB

Although the three pocket proteins share extensive sequence homology and there is some redundancy between the proteins, gene knockout experiments revealed that some functions of RB cannot be performed by p107 or p130 (95, 245, 322). Some of these unique functions of RB may be essential for its function as a potent tumour suppressor. Overexpression of p107 or p130 can induce a G1 arrest in some cell lines; however, the genes encoding p107 and p130 do not seem to be targeted for inactivation in cancers. It is thus unclear whether p107 and p130 are *bona fide* tumour suppressors. It was therefore interesting to determine whether p107 and p130 can also repress pol III transcription or whether this is a unique property of RB among the pocket proteins. Primary embryonic fibroblasts from $RB^{-/-}$ mice, which are wild type for p107 and p130, display substantially elevated levels of pol III transcription suggesting that the repression of pol III transcription by RB cannot be compensated for by p107 or p130 (579). This does not exclude the possibility that p107 and p130 also repress pol III transcription, but suggests that p107 and p130 may target a different component of the pol III transcriptional apparatus to RB or they may function at different times of the cell cycle.

Recently, it has been shown that p107 and p130 can also repress pol III transcription, both in vitro and in vivo (504). Thus, this is a function shared by all three pocket proteins. Pull-down assays and immunoprecipitation assays using recombinant components have demonstrated that the BRF subunit of TFIIIB can interact with p107 and p130 (504). This suggested that p107 and p130, like RB, might specifically target TFIIIB. However, it was important to determine whether p107 and p130 interact with BRF in a physiological context. Immunoprecipitations were therefore carried out using endogenous extracts to which no exogenous factors had been added (504). Using antisera raised against the different pocket proteins, endogenous RB, p107 and p130 have been immunoprecipitated from HeLa nuclear extract. The precipitated material was then washed extensively, resolved on SDS-

PAGE and probed for the presence of BRF by Western blotting (504). Figure 4.7 shows the results of such an immunoprecipitation. As expected, the antiserum against RB, which was used as a positive control, coprecipitated BRF (lane 1). The p107 antiserum also coprecipitated BRF, at similar levels to the RB antiserum (compare lanes 1 & 2). In contrast, an antiserum against the $TAF_I48$ subunit of SL1 was unable to immunoprecipitate BRF (lane 3), demonstrating the specificity of the coimmunoprecipitation of BRF observed with the RB and p107 antisera. The presence of BRF in the material that coprecipitated with RB and p107 was confirmed by using a second antiserum raised against a different region of BRF (data not shown). BRF is also coprecipitated by an antiserum against p130 (504). These results have been confirmed by the converse immunoprecipitation, using a BRF antiserum or the corresponding preimmune serum, in which the coprecipitation of p107 and 130 was specifically probed for (504).

Since endogenous p107 and p130 were found to specifically interact with endogenous BRF, it was postulated that a population of endogenous p107 and p130 might copurify with TFIIIB depending on the stability of the interaction. The immunopurified TFIIIB and TFIIIC, and DNA-affinity purified TFIIIC generated as described in the previous chapter, were probed by Western blotting for p107 and p130 to see if these two proteins have specifically copurified with TFIIIB. Unfortunately, however, no p107 or p130 was detected in any of these purified fractions (data not shown). It may be the fractions are too dilute to detect the presence of p107 or p130 with the available antisera (data not shown). Some other partially purifed fractions have been tested, however. p107 and p130 were found to copurify with TFIIIB, but not with pol III or TFIIIC2 (504).

As a further test for a stable interaction between TFIIIB and p107 or p130, it was examined whether endogenous p107 and p130 would cofractionate with TFIIIB during gradient chromatography of a PC-B fraction on heparin-Sepharose. Bound protein was eluted with a linear salt gradient and the individual fractions were then probed for the presence of TFIIIB and p107 or p130 (504). As Figure 4.8 shows, TFIIIB (upper panel) and p130 (lower panel) cofractionated very closely on the heparin-Sepharose salt gradient. The same was found to be true of p107 (data not shown). Both pocket proteins and TFIIIB were found to peak in fractions 20 to 22 and then tail off sharply (Figure 4.8 & data not shown). This close cofractionation of p107 and p130 with TFIIIB was confirmed using alternative antisera against both

pocket proteins (data not shown). The fractionation of cyclin A on the gradient was also assayed and was found to fractionate differently (data not shown). Thus, like RB, it appears that p107 and p130 also specifically target TFIIIB and that the association of p107 and p130 with TFIIIB is relatively stable.

**Figure 4.7**

**Endogenous BRF coimmunoprecipitates with endogenous pocket proteins**

HeLa nuclear extract (150μg) was immunoprecipitated using anti-RB antibody C-15 (lane 1), anti-p107 antibody C-18 (lane 2), and anti-$TAF_I48$ antibody M-19 (lane 3). Precipitated material was resolved on a SDS-7.8% polyacrylamide gel and then analysed by Western blotting with anti-BRF antiserum 128.

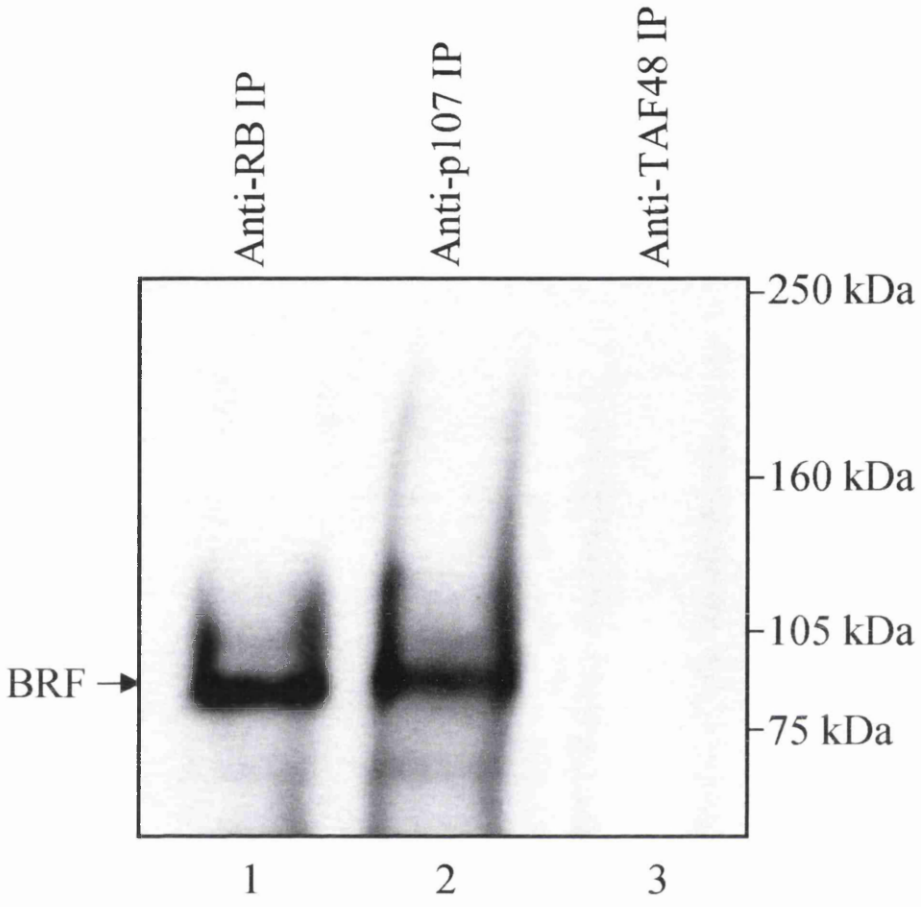
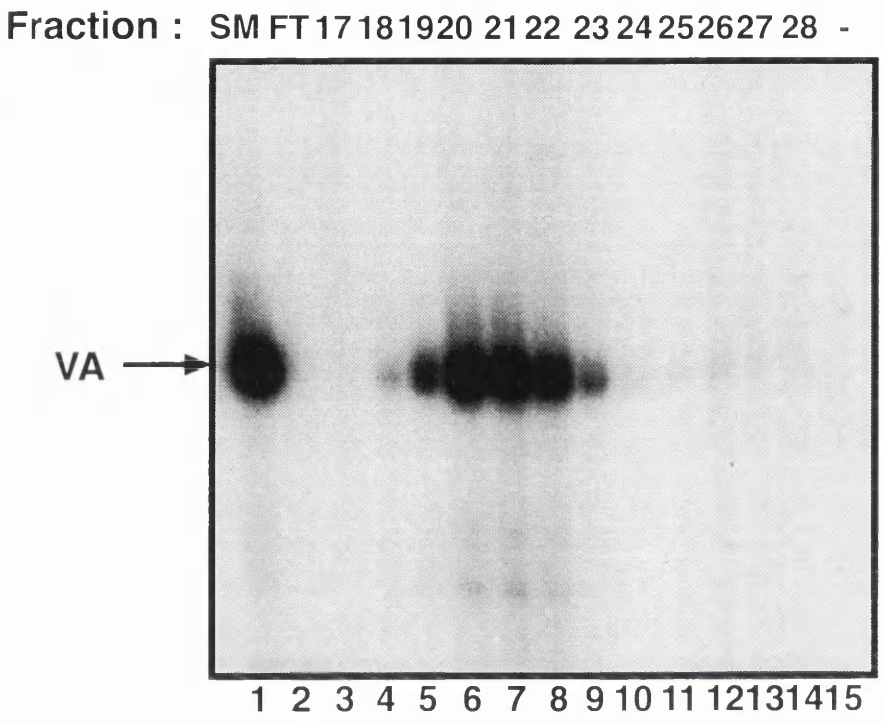
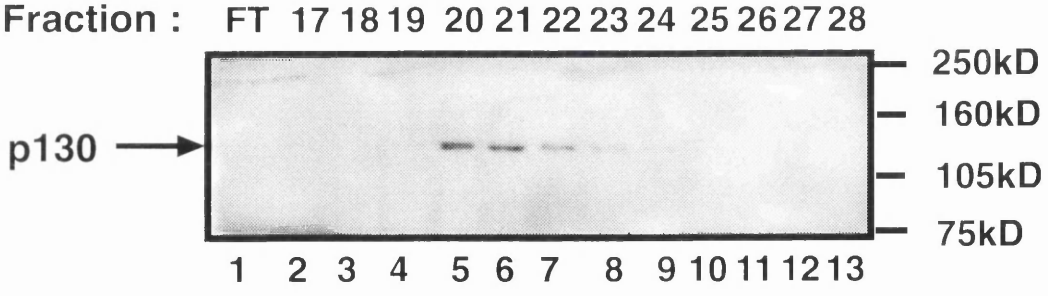

Anti-RB IP
Anti-p107 IP
Anti-TAF48 IP
250 kDa
160 kDa
105 kDa
75 kDa
BRF
1
2
3

**Figure 4.8**

**p130 cofractionates with TFIIIB during gradient chromatography of PC-B on heparin-Sepharose**

The upper panel shows the TFIIIB activity of individual fractions, and the lower panel shows the p130 content of the same fractions. Fraction numbers are indicated. SM, starting material. FT, flowthrough. TFIIIB activity was assayed by reconstituted pol III transcription reactions using 4 μl of the individual fractions, 2μl of PC-C and 250 ng of $pVA_L$. The p130 content of fractions was determined by resolution of equal volumes (15μl) of the individual fractions by SDS- 7.8% PAGE followed by immunoblotting with the anti-p130 antiserum Rb2.


Fraction : SM FT 17 18 19 20 21 22 23 24 25 26 27 28 -
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


Fraction : FT 17 18 19 20 21 22 23 24 25 26 27 28
p130
250kD
160kD
105kD
75kD
1 2 3 4 5 6 7 8 9 10 11 12 13

## Discussion

### 4.3.1 Mechanisms of repression of TFIIIB by RB

Paradoxically, E2F has been described both as an oncoprotein and as a tumour suppressor (556). Ectopic expression of E2F-1 can drive quiescent cells all the way through G1 into S phase. Furthermore, when E2F-1 is overexpressed with other known oncogenes it leads to cellular transformation, thereby conforming to a widely accepted operational definition of an oncogene. It therefore came as something of a surprise when E2F-$1^{-/-}$ mice started to exhibit hyperplasia of certain tissues and even neoplasia as they aged (556). There has been considerable speculation as to how E2F can act both as an oncoprotein and as a tumour suppressor. One of a variety of speculations is that it is related to the ability of RB to convert E2F from a transcriptional activator to a transcriptional repressor (556). E2F activates the expression of a variety of genes required for cellular proliferation. However, when associated with RB, E2F may actively repress these very same genes. Loss of E2F in a RB-positive background may therefore actually cause increased expression of these proliferation-promoting genes.

The active repression of some E2F-regulated genes by RB is dependent on histone deacetylase activity (346). HDAC1 has been found to associate with RB and it is thought to be recruited by RB to E2F-regulated promoters where it may then deacetylate nucleosomal histones resulting in a more compact chromatin structure that is repressive to transcription (52, 53, 346, 349). Since RB stably interacts with TFIIIB, which forms part of the pol III preinitiation complex, it seemed plausible that RB may also recruit deacetylase activity to class III genes, and that this may be required for the ability of RB to repress pol III transcription.

Support for a role of histone deacetylation in the regulation of pol III transcription was provided by recent reports that human TFIIIC2 has intrinsic HAT activity (230, 298). Furthermore, human TFIIIC was shown to relieve chromatin-mediated repression of a tRNA gene in vitro (298). Additionally, histone acetylation has previously been shown to facilitate the transcription of a 5S gene assembled into chromatin in vitro (321, 529, 534). Nonetheless, the presence of the potent and irreversible histone deacetylase inhibitor TSA had no effect on the repression of pol III transcription by RB either in vitro, as described here, or in vivo (503). It is

unclear though whether or not the RB molecules that interact with TFIIIB are also associated with histone deacetylase. TSA has been found to stimulate transcription of chromosomal B2 genes in vivo in an RB-independent manner, suggesting that histone acetylation and deacetylation does have an important physiological role in regulating pol III transcription (503). However, since RB disrupts the interaction between TFIIIB and TFIIIC2, for the majority of class III genes it is unlikely that any TFIIIB recruited to the promoter will remain associated. Thus, any deacetylase activity recruited to TFIIIB indirectly through association with RB is also unlikely to stay associated with a class III promoter and may therefore not affect the chromatin structure of these genes.

All three of the pocket proteins can repress pol III transcription and they each specifically target the same factor, TFIIIB, for repression (314, 504, 579). This implies redundancy between the proteins; however, p107 and p130 are unable to compensate for the loss of RB on pol III transcription (579). p107 and p130 may utilise a different, and possibly less potent, mechanism to repress TFIIIB. Indeed, p107 and p130 have also been found associated with histone deacetylase activity (144). Unlike RB, it may be that p107 and p130 interact with TFIIIB but do not displace it from the promoter, which may enable the stable recruitment of deacetylase activity to class III promoters. The tumour suppressor p53, which also interacts with TFIIIB and represses pol III transcription, has also been found to associate with deacetylase activity, which is essential for its ability to repress certain pol II-transcribed genes (388).

In the pol II system, RB blocks E2F recruiting TFIID to promoters (448). This is analogous to the disruption of the interaction between TFIIIB and TFIIIC2 by RB, which is likely to prevent the recruitment of TFIIIB to class III promoters. Although the "holoenzyme" studies and the subsequent immunoprecipitations that were performed provide compelling evidence that RB disrupts the interaction between TFIIIB and TFIIIC2, a few class III genes that have previously been shown to be repressed by RB are TFIIIC2-independent. Thus, the disruption of this interaction cannot be the sole mechanism by which RB is able to repress transcription. Indeed, some other immunoprecipitations performed suggested that RB also disrupts the interaction between TFIIIB and pol III (503). This provides a potential mechanism by which RB represses TFIIIC2-independent genes. However, there was little discernible effect of RB on the interaction of TFIIIB and pol III in the

"holoenzyme" studies. The reason for this discrepancy is unknown. It may be that RB can more easily disrupt the interaction between TFIIIB and TFIIIC2 than that between TFIIIB and pol III. Perhaps insufficient RB was recirculated through the column and the interaction with TFIIIC2 was preferentially disrupted. In contrast to the immunoprecipitation, a small amount of TFIIIC2 remained associated with the immunoaffinity column after exposure to RB, suggesting that disruption was generally less effective in this system.

There may be other mechanisms by which RB can repress pol III transcription in addition to these. Recent work by Hirsch et al. suggests that RB might disrupt the interaction between TFIIIB and SNAPc (215). The intramolecular interaction between BRF and TBP within TFIIIB seems to be unaffected by RB (314, 503). Unfortunately, a lack of knowledge as to the composition of TFIIIB largely prevents any effect of RB on any other intramolecular interactions within TFIIIB from being studied. Human TFIIIB is thought to contain a human homologue of yeast B″, which has recently been cloned; however, as yet we have no molecular reagents against this component, so any effects RB may have on it cannot be properly tested (463).

An interesting question is the relative contribution of the different mechanisms by which RB can repress pol III transcription, in a physiological context. This may differ for different genes. TFIIIC2-dependent genes are predicted to be completely repressed either by the disruption of the interaction between TFIIIB and TFIIIC2 or that between TFIIIB and pol III. In vivo, RB may utilise both of these mechanisms or one may predominate. If both mechanisms are used the question arises as to whether this is necessary or whether there is redundancy, such that if only one of the mechanisms is lost this would not have any effect on the ability of RB to repress the gene. The utilisation of more than one mechanism by RB in vivo may act as a kind of a fail-safe; it may also allow some local control to be exerted on the global repression of pol III transcription by RB if different class III genes utilise distinct mechanisms to different extents. However, it is presently unclear to what extent this is the case or whether the different mechanisms can be individually regulated. Clearly, a lot of unanswered questions remain regarding the mechanisms by which RB represses pol III transcription.

### 4.3.2 Implications of a pol III holoenzyme

The immunoaffinity purification of a complex containing TFIIIB, TFIIIC and pol III, as described in this chapter, enabled the effect of RB on the intermolecular interactions of TFIIIB to be tested. It also provides support for a growing body of evidence suggesting that a pol III holoenzyme may exist in vivo, or at the very least, associations between components of the pol III preinitiation complex off the DNA, which may represent PIC assembly intermediates. This has important implications for the regulation of pol III transcription. Much of the regulation of pol III transcription is thought to occur at the initiation stage. Once a preinitiation complex has been formed it is extremely stable and can support multiple rounds of transcription (73). In yeast there is a facilitated recycling pathway in which polymerase is recycled without being released from the template, allowing very rapid multiple round transcription (123). Thus, clearly the regulation of the formation of the preinitiation complex is extremely important.

The ordered and stepwise assembly pathway defined in vitro provides multiple opportunities at which preinitiation complex formation at a specific class III gene may be regulated, namely each stage that a component is recruited. In contrast, if the components of the preinitiation complex are preassembled into a functional holoenzyme prior to recruitment, the regulation of the assembly of a preinitiation complex at a specific gene is limited to a single step. However, controlling the formation of the pol III holoenzyme off the DNA will allow the global regulation of pol III transcription. Although the concomitant recruitment of all the essential components that constitute the preinitiation complex will result in control of assembly at a specific gene being restricted to a single step, this does not necessarily constitute less control being exerted.

Regulation of preinitiation complex formation at a specific class III gene achieved through the recruitment of a holoenzyme is likely to occur less through modulation of protein-protein interactions, as for a stepwise assembly pathway, and more through modulation of protein-DNA interactions. The holoenzyme is likely to be hampered in mobility and its ability to find a gene to which it can bind. Control of local chromatin structure is likely to play a very significant role in the regulation of recruitment of a holoenzyme. The recruitment of a holoenzyme will probably

require multiple interactions between components in the complex and a large segment of DNA. This may be necessary for the high degree of sequence specificity to enable the appropriate target genes to be found in the vast excess of non-specific sites in the eukaryotic genome. The multiple protein-DNA interactions are all potential targets for regulating the recruitment of the holoenzyme.

It may be that preinitiation complex formation can occur both by a stepwise pathway and through recruitment of a preassembled "holoenzyme" in vivo. Since the transcription factor requirements are not the same at all class III genes, it may be that there are several types of pol III holoenzymes or some genes may utilise a holoenzyme, whereas others do not. When pol III transcription is reconstituted in vitro using fractionated factors, a time lag occurs prior to the attainment of a linear transcription rate (174, 568). This is because of the time it takes for assembly of the preinitiation complex. However, when the pol III "holoenzymes" isolated by density gradient centrifugation or an unfractionated extract is tested, no lag is obtained; this is consistent with the presence of preassembled complexes fully active for transcription (174, 248, 585). This is strongly supportive of the existence of a physiologically relevant pol III holoenzyme in vivo that can start transcription as soon as it has been recruited to an appropriate gene. Nonetheless, any pol III holoenzymes that do exist in vivo seem to be of quite low abundance; Wang et al. estimated that approximately 10% of the total pol III in their extract was in a holoenzyme form (551). Consistent with this, my immunopurifications resulted in a very poor yield of the "holoenzyme" relative to the yield of the component to which the antibody was raised, although this may also reflect disruption of some "holoenzyme" by the antibody and poor epitope accessibility.

### 4.3.3 p107 and p130 also target TFIIIB

Since the three pocket proteins share their most extensive homology in the pocket domain and it is this same domain which is essential for the ability of RB to repress pol III transcription, the finding that p107 and p130 also repress pol III transcription was not unexpected (504). Furthermore, they seem to target the same transcription factor, TFIIIB, with which they can stably associate at physiological concentrations, as described in this chapter (504). Since TFIIIB is required for the

expression of all pol III templates, by specifically targeting this factor, like RB, p107 and p130 may be able to repress the transcription of every class III gene.

The discovery that endogenous RB, p107 and p130 all bind to endogenous TFIIIB raises questions as to whether there is competition between the pocket proteins for binding to TFIIIB. The proportion of TFIIIB that is bound by each of the pocket proteins in vivo is likely to affect the relative contributions of RB, p107 and p130 to the repression of pol III transcription, as well as their relative affinities.

The contributions of the pocket proteins to transcriptional repression have been analysed by transfecting cycling NIH 3T3 cells either with wild type HPV16 E7 oncoprotein, which potently binds to all three pocket proteins, or with an E7 GLY26 mutant, which can only bind p107 and p130 (504). Binding of E7 prevents the pocket proteins from interacting with some of their cellular targets. Transfection with the wild type E7 protein resulted in a substantial increase in pol III transcription, suggesting that the interactions between the pocket proteins and TFIIIB were disrupted. The GLY26 mutant also stimulated transcription, but the activation obtained was only 27% of that observed with wild type E7 protein (504). Thus, although p107 and p130 contributed to repression, RB seemed to have the much more dominant role in these cycling NIH 3T3 cells.

The binding of the three pocket proteins to the same transcription factor suggests that they repress pol III transcription in a very similar, if not an identical manner, which in turn implies that there may be some redundancy between the proteins. The phenotypes of knockout mice suggest otherwise; synthesis of tRNA and 5S rRNA is five fold more active in primary fibroblasts from $Rb^{-/-}$ mice, with functional p107 and p130, than the corresponding cells from $Rb^{+/+}$ mice (579). The inability of p107 and p130 to compensate for loss of RB suggests that the pocket proteins are functionally distinct. The mechanism(s) by which p107 and p130 repress pol III transcription have yet to be investigated. Indeed, although p107 and p130 have been shown to stably bind TFIIIB, it has not yet been demonstrated that TFIIIB is actually inhibited by these two pocket proteins. It may be that p107 and p130 repress transcription by a different mechanism to RB and perhaps less potently. Although loss of RB may enable more p107 and p130 to bind more of their molecular target, TFIIIB, because of a lack of competition from RB, this clearly is not sufficient to enable p107 and p130 to fully compensate. However, this may

simply be a reflection of the relative amounts of the pocket proteins, since there is considerably more RB than p107 or p130 in a normal cell.

Although the protein levels of RB stay relatively constant through the cell cycle, levels of p107 and p130 change dramatically (173). p130 is highly abundant in quiescent cells, but is rapidly downregulated when cells re-enter the cell cycle. In contrast, p107 is present at very low levels in G0, and is present in highest amounts in S, G2 and M phase. In addition to varying protein levels, the activity of p107 and p130, like RB, is regulated by phosphorylation (173). After the G1/S transition, all three pocket proteins are hyperphosphorylated and remain inactive until the next G1 phase. The cell cycle-dependent changes in the relative abundance of the different pocket proteins means that the relative contributions of the individual pocket proteins to repression are likely to differ temporally. Support for this is provided by the regulation of E2F by the pocket proteins (131, 173). p107 and p130 can also bind E2F. However, the temporal association of each pocket protein with E2F during the cell cycle is quite different. The p130-E2F complex predominates in quiescent fibroblasts, whereas a RB-E2F complex occurs mainly in G1 phase. The p107-E2F complex occurs predominantly in S phase. A similar situation probably exists for the regulation of pol III transcription by the pocket proteins. Each pocket protein may have its own characteristic temporal profile of interaction with TFIIIB. In support of this, targeted disruption of the genes encoding p107 and p130 made little difference to pol III transcript levels in cycling mouse embryonic fibroblasts (504). However, when these cells were made quiescent by serum withdrawal a significant increase in pol III transcript levels was observed in the $p107^{-/-}$ $p130^{-/-}$ cells compared to pol III transcript levels of the corresponding cells expressing wild-type p107 and p130 (504). Thus, during the G0 phase p107 or p130 exert a repressive effect on pol III transcription that cannot be compensated for by RB. Recently, Scott et al. reported that in G0 phase cells, ~70% of TFIIIB is bound by RB and ~30% is bound by p130 (471). Although the three pocket proteins all repress pol III transcription and they target the same factor, TFIIIB, their relative contributions to the repression of TFIIIB appear to differ at different times of the cell cycle. Thus, whereas RB can exert an inhibitory influence on TFIIIB both in G0 and G1, the repressive effects of p130 upon pol III activity are largely limited to G0. The levels of p130 are substantially reduced following exit from G0 phase and thus the protein likely makes little or no

contribution to the repression of pol III transcription by the pocket proteins when cells are cycling.

Recently, fibroblasts from *Rb*$^{-/-}$ *p107*$^{-/-}$*p130*$^{-/-}$ triple knockout mice have been analysed for pol III activity. The activation of pol III transcription in these triple knockout cells was found to be greater than that in fibroblasts from mice lacking either one, or any two, of the pocket proteins (470). Thus, regardless of the mechanism, it is clear that all three pocket proteins have a physiological role in the repression of pol III transcription.

# Chapter 5.

# Deregulation of pol III transcription in ovarian cancer

## 5.1 Introduction

Transfer RNA (tRNA) and 5S ribosomal RNA (5S rRNA) have essential enzymatic and/or structural roles in translation. These pol III transcripts perform a housekeeping function and it was thus expected that any genes encoding such products be constitutively active. However, it is now clear that the transcription of tRNAs and 5S rRNA, and most other pol III templates, is strongly regulated (568, 569). This enables the production of such transcripts to be adjusted to meet the metabolic demands of the cell.

The retinoblastoma protein and the unrelated tumour suppressor p53 both repress pol III transcription (70, 579). Missense mutations in p53 occur in approximately half of all human tumours (222, 223). The effects of such mutations on pol III transcription have yet to be reported, but it seems plausible that many will impair the ability of p53 to repress pol III transcription. It has been suggested that RB function may be compromised in all human malignancies (557). Although clearly a limited survey, several subtle mutations that have arisen in RB in small cell lung carcinomas have been shown to prevent it from repressing pol III transcription (59, 579). This demonstrates that mutations that arise in RB in tumours can compromise its ability to regulate pol III transcription.

The constraint imposed upon pol III transcription by these two key tumour suppressors and their frequent loss of function in human cancers suggests that elevated pol III transcription may be a common feature of human malignancies. Indeed, it has been suggested that elevated pol III transcription may constitute an important step in tumour development (313, 569). tRNAs and 5S rRNA are important determinants of the biosynthetic capacity of the cell (394, 567). Following mitogenic stimulation of resting cells, rRNA and tRNA synthesis increases, ribosomes assemble into polysomes, translation factors are activated and a net

increase in protein synthesis is observed (445). The elevated synthesis of tRNAs and rRNA in response to mitogens suggests that these RNA species are not normally in excess and that their synthesis is tightly controlled according to the biosynthetic needs of the cell. The unrestrained proliferation of tumour cells requires sustained growth, which in turn is dependent upon active protein synthesis (566). It may be that the levels of tRNA and 5S rRNA in a normal proliferating cell are insufficient to support the increased or sustained protein synthesis required by tumour cells in order to maintain cell size during rapid proliferation. It has yet to be proven that tRNA and 5S rRNA levels are limiting, but this provides a plausible explanation for the targeting of pol III transcription for repression by RB and p53. It would also explain the targeting of pol III transcription for activation by a number of viral oncoproteins (569).

The growth-suppressive function of RB may be mediated, at least in part, by its repressive effect on the synthesis of tRNA and 5S rRNA. Cells are unable to enter S phase and replicate their DNA until they have accumulated an adequate level of protein (273, 517). Therefore, by restraining growth the cell is prevented from proliferating uncontrollably. Loss of growth control is thus essential for tumour development. The contention that the control of protein synthesis is an important aspect of growth regulation is supported by a multitude of evidence demonstrating the deregulation of protein synthesis in transformed cells (445, 494). Indeed, abnormal activation of translation factors is sufficient to cause neoplastic transformation. Overexpression of the translation initiation factor eIF4E in primary fibroblasts induces morphological transformation; moreover, these cells can induce tumours in nude mice (319, 320). Mutational activation of another translation factor, eIF2$\alpha$ can also cause malignant transformation (128). Activation of the oncoprotein c-myc causes an increase in the abundance of both of these factors; this is also accompanied by an increase in the rate of protein synthesis and cellular growth (446, 447). The protein synthesis apparatus is also deregulated in real tumours. The expression profile of some 45,000 genes in gastrointestinal tumours has been studied using the recently developed methodology of serial analysis of gene expression (SAGE) (614). Only 108 pol II transcripts were found to be expressed at increased levels in the tumours relative to normal colonic epithelium. 48 of these transcripts encode ribosomal proteins and 5 encode translation factors (614). Together, the

available evidence strongly suggests that the deregulation of protein synthesis is intimately linked to carcinogenesis. The deregulation of pol III transcription may represent another manifestation of a selection for changes in the protein synthetic machinery during tumour development.

If pol III transcription truly does have an important role in tumour development, it is predicted that it will be abnormally elevated in a range of transformed and tumour cells. Indeed, pol III products are found to be overexpressed in many types of transformed cell line (16, 168, 292, 350, 469, 487, 548, 549, 578). Fibroblasts transformed with a temperature-sensitive mutant of the SV40 large T antigen downregulate pol III transcription at the non-permissive temperature whilst reverting to a normal phenotype and morphology, suggesting a tight link between transformation and pol III activation (469). Moreover, when different SV40-transformed cell lines are compared, those which most efficiently induce tumours in nude mice display the highest levels of pol III products, whereas in the less tumorigenic cell lines there are lower levels of pol III transcripts (469, 578). Pol III transcription is activated by a broad range of transforming agents, including DNA tumour viruses, RNA tumour viruses and chemical carcinogens (569). Hepatitis B virus (HBV), a DNA virus, and a RNA virus called human T-cell leukaemia virus type 1 (HTLV-1) are both important causative agents in human cancers and both stimulate an increase in pol III transcript levels following transformation (569). The activation of pol III transcription in transformed cells is very general but it is not universal. There are a few reported cases in which pol III transcriptional activity is not abnormally elevated in transformed cells, such as in the $Rb^{+/+}p53^{+/+}$ osteosarcoma cell line U20S (579).

A recent SAGE study has shown that only 21% of genes overexpressed in colorectal cancer cell lines are also overexpressed in primary colorectal carcinomas (614). Clearly, substantial differences in behaviour can exist between transformed cells in culture and tumours in vivo. Transformed cells in culture provide a useful model for real tumours; however, it is of paramount importance to determine how closely the behaviour of the transformed cells studied mimics that of the tumours in vivo. Recently, it has been shown that pol III transcripts are elevated in rodent and human tumours in vivo, verifying the physiological significance to real tumours of the abnormally elevated levels of pol III transcription consistently observed in many transformed cell lines (87, 88). In one study, samples from eighty human tumours,

representing nineteen different types of cancer, were tested (87). BC200, a pol III product of unknown function, was found to be overexpressed in many, but not all, of the primary human tumours. The levels of 7SL RNA, an essential pol III product that forms part of the signal recognition particle, were elevated in every tumour examined relative to the corresponding normal tissue (87).

Recently, we have been investigating pol III transcription in ovarian cancer, the leading cause of death from gynaecological malignancies in the United States (159). Ovarian tumour samples and corresponding normal healthy tissue from the same patients were examined by RT-PCR for the levels of 5S rRNA, 7SL RNA and two different types of tRNA (586). For each of the four patients whose tissue samples were tested, a substantial increase in the levels of each of these pol III transcripts was observed in the tumour samples (586). In contrast, no change was detected in the levels of the mRNA encoding acidic ribosomal phosphoprotein (ARPP P0) or glyceraldehyde phosphate dehydrogenase (GAPDH), demonstrating the specificity of the overexpression of pol III products. The genes encoding tRNA, 5S rRNA and 7SL RNA have distinct promoter structures and transcription factor requirements. Despite these differences, each of these transcripts was found to be abnormally elevated in these tumour samples, suggesting that there may be a general elevation of pol III transcript levels in ovarian cancer. The use of intron-specific primers for determining the levels of tRNA ensured that they provided an accurate indication of the rate of ongoing transcription, as the introns are rapidly processed from primary transcripts and degraded (586). Thus, the substantial elevation of pol III transcript levels seems to be caused by an activation of pol III transcription rather than an increase in the stability of transcripts (586). Consistent with our findings of increased levels of pol III transcripts in ovarian cancer, Chen et al. reported an increase in BC200 RNA levels in the one ovarian carcinoma sample that they tested (87).

Although abnormally elevated levels of pol III transcripts have been observed in a broad range of transformed cell lines, in most cases the mechanistic basis of this effect has not been elucidated. Similarly, in the studies of tumours in vivo by Chen et al. the mechanism(s) responsible for the substantial increase in the levels of pol III transcripts was not investigated. Loss of function of RB and p53 and the release of TFIIIB from repression by these two pocket proteins may account for pol III activation in many tumours. However, TFIIIB activity has been shown to be in

relative excess in several types of mammalian cell, so the deregulation of TFIIIB in these cells may have little or no effect on pol III transcriptional output (74, 151, 218, 571, 577, 578). In such cells, pol III or TFIIIC may be targeted for activation. In support of this, higher levels of pol III have been found in mouse myeloma cells relative to healthy tissue; in contrast, the levels of pol II were unchanged (467). The DNA-tumour viruses SV40 and adenovirus seem to utilise multiple mechanisms to ensure high levels of pol III transcription (569). The adenoviral E1a oncoprotein and the SV40 large T antigen both bind to RB relieving repression of TFIIIB. Additionally, both viruses increase the TFIIIC activity of infected cells. SV40-transformed fibroblasts overexpress TFIIIC2, which is the rate-limiting factor for pol III transcription in these cells (315, 578). The adenoviral infection of HeLa cells has been reported to result in an increase in the concentration of TFIIIC2 (606). Independently, it has been reported to result in a specific increase in the proportion of TFIIIC2 that exists in the transcriptionally active TFIIIC2a form (488).

The targeting of TFIIIC for activation is seemingly not restricted to transformed cells. We have recently been investigating the mechanistic basis for the observed activation of pol III transcription in human ovarian carcinomas. In nine out of nine ovarian tumours examined, substantially elevated TFIIIC2 activity was observed relative to that of normal healthy tissue from the same women (586). Thus, the activation of pol III transcription in ovarian cancer may be caused by abnormally high levels of TFIIIC2 activity (586). This will depend, however, on whether or not TFIIIC2 activity is limiting in normal ovarian cells; if it is in vast excess, increasing it further is unlikely to have much effect on transcriptional output. The investigation of the physiological significance of elevated TFIIIC2 activity on pol III transcription in ovarian cancer is described in this chapter.

## 5.2 Results

### 5.2.1 TFIIIB is limiting for pol III transcription in nuclear extract from a human ovarian tumour cell line

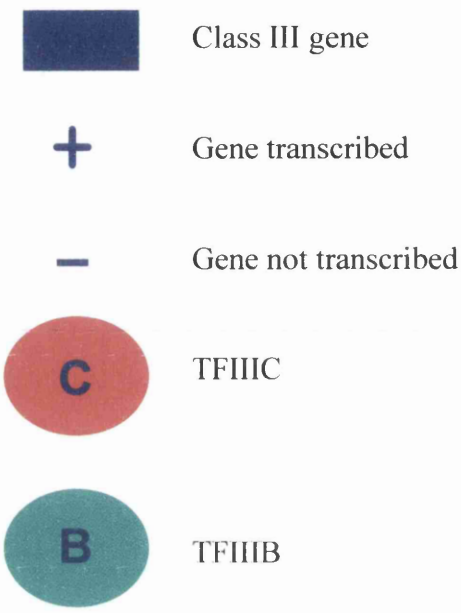
TFIIIC2 must be a limiting factor in normal ovarian epithelial cells for the abnormally high levels of TFIIIC2 in the ovarian tumours analysed to be at least partly responsible for the activation of pol III transcriptional output consistently observed in these tumour samples. Figure 5.1 is a schematic diagram explaining the concept of a limiting factor. A limiting factor may be defined as a factor that stimulates the process when its concentration or activity is increased whilst that of any other factors remains constant. In the upper diagram of Figure 5.1 there is a relative deficiency of TFIIIC compared to TFIIIB and pol III. Addition of more TFIIIC stimulates transcription; therefore, TFIIIC can be regarded as a limiting factor. In contrast, in the lower diagram TFIIIC is in relative excess and although addition of more TFIIIC increases promoter occupancy, because of a deficiency in TFIIIB it does not increase transcriptional output. Therefore, in this situation TFIIIC is not a limiting factor.

Unfortunately, there were insufficient amounts of extract from the human ovarian tumour samples and healthy ovarian epithelial tissue from the same patients to investigate which factors are limiting in these extracts. Nuclear extract from a human ovarian tumour cell line, A27-80, was tested to see if TFIIIB or TFIIIC is limiting. To determine which factor or factors of the basal pol III transcriptional machinery are limiting in a particular cell type, add-back experiments are performed. This involves carrying out in vitro transcription reactions using a constant amount of cell extract in each reaction and separately titrating in increasing amounts of purified sources of each factor to see if they stimulate transcription. Figure 5.2 shows the results of such add-back experiments using nuclear extract from the human ovarian tumour cell line A27-80. As highly purified sources of TFIIIB and TFIIIC, immunoaffinity-purified TFIIIB and TFIIIC were used. A conventionally purified source of pol III was used. As Figure 5.2 **A** shows, the addition of partially purified TFIIIB strongly stimulated transcription. Although the immunopurified TFIIIC also stimulated transcription, this effect was much less than with the TFIIIB. The purified source of pol III had no stimulatory effect; in fact at the higher doses there seemed to

**Figure 5.1**

**Schematic diagram explaining the concept of a limiting factor**

Key:-




For simplicity pol III is assumed to be in vast excess and associated with any TFIIIB that associates with TFIIIC.

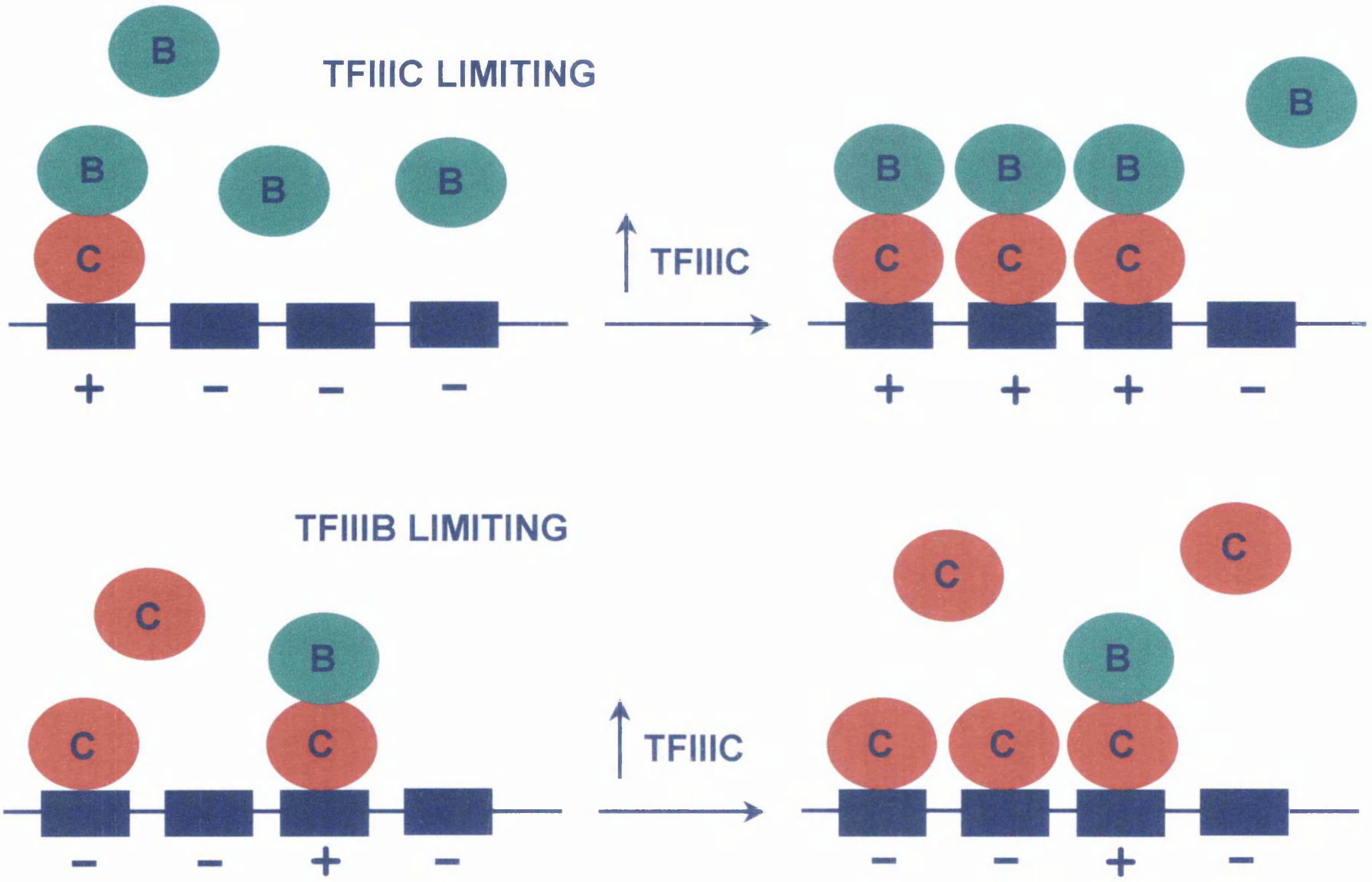

TFIIIC LIMITING
B
B
B
B
C
+
−
−
−
TFIIIC
B
B
B
B
C
C
C
+
+
+
−
TFIIIB LIMITING
C
B
C
C
−
−
+
−
TFIIIC
C
C
B
C
C
C
−
−
+
−

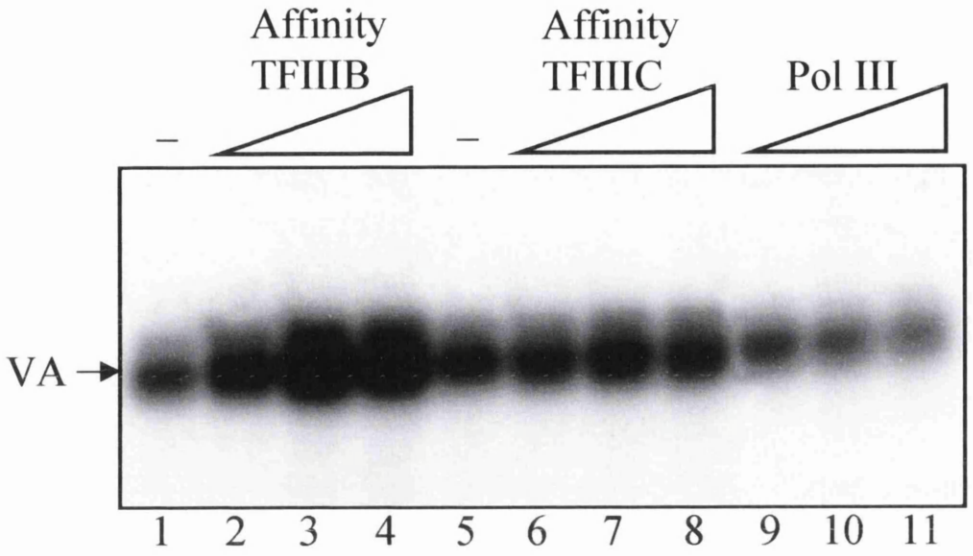
**Figure 5.2**

**Raising the level of TFIIIB stimulates pol III transcription reconstituted with extract from a human ovarian tumour cell line**

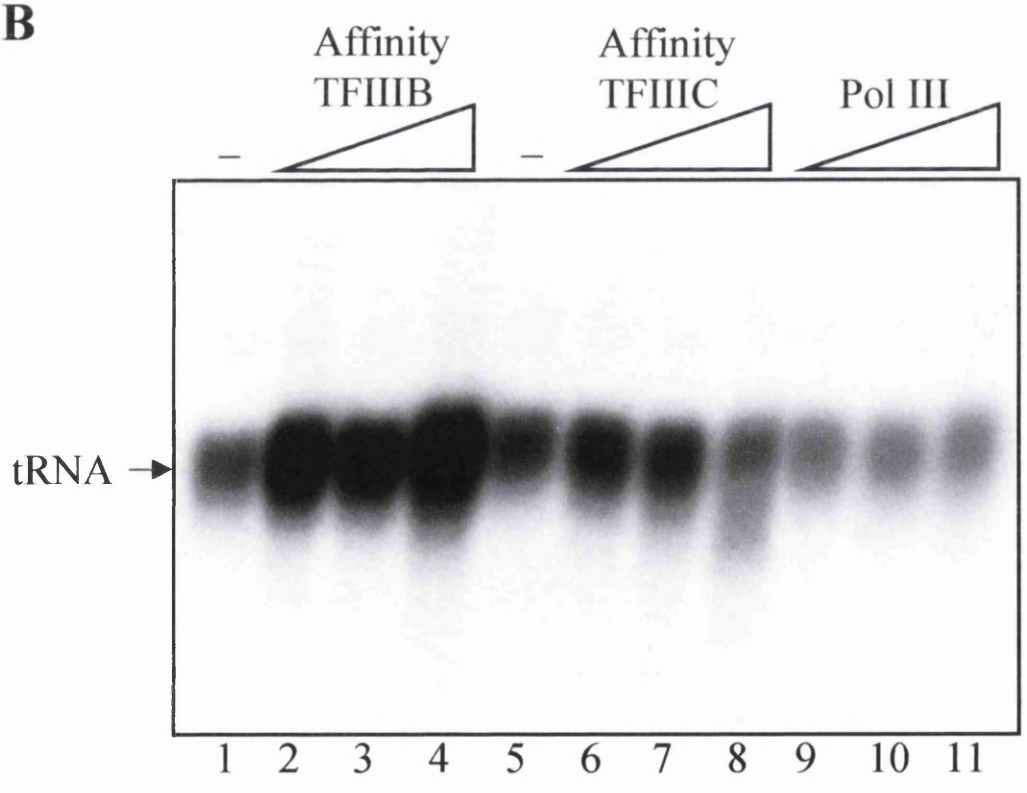
**A)** Transcription reactions were reconstituted using 10μg of A27-80 nuclear extract and 250 ng of $pVA_I$. Reactions 2, 3 and 4 were supplemented with 2, 4 and 8μl, respectively, of TFIIIB that had been immunopurified from PC-BHep using the antiserum 330 against BRF. Reactions 6, 7 and 8 were supplemented with 2, 4 and 8μl, respectively, of TFIIIC that had been immunopurified from PC-C using the antiserum 4286 against TFIIICβ. Reactions 9, 10 and 11 were supplemented with 2, 4 and 8μl, respectively, of an A25(1.0) fraction which is highly enriched in RNA polymerase III, generated by DEAE Sephadex chromatography of a PC-B fraction.

**B)** As for **(A)** except each reaction contained 250ng of pLeu template instead of $pVA_I$.

**A**


Affinity
TFIIIB
Affinity
TFIIIC
Pol III
–
–
VA
1 2 3 4 5 6 7 8 9 10 11

**B**


Affinity
TFIIIB
Affinity
TFIIIC
Pol III
–
–
tRNA
1 2 3 4 5 6 7 8 9 10 11

be a slight inhibitory effect. This may be due to quenching, the pol III may sequester active TFIIIB or TFIIIC which may otherwise have been able to participate in transcription. These results obtained using the $VA_I$ gene were confirmed using a tRNA gene as template (Figure 5.2 **B**). Thus, for both promoters tested TFIIIB was the major limiting factor for pol III transcription. This is consistent with the elevated levels of TFIIIC2 observed in ovarian tumour samples. It may be that TFIIIC is the limiting factor in normal ovarian cells, but the increase in the levels of TFIIIC2 in human ovarian tumour cells result in TFIIIB becoming more limiting in place of TFIIIC. However, it is equally plausible that TFIIIC is not limiting in normal non-tumorigenic ovarian epithelial cells.

### 5.2.2 Extracts from *neu*-transformed rat ovarian epithelial cells have higher pol III transcriptional activity than extracts from untransformed control cells

Since normal healthy ovarian epithelial tissue was unavailable for analysis, an ovarian epithelial cell line, ROSE 199-*βgal*, was used. Two different cell lines were examined, ROSE 199-*βgal* and ROSE 199-*neu*. The parental cell line, ROSE 199, is an established cell line of rat ovarian surface epithelium derived from normal, non-tumorigenic rat ovarian epithelium (2). ROSE 199-*neu* was generated by retroviral transfection of the parental cell line with mutationally activated *neu* oncogene (115). The cellular *neu* gene encodes a 185 kD protein related to, but distinct from, the epidermal growth factor receptor (EGFR) (459). Neu is a member of the erbB family of receptor tyrosine kinases (241). It is amplified and/or overexpressed in approximately one-third of human ovarian epithelial cancers and is associated with a poor prognosis (30, 491). ROSE 199-*neu* cells exhibit transformed phenotypes in vitro and the tumorigenic phenotype in vivo and are regarded as a useful model of human ovarian cancer (115). ROSE 199-*βgal* was generated by transduction of ROSE 199 with the *β-galactosidase* gene (115). In contrast to the potent oncogenicity of *neu*, the *β-galactosidase* gene does not induce transformed phenotypes nor tumorigenicity and ROSE 199-*βgal* cells have been used as a model of normal ovarian epithelial cells (115).

If ROSE 199-*βgal* and ROSE 199-*neu* truly are good models of normal ovarian epithelial cells and cancerous ovarian epithelial cells, respectively, and pol III activation does have an essential role in ovarian cancer, one may expect ROSE 199-*neu* cells to support higher levels of pol III transcription than ROSE 199-*βgal* cells. This was investigated by carrying out in vitro transcription assays using equal amounts of extract of actively cycling ROSE 199-*βgal* cells and ROSE 199-*neu* cells that were prepared in parallel. As Figure 5.3 shows, the *neu* extract supported a significantly higher level of pol III transcription than the *βgal* extract at all the amounts of extract tested, both using the $VA_I$ gene and a $tRNA^{leu}$ gene. This behaviour mimics the elevated levels of pol III transcription observed in the human ovarian tumour samples relative to healthy tissue. The same result has consistently been obtained using four different preparations of *βgal* and *neu* extracts (data not shown).

### 5.2.3 *Neu*-transformed ROSE 199 cells mimic human ovarian epithelial tumours by displaying elevated TFIIIC2 activity

Although the ROSE 199-*neu* cells have higher levels of pol III transcriptional activity than the ROSE 199-*βgal* cells, the mechanistic basis for this may be very different to that for the elevated levels of pol III transcripts observed in the human ovarian tumour samples. It was therefore tested whether the ROSE 199-*neu* cells also display elevated TFIIIC2 activity. As Figure 5.4 **A** shows, extracts from ROSE 199-*neu* cells have higher TFIIIC2 activity than an equal amount of *βgal* extract, as assayed by its specific B-block oligonucleotide DNA-binding activity. This result has been confirmed using four different preparations of *βgal* and *neu* extracts (data not shown). As a control, extracts were also analysed by electrophoretic mobility shift assay using an oligonucleotide carrying a Sp1 binding site as probe. Sp1, Sp2 and Sp3 all specifically bind to this oligonucleotide and produce protein-DNA shifts of different mobilities, as shown in Figure 5.4 **B**. The Sp1 oligonucleotide DNA-binding activities of the same two pairs of *βgal* and *neu* extracts shown in Figure 5.4 **A**, were tested and are shown in Figure 5.4 **B**. For both pairs of extracts, Sp1 oligonucleotide binding is slightly higher for the *βgal* extracts, despite these extracts

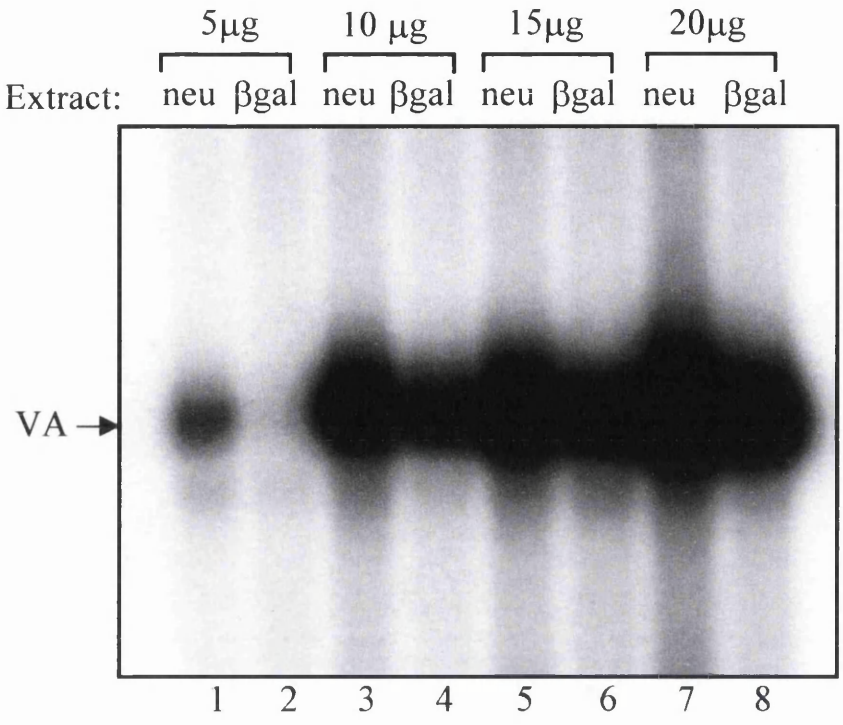
**Figure 5.3**

**Neu-transformed ROSE 199 cell extracts have higher RNA polymerase III transcriptional activity than untransformed βgal-transfected ROSE 199 cell extracts**

**A)** In vitro transcription assay using ROSE 199-*neu* cell extract (odd-numbered lanes) and ROSE 199-*βgal* cell extract (even-numbered lanes). Lanes 1 and 2 contained 5μg of protein extract, lanes 3 and 4 contained 10μg of protein extract, lanes 5 and 6 contained 15μg of protein extract and lanes 7 and 8 contained 20μg of protein extract. All reactions contained 250 ng of $pVA_I$ template. Transcription was initiated by the addition of nucleotides and allowed to proceed for 1 h at $30^0$C.

**B)** As for **(A)**, except that 250 ng of pLeu was used as the specific pol III template rather than $pVA_I$.

**A**


5μg
10 μg
15μg
20μg
Extract:
neu βgal neu βgal neu βgal neu βgal
VA
1 2 3 4 5 6 7 8

**B**

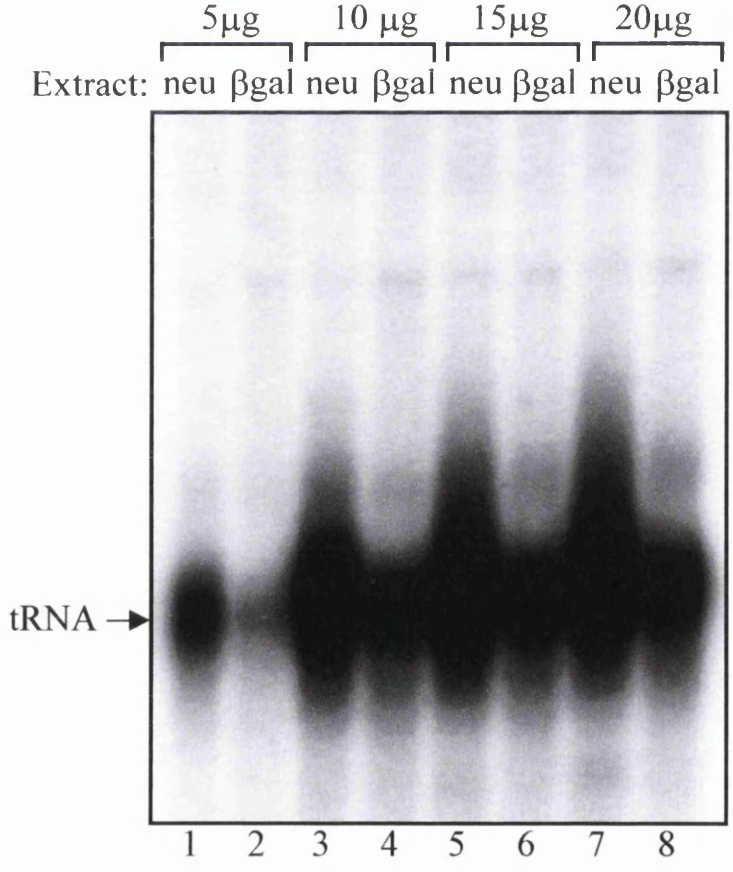
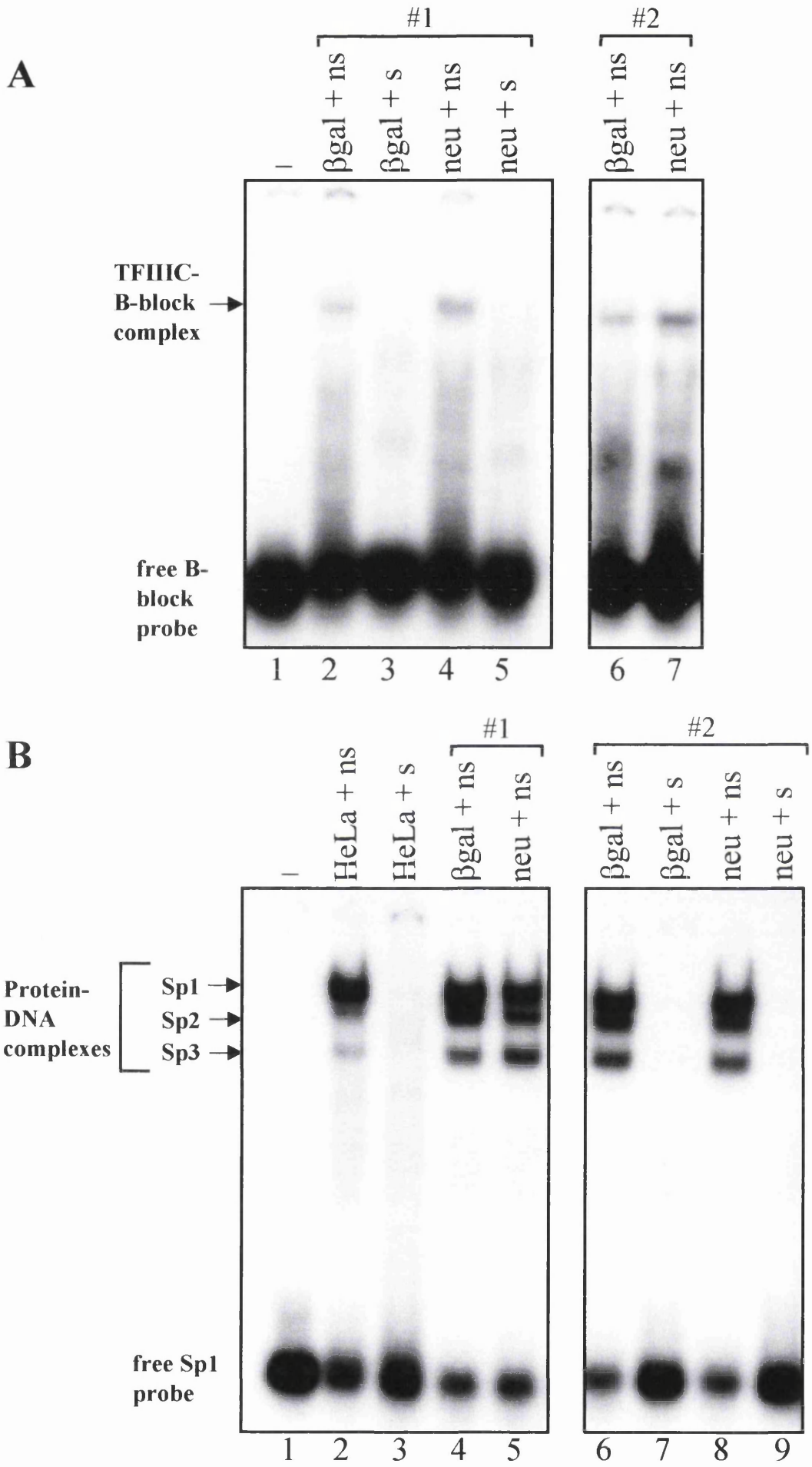

5μg
10 μg
15μg
20μg
Extract: neu βgal neu βgal neu βgal neu βgal
tRNA
1 2 3 4 5 6 7 8

**Figure 5.4**

**Extracts from *neu*-transformed ROSE 199 cells display elevated TFIIIC2 activity**

**A)** EMSA using 0.5 ng of B-block oligonucleotide probe, 1μg of poly(dI.dC) competitor and no protein (lane 1), or 10μg of protein extracts from ROSE 199-*βgal* cells (lanes 2, 3 and 6), or ROSE 199-*neu* cells (lanes 4, 5 and 7). Reactions 2-5 were carried out using one set of *βgal* and *neu* extracts that were prepared in parallel (#1) and reactions 6 and 7 were performed using a different set of *βgal* and *neu* extracts (#2). Reactions 2, 4, 6 and 7 also contained 100ng of unlabelled non-specific oligonucleotide. Reactions 3 and 5 also contained 100ng of unlabelled B-block oligonucleotide as specific competitor.

**B)** EMSA using 0.35ng of Sp1 oligonucleotide probe, 1μg of poly(dI.dC) competitor and no protein (lane 1), or 10μg of protein extracts from HeLa cells (lanes 2 & 3), ROSE 199-*βgal* cells (lanes 4, 6 and 7), or ROSE 199-*neu* cells (lanes 5, 8 and 9). Reactions 4 and 5 were carried out using the set of *βgal* and *neu* extracts used in reactions 2-5 of **(A)** (#1) and reactions 6-9 were performed using the set of *βgal* and *neu* extracts used in reactions 6 and 7 of **(A)** (#2). Reactions 2, 4, 5, 6 and 8 also contained 100ng of unlabelled non-specific oligonucleotide. Reactions 3, 7 and 9 also contained 100ng of unlabelled Sp1 oligonucleotide as specific competitor.


A
#1
#2
–
βgal + ns
βgal + s
neu + ns
neu + s
βgal + ns
neu + ns
TFIIIC-
B-block
complex
free B-
block
probe
1 2 3 4 5 6 7
B
#1
#2
–
HeLa + ns
HeLa + s
βgal + ns
neu + ns
βgal + ns
βgal + s
neu + ns
neu + s
Protein-
DNA
complexes
Sp1
Sp2
Sp3
free Sp1
probe
1 2 3 4 5 6 7 8 9

displaying lower TFIIIC2 DNA-binding activity than the corresponding *neu* extracts. The other extracts were also tested and in every case protein binding to the Sp1 oligonucleotide was about the same for the *βgal* and *neu* extracts or slightly lower for the *neu* extracts (data not shown). This clearly demonstrates that the increase in TFIIIC2 DNA-binding activity consistently observed in the *neu* extracts is a specific phenomenon and is not caused by a general loss of DNA-binding proteins during preparation of *βgal* extracts or a general increase in DNA-binding proteins following transformation of ROSE 199 cells by *neu*.

### 5.2.4 TFIIIC is a limiting factor for pol III transcription in extracts of the untransformed ovarian epithelial cell line ROSE 199-*βgal*

ROSE 199-*βgal* and ROSE 199-*neu* seem to mimic the behaviour of normal human ovarian epithelium and human ovarian tumours respectively, at least in terms of some aspects of pol III transcription. It was therefore of interest to see if TFIIIC is limiting in extracts of the ROSE 199-*βgal* cells, as this would provide an indication as to whether the increase in TFIIIC2 activity in ovarian tumours may be responsible for the increase in pol III transcriptional output.

In the initial add-back experiments, the phosphocellulose fractions PC-B and PC-C, which are enriched in TFIIIB and TFIIIC respectively, were used (Figure 5.5). The PC-C fraction strongly stimulated transcription in a dose-dependent manner. The highest dose of PC-C added stimulated transcription ~10-fold compared to that obtained with the same amount of *βgal* extract without addition of any PC fraction (Figure 5.5, compare lanes 1 & 5 with lanes 6-8). Addition of PC-B also stimulated transcription (Figure 5.5, compare lanes 1 & 5 with lanes 2-4), but to a much lesser extent than with comparable amounts of PC-C; the highest dose of PC-B titrated in increased transcription only 2.6 fold. Although this result suggests that TFIIIC is limiting, PC-B and PC-C are relatively crude fractions and contain a lot of other proteins; one or more of these proteins that have cofractionated with TFIIIC may be responsible for the stimulatory effect of PC-C, instead of TFIIIC itself. To test this possibility, add-back experiments were carried out using PC-C and heat-treated PC-C (HT PC-C). TFIIIC1 and TFIIIC2 are both unusually sensitive to mild heat

treatment and are inactivated by warming at 47$^0$C for 15 min (291, 485, 575). Prior to use in the add-back, the inactivation of the TFIIIC in the HT PC-C fraction was verified by its inability to reconstitute in vitro transcription in the presence of an active PC-B fraction (data not shown). As Figure 5.6 shows, the inactivation of TFIIIC in the HT PC-C fraction completely abolished its ability to stimulate transcription when added to the *βgal* extract. Thus, the stimulatory effect of PC-C on transcription reconstituted with *βgal* extracts does seem to be due to TFIIIC rather than some other protein(s) which also fractionated on phosphocellulose in the PC-C fraction.

Immunoaffinity-purified TFIIIC, generated as described in Chapter 3, was also tested, as shown in Figure 5.7. This highly purified source of TFIIIC stimulated transcription in a dose-dependent manner when titrated into *βgal* extracts (compare lane 1 with lanes 2-4). In contrast, mock immunopurified TFIIIC made in parallel as described in Chapter 3 using the 4286 preimmune serum, had little or no effect on transcription (Figure 5.7, lanes 5-7). This provides further support that TFIIIC is limiting in *βgal* extracts.

The same 4286 antiserum used to immunopurify TFIIIC from PC-C was also capable of purifying a pol III "holoenzyme" from nuclear extract, as described in Chapter 4. It was therefore important to verify that the immunopurified TFIIIC was not contaminated with any TFIIIB or pol III. As Figure 5.8 shows, the immunoaffinity-purified TFIIIC can reconstitute transcription when combined with a PC-B fraction that provides a source of TFIIIB and pol III (lanes 2 & 3). However, it is unable to reconstitute transcription when combined with a PC-C fraction, which provides a source of TFIIIC and pol III (lanes 6 & 7). Thus, the immunopurified TFIIIC does not seem to be contaminated with TFIIIB. RNA polymerisation assays revealed that the immunopurified TFIIIC is also free of pol III (data not shown). This result is unsurprising, as any interaction between TFIIIC and pol III is thought to be mediated by TFIIIB and phosphocellulose chromatography effectively separates TFIIIB and TFIIIC (473, 568).

The ability of DNA-affinity purified TFIIIC (see Chapter 3) to stimulate transcription when added to *βgal* extracts was also tested (Figure 5.9). This source of TFIIIC, purified on the basis of the specific B-block DNA-binding activity of TFIIIC2, strongly stimulated transcription up to ~5-fold of that obtained in the

**Figure 5.5**

**TFIIIC is a limiting factor in ovarian epithelial cell extracts**

In vitro transcription assay using 10μg of ROSE 199-*βgal* cell extract and 250ng of $pVA_I$ template. Reactions 2, 3 and 4 were supplemented with 2, 4 and 8μl of PC-B, respectively. Reactions 6, 7 and 8 were supplemented with 2, 4 and 8μl of PC-C, respectively. Reactions 1 and 5 contained no additional protein. Transcription was initiated by addition of nucleotides following a 15 min preincubation at $30^0$C.

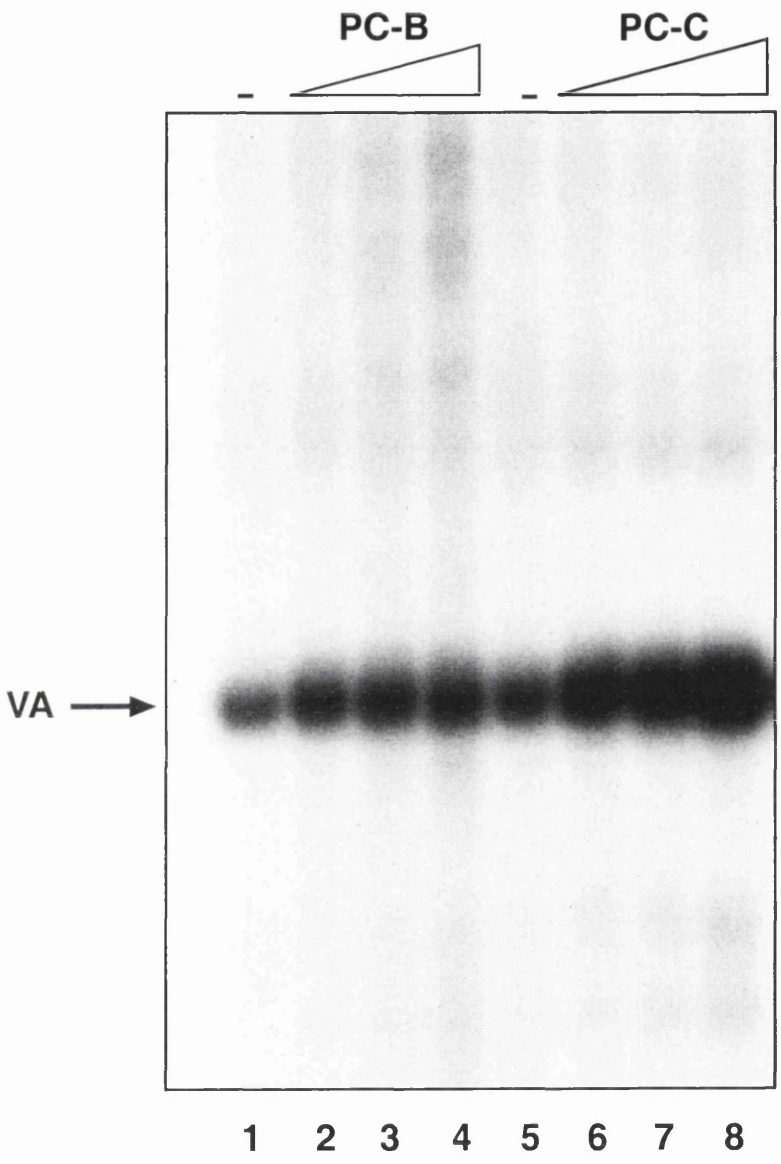

PC-B
PC-C
-
-
VA
1
2
3
4
5
6
7
8

**Figure 5.6**

**Heat inactivation of TFIIIC prevents PC-C from stimulating pol III transcription in ROSE 199-*βgal* cell extracts**

Transcription reactions were reconstituted using 10μg of ROSE 199-*βgal* cell extract and 250ng of $pVA_I$ template. Reactions 2, 3 and 4 were supplemented with 2μl, 4μl and 6μl of PC-C, respectively. Reactions 5, 6 and 7 were supplemented with 2μl, 4μl and 6μl, respectively, of the same PC-C fraction as in reactions 2-4, except that it was warmed at $47^0$C for 15 min prior to use.

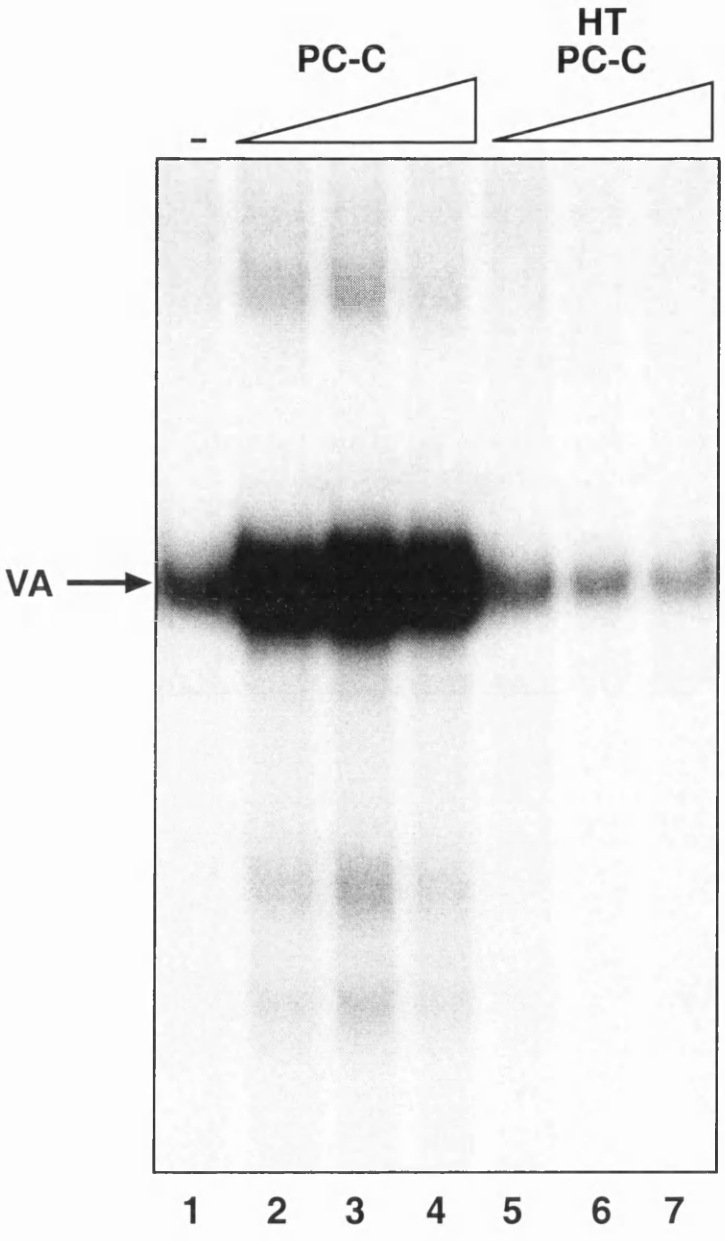

HT
PC-C
PC-C
-
VA
1
2
3
4
5
6
7

**Figure 5.7**

**Raising the level of TFIIIC stimulates pol III transcription in ovarian epithelial cell extracts**

In vitro transcription assay using 10μg of ROSE 199-*βgal* cell extract and 250ng of $pVA_I$ template. Reactions 2, 3 and 4 were supplemented with 3, 6 and 9μl, respectively, of TFIIIC that had been immunopurified from PC-C using antiserum 4286 against TFIIICβ. Reactions 5, 6 and 7 were supplemented with 3, 6 and 9μl of the material that had been mock immunopurifed from PC-C using the 4286 preimmune serum.

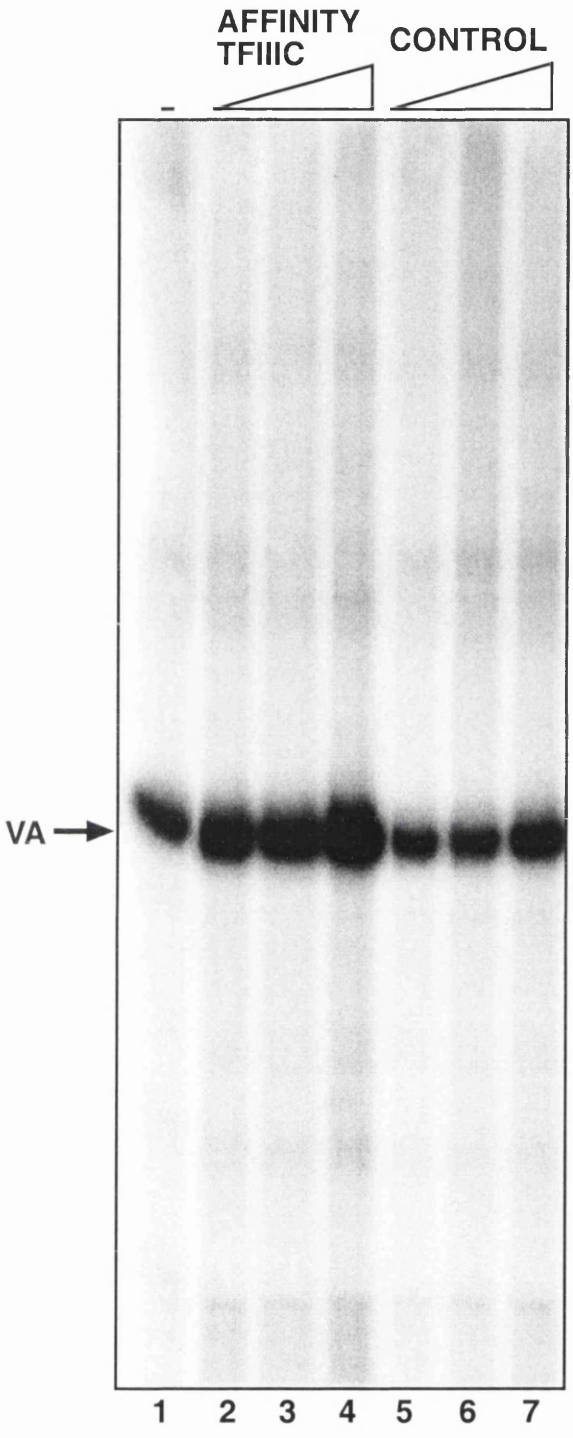

AFFINITY
TFIIIC
CONTROL
-
VA
1
2
3
4
5
6
7

**Figure 5.8**

**Immunopurified TFIIIC contains no trace of TFIIIB**

In vitro transcription assay using 250 ng of $pVA_I$ template, 1μl of PC-B (all lanes except 5-7) and 1μl of PC-C (lanes 4-8). TFIIIC that had been immunopurifed using antiserum 4286 against TFIIICβ was added to reactions 2 & 6 (6μl), and also, 3 & 7 (12μl).

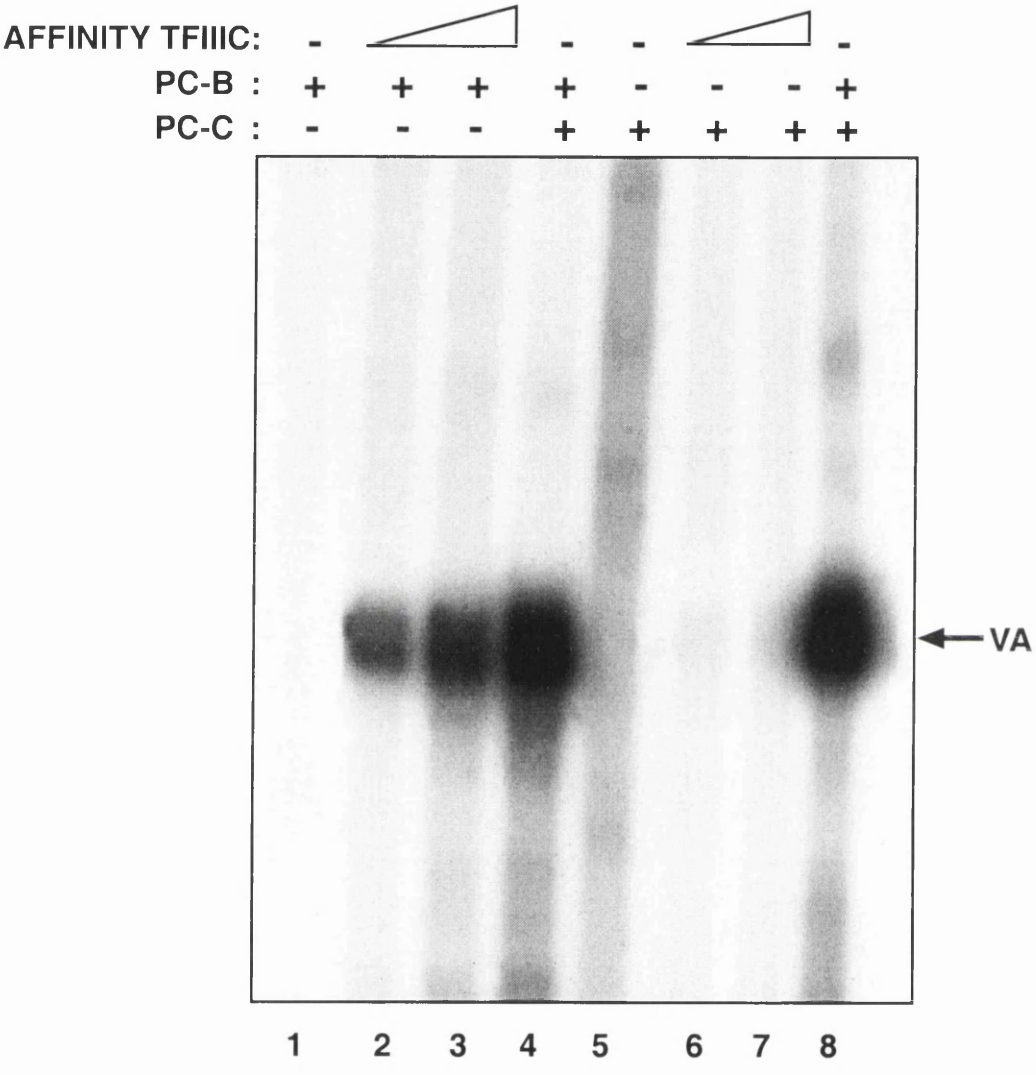
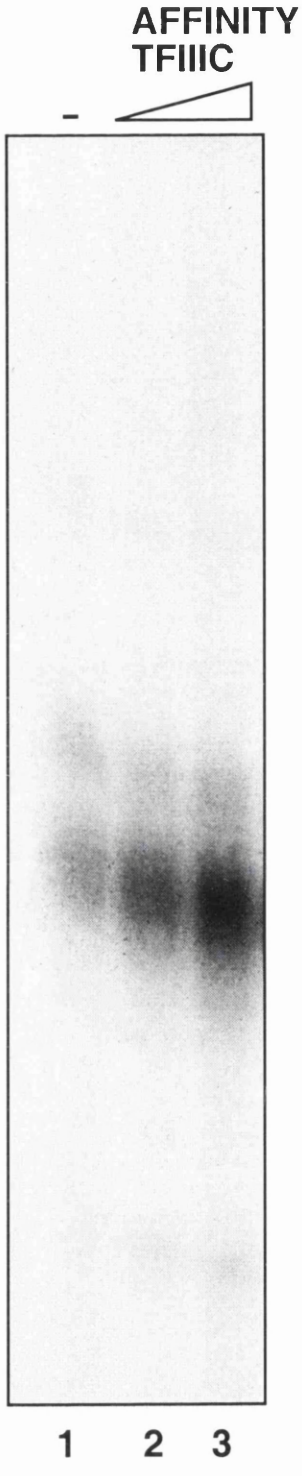

AFFINITY TFIIIC: - - - - -
PC-B : + + + + - - - +
PC-C : - - - + + + + +
VA
1 2 3 4 5 6 7 8

**Figure 5.9**

**tRNA synthesis is stimulated by raising the level of TFIIIC in ovarian epithelial cell extracts**

Transcription reactions were reconstituted using 10μg of ROSE 199-*βgal* cell extract and 250ng of pLeu template. Reactions 2 and 3 were supplemented with 3μl and 6μl, respectively, of TFIIIC that had been affinity-purified using a B-block oligonucleotide column.


AFFINITY
TFIIIC
-
1
2
3

absence of added TFIIIC (Figure 5.9, compare lane 1 with lanes 2 & 3). This transcription assay was carried out using a $tRNA^{leu}$ gene as the specific template. The different sources of TFIIIC (PC-C, immmunopurified TFIIIC and DNA-affinity purified TFIIIC) have all been tested and found to stimulate transcription reconstituted with *βgal* extracts both using the $VA_I$ gene and the $tRNA^{leu}$ gene as the specific pol III template. Together, these results strongly suggest that TFIIIC is a limiting factor for pol III transcription in the untransformed rat ovarian epithelial cell line, ROSE 199-*βgal*. Thus, the elevated TFIIIC2 activity of ROSE 199-*neu* cells is likely to contribute to the elevated pol III transcriptional output of these cells. That TFIIIC is limiting in ROSE 199-*βgal* cells clearly does not mean that the same is true in human ovarian epithelium. However, it does raise this possibility and suggests that the elevation of TFIIIC2 activity in human ovarian cancers may be of functional significance to the increase in pol III transcript levels.

The promoters of class III genes are quite diverse in structure and can be loosely categorised into one of three types, each of which has distinct transcription factor requirements (568). Only types I and II promoters are TFIIIC2-dependent. Within each of these three types there is still considerable variation in promoter structure and also the strength of the promoter. The consequence of this variability is that the limiting factor(s) for pol III transcription in a given cell extract may be different for different class III genes, even if they are of the same promoter type. The results obtained suggest that TFIIIC is limiting in ROSE 199-*βgal* cell extracts at least for the transcription of some class III genes. The $VA_I$ gene and a $tRNA^{leu}$ gene were primarily tested; these genes both have a type II promoter but the promoter of the tRNA gene is much weaker. Affinity-purified TFIIIC also stimulated transcription reconstituted using *βgal* extract when using the 7SL gene as the specific pol III template (data not shown).

The evidence suggesting that TFIIIC can stimulate tRNA synthesis in untransformed ovarian epithelial cells is of particular physiological significance because of the fundamental role of tRNA in translation and the fact that elevated levels of TFIIIC2 were found in nine out of nine human ovarian tumours analysed. The deregulation of TFIIIC2 has never previously been reported in human malignancies. It has not as yet been demonstrated that tRNA and 5S rRNA synthesis has an essential role in tumorigenesis. If this is found to be the case, it may be that

the deregulation of TFIIIC2 is a widespread phenomenon in ovarian cancer and perhaps other cancers in which TFIIIC2 is a major limiting factor in the normal nontumorigenic cell type.

### 5.2.5 TFIIIB and TFIIIC are both limiting for pol III transcription in cell extracts of the transformed cell line ROSE 199-*neu*

In the extract from a human ovarian tumour cell line that was tested TFIIIB was found to be the major limiting factor for pol III transcription (Figure 5.2). It was of interest to determine if this was also the case in the transformed cell line ROSE 199-*neu*, or whether TFIIIC was still limiting as in the *βgal*-transfected cells, despite the significant increase in TFIIIC2 activity in the *neu*-transformed cells. As Figure 5.10 **A** shows, PC-B (lanes 2-4) and PC-C (lanes 9-11) both stimulated transcription when separately titrated into extracts of ROSE 199-*neu* cells (compare with lanes 1 & 8). However, PC-C stimulated transcription to a greater extent than equal volumes of PC-B. The PC-B and PC-C fractions were of comparable TFIIIB and TFIIIC activity, respectively. As previously, the effect of HT PC-C was also tested and was found unable to stimulate transcription (lanes 12-14), suggesting that TFIIIC rather than some contaminant present in the PC-C was responsible for the ability of PC-C to stimulate transcription.

In support of this, immunopurified TFIIIC was able to stimulate transcription reconstituted with unfractionated *neu* extracts (Figure 5.10 **B**, compare lanes 2 & 3 with lane 1) whereas mock-immunopurified TFIIIC was not (Fig. 5.10 **B**, lanes 4 & 5). Immunopurified TFIIIB also stimulated transcription (lanes 6 & 7) but mock immunopurified TFIIIB was unable to (lanes 8 & 9). As for the immunopurified TFIIIC, the immunopurified TFIIIB used was tested for contamination with pol III and, in this case, TFIIIC. None of the different preparations of purified TFIIIB contained pol III, as assayed by the ability of the fractions to support transcription of poly(dA.dT) (data not shown). Immunopurified TFIIIB also lacked TFIIIC as Figure 5.11 shows for a variety of different preparations of TFIIIB of varying activity. The immunopurified TFIIIB preparations reconstituted transcription when combined with PC-C but were consistently unable to do so when combined with PC-B. The PC-B

fraction was able to reconstitute some transcription when combined with a weakly active immunopurified TFIIIC fraction, however. Thus, TFIIIB and TFIIIC both seem to be limiting in extracts of ROSE 199-*neu*. This result has been confirmed with several independent preparations of *neu* extract (data not shown).

Heat treatment of PC-B, as done for PC-C, selectively inactivates TBP (575). The ability of HT PC-B to stimulate transcription reconstituted with *neu* cell extracts was tested to see if TBP, an essential component of TFIIIB, was required for the ability of PC-B to stimulate transcription. As Figure 5.10 **A** shows, HT PC-B actually stimulated transcription more strongly than equal amounts of the same PC-B fraction that was not heat treated. This suggests that TBP is not required for the stimulatory effect of PC-B. Indeed, the result suggests that TBP may have a slight inhibitory effect; this is probably caused by the TBP being in excess and sequestering components that are limiting into inactive complexes. That PC-B and immunopurified TFIIIB have a stimulatory effect on transcription reconstituted with *neu* extracts is therefore likely to be caused by a relative deficiency in one or more of the TAFs of TFIIIB, such as BRF, in the *neu* extracts.

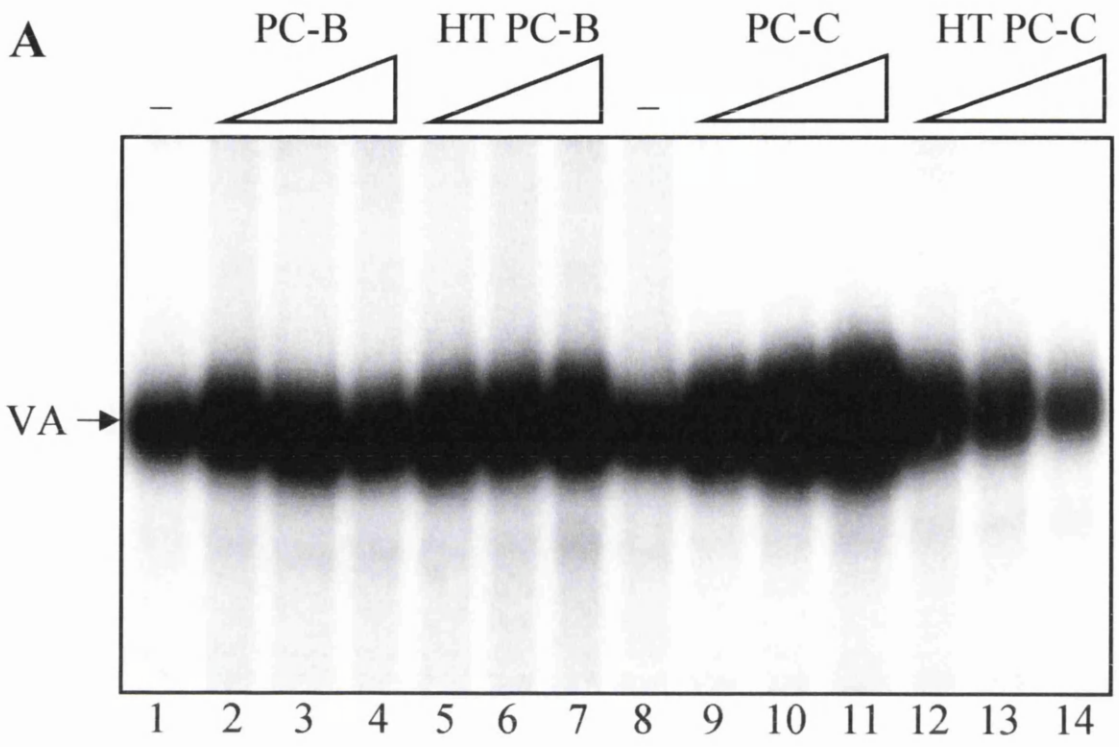
The inactivation of TBP in the HT PC-B was verified by testing the ability of the fraction to reconstitute transcription when combined with a PC-C fraction, as shown in Figure 5.12 **A**. Only a trace amount of specific pol III transcript was produced compared to that obtained using untreated PC-B. In support of the stimulation observed with HT PC-B (Fig. 5.10 **A** & Fig. 5.12 **B**) suggesting that TBP may not be limiting, the titration of increasing amounts of recombinant TBP into *neu* extracts actually reduced the level of transcription to below that obtained with *neu* extract alone (Figure 5.12 **B**). This dose-dependent decrease in transcription in response to recombinant TBP suggests that TBP is in relative excess in the *neu* extracts and that titrating in more results in a quenching effect whereby essential components that are limiting are prevented from participating in transcription.

**Figure 5.10**

**TFIIIB and TFIIIC both stimulate pol III transcription when independently titrated into *neu*-transformed ROSE 199 ovarian epithelial cell extracts**

**A)** In vitro transcription assay using 250ng of $pVA_I$ template and 10μg of ROSE 199-*neu* cell extract (all reactions). PC-B was added to reactions 2-7: reactions 2 and 5, 2μl; reactions 3 and 6, 4μl; reactions 4 and 7, 6μl. The PC-B that was added to reactions 5-7 is the same as that added to reactions 2-4 except that it was heated at $47^0$C for 15 min prior to use (HT PC-B). PC-C was added to reactions 9-14: reactions 9 and 12, 2μl; reactions 10 and 13, 4μl; reactions 11 and 14, 6μl. The PC-C added to reactions 12-14 was heated at $47^0$C for 15 min prior to use.

**B)** In vitro transcription add-back experiment using immunopurified fractions. All the reactions contained 250ng of $pVA_I$ template and 10μg of ROSE 199-*neu* cell extract. TFIIIC that had been purified using the 4286 antiserum against TFIIICβ was added to reactions 2 (3μl) and 3 (6μl). Reactions 4 and 5 contained 3μl and 6μl respectively, of material that was mock immunopurified using the 4286 preimmune serum. TFIIIB that had been purified using the 330 antiserum against BRF was added to reactions 6 (3μl) and 7 (6μl). Reactions 8 and 9 contained 3μl and 6μl, respectively, of material that was mock immunopurified using the 330 preimmune serum.


A
PC-B
HT PC-B
PC-C
HT PC-C
–
–
VA
1 2 3 4 5 6 7 8 9 10 11 12 13 14

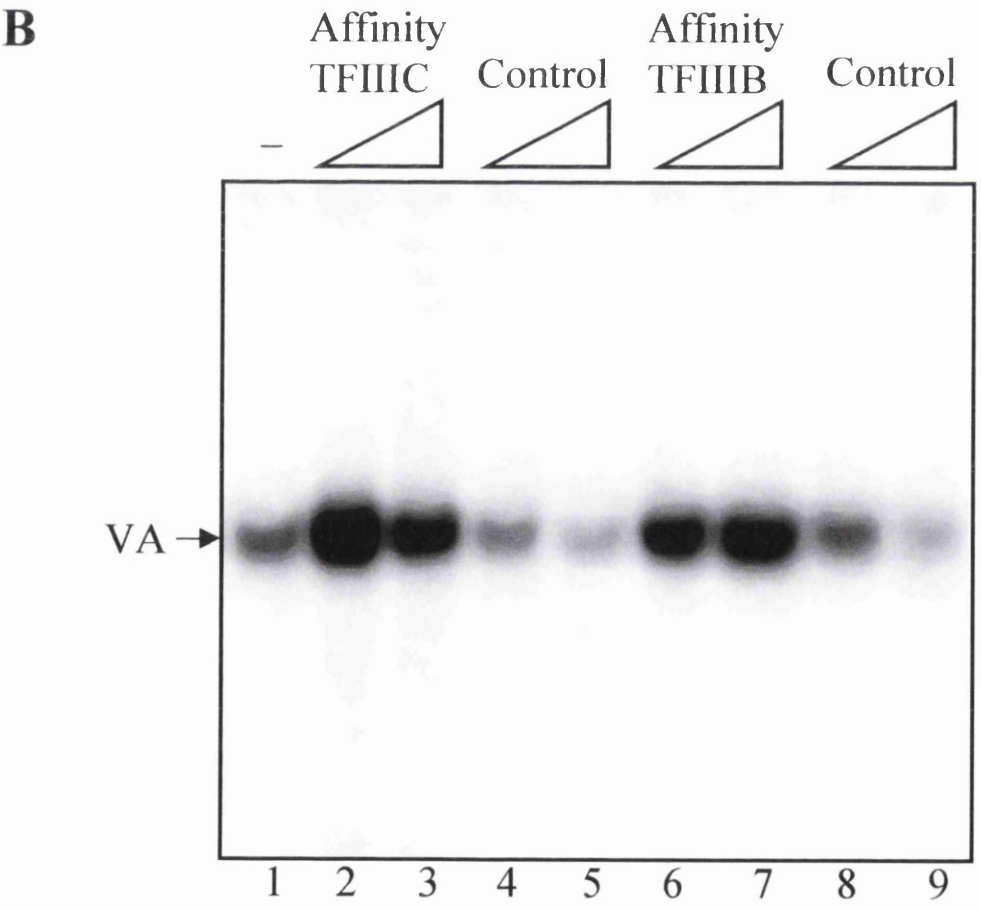

B
Affinity TFIIIC
Control
Affinity TFIIIB
Control
–
VA
1 2 3 4 5 6 7 8 9

**Figure 5.11**

**Immunopurified TFIIIB is free of TFIIIC**

In vitro transcription assay using 250ng of $pVA_I$ template, 1μl of PC-B fraction (lanes 2-4, 6 and 8) or PC-C fraction (lanes 1, 5, 7 and 9), and 12μl of TFIIIB that had been immunopurified using antiserum 330 against BRF (lanes 4-9). Lane 2 contained 12μl of TFIIIC that had been immunopurified using antiserum 4286 against TFIIICβ. Three independent preparations of immunoaffinity TFIIIB of differing activity were assayed for contamination with TFIIIC: Preparation A, lanes 4 and 5; Preparation B, lanes 6 and 7; Preparation C, lanes 8 and 9.

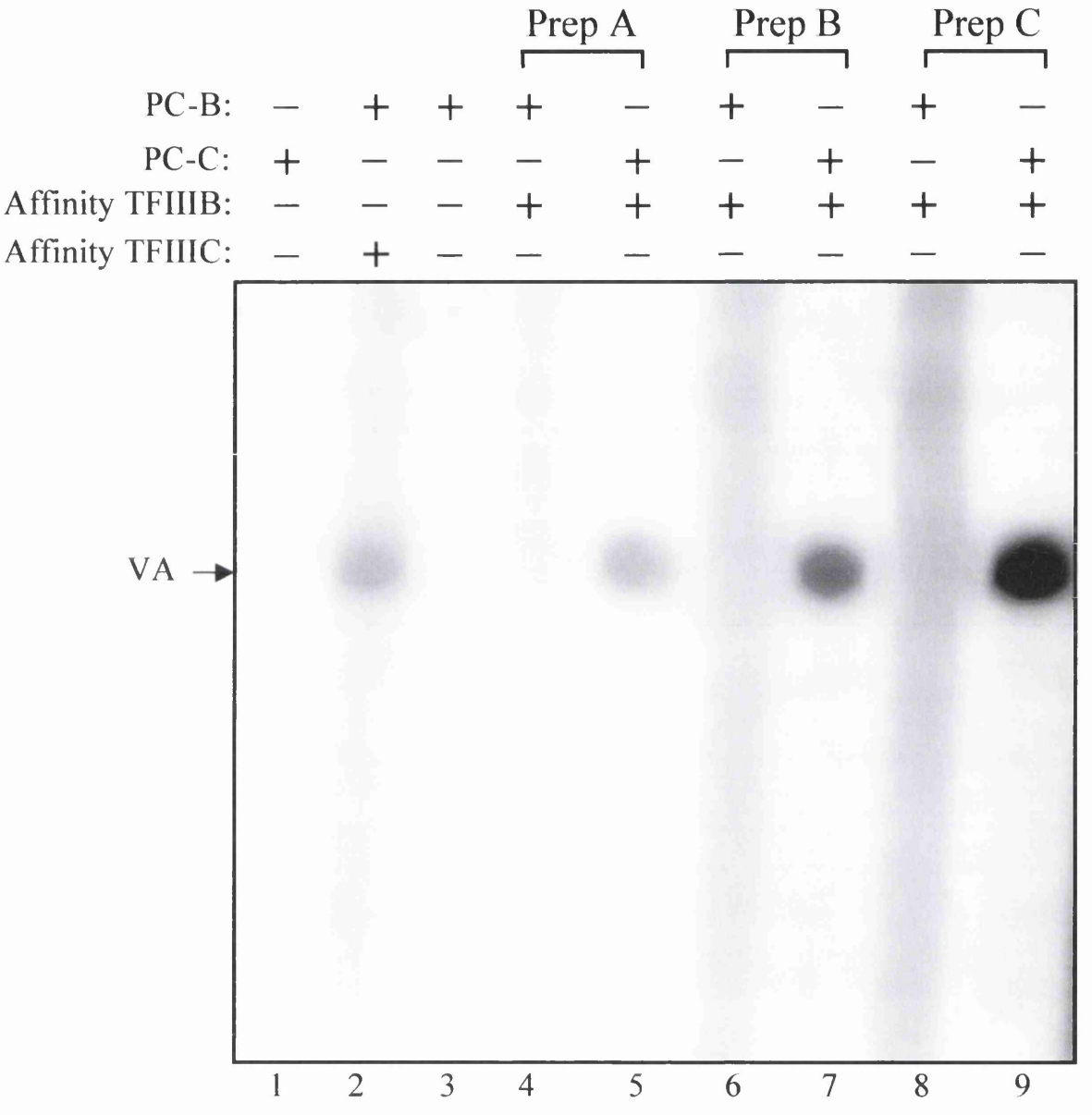
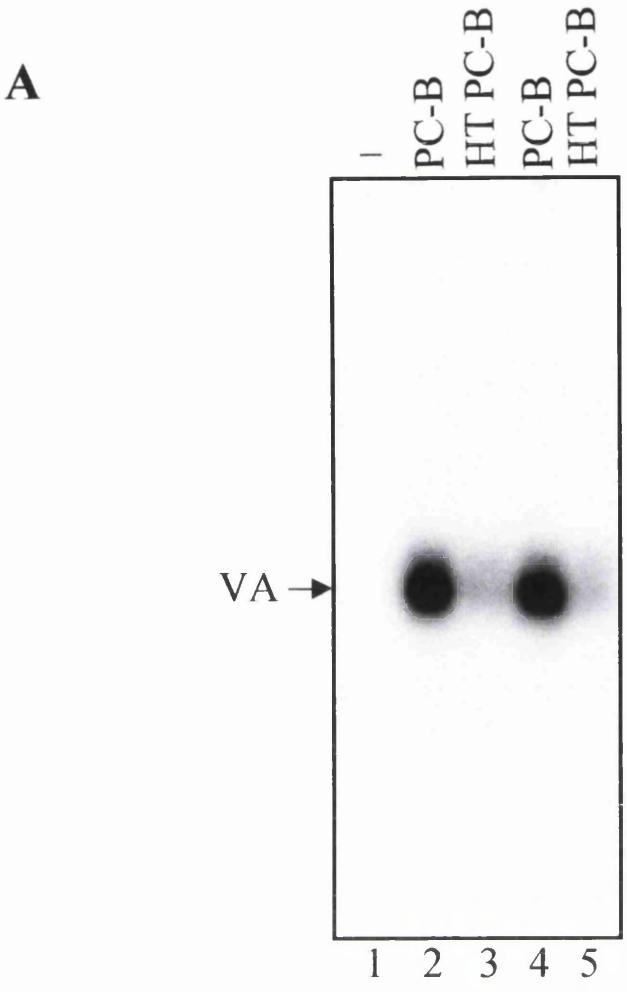

Prep A
Prep B
Prep C
PC-B: – + + + – + – + –
PC-C: + – – – + – + – +
Affinity TFIIIB: – – – + + + + + +
Affinity TFIIIC: – + – – – – – – –
VA
1 2 3 4 5 6 7 8 9

**Figure 5.12**

**TBP is not required for the stimulatory effect of TFIIIB on pol III transcription reconstituted with ROSE 199-*neu* cell extracts**

**A)** In vitro transcription assay reconstituted with 250ng $pVA_I$ template, 2μl of PC-B (reactions 2-5) and 2μl of PC-C (all reactions). The PC-B added to reactions 3 and 5 was heated at $47^0$C for 15 min prior to use.

**B)** Transcription of $pVA_I$ (250ng) using ROSE 199-*neu* cell extract (10μg) preincubated (15 min at $30^0$C) with no addition (reactions 1 and 10) or with 1, 2, 3 or 4μl of recombinant TBP (reactions 2, 3, 4 and 5, respectively) or with 1, 2, 3 or 4μl of HT PC-B (reactions 6, 7, 8 and 9, respectively).


A
–
PC-B
HT PC-B
PC-B
HT PC-B
VA
1 2 3 4 5
B
TBP
HT PC-B
–
–
VA
1 2 3 4 5 6 7 8 9 10

## 5.3 Discussion

### 5.3.1 Deregulation of TFIIIC2

The activation of pol III transcription in the transformed ovarian epithelial cell line ROSE 199-*neu* provides another example to add to a long and growing list of transformed cell types that display elevated pol III transcription. Furthermore, extracts of ROSE 199-*neu* cells showed a specific increase in TFIIIC2 activity. The significance of this is that this same molecular abnormality was observed in nine out of nine human ovarian tumours analysed, each of which also displayed elevated pol III transcription (586). This analysis of human ovarian tumours represents a limited survey. However, the finding that the transformed rat ovarian epithelial cell line ROSE 199-*neu*, which is considered a useful model of certain aspects of human ovarian cancer (115), also has substantially elevated TFIIIC2 activity suggests that this phenomenon may be widespread and, perhaps, fundamental to ovarian cancer. Clearly there is a need to analyse a larger number of ovarian tumour samples for changes in pol III transcript levels and also the mechanistic basis for any changes. Increased TFIIIC2 activity has previously been observed in SV40-transformed murine cell lines and also in adenoviral-infected HeLa cells (315, 488, 578, 606). Although elevated pol III transcript levels have recently been reported in a number of human cancers the molecular basis for this has not been studied (87). It remains to be determined whether changes in TFIIIC2 activity are also found in other human malignancies.

The mechanism by which TFIIIC2 activity is increased in ROSE 199-*neu* cells has not yet been investigated. It may be that TFIIIC2 is overexpressed as seems to be the case in the human ovarian tumours that were analysed (586). Alternatively, perhaps there is an increase in the proportion of TFIIIC2 that is in the active TFIIIC2a form. This may be caused by a specific increase in TFIIICβ concentration or by phosphorylation induced changes (217, 488). The stable transfection of ROSE 199 cells with activated *neu* oncogene activates pol III transcription and elevates TFIIIC2 activity when compared to the same cell line stably transfected with a *β-galactosidase* gene. This suggests that the *neu* oncogene and its normal cellular

counterpart impinge directly or indirectly on pol III transcription somehow, and that at least one of the targets is TFIIIC2.

The *neu/c-erbB2* gene encodes an EGFR-like receptor tyrosine kinase (RTK) (241, 399). erbB2 is a member of the erbB family of RTKs, which also includes EGFR (erbB), erbB3 and erbB4 (241, 399). Activation of the tyrosine kinase activity of the erbB family members occurs through receptor dimerisation induced by ligand binding. No direct ligand for erbB2 has yet been discovered despite extensive efforts (241, 399). However, the agonists for the other erbB family members are bivalent and erbB2 is the preferred heterodimerisation partner for all the other erbB family members (399). erbB2 kinase activity can also be activated by overexpression of erbB2, which is a frequent occurrence in human ovarian cancers, and causes spontaneous dimerisation (399). Activation of tyrosine kinase activity of the receptors triggers autophosphorylation of specific tyrosine residues within the cytoplasmic domain. These phosphotyrosines act as docking sites for specific downstream proteins (399). The erbB2 receptor seems to influence a diverse range of intracellular signalling pathways; this will also depend on its dimerisation partner (241, 399). Recently it has been shown that cyclin D1 is a critical downstream target of activated neu (323). The induction of cyclin D1 expression by neu is dependent on E2F (323). Furthermore, Ras, Rac, Rho, extracellular signal-regulated kinase, c-Jun N-terminal kinase and p38 are all involved (323). Perhaps, neu raises TFIIIC2 activity indirectly via the phosphorylation of TFIIIC2 by an intracellular kinase that is part of one of the kinase signalling cascades induced by neu. Alternatively, as for the cyclin D1 promoter, perhaps the TFIIIC2 promoter contains E2F-binding sites and increased expression of TFIIIC2 mRNA induced by E2F is responsible for the increased TFIIIC2 activity of *neu*-transformed ROSE 199 cells.

The identification of cyclin D1 as a critical downstream effector of *neu*-induced transformation suggests that TFIIIC2 may not be the only pol III transcription component that is targeted in the ROSE 199-*neu* cells. Cyclin D1, in combination with its kinase partner cdk4 or cdk6, has a critical role in the phosphorylation and inactivation of RB (482). Since RB represses TFIIIB, TFIIIB activity may also be elevated in the *neu*-transformed cells. This has not yet been investigated.

Neu is amplified and/or overexpressed in approximately one-third of human ovarian cancers (30, 159, 491). However, the neu status of the nine human ovarian

tumour samples that displayed elevated TFIIIC2 activity has not been determined. It may be that the deregulation of TFIIIC2 occurs independently of neu in some or perhaps all of these tumours. The molecular basis of human ovarian cancer is poorly understood, largely owing to the inaccessible position of the ovary and the fact that the tumours are often very advanced at the time of detection (159). However, in addition to the changes in neu, mutations in *K-ras* and *p53* have also frequently been detected (159). Ras has previously been shown to increase TBP abundance and is also involved in neu signalling (323, 548, 549). Like RB, p53 can inhibit TFIIIB. TFIIIB activity may therefore be elevated in some ovarian cancers. Perhaps multiple components of the pol III transcriptional apparatus are deregulated in ovarian cancer, as has previously been reported for several transformed cell types (569). The increased TFIIIC2 activity of the nine ovarian tumour samples analysed seems to have resulted from the overexpression of its subunits (586). The molecular basis for this overexpression has yet to be elucidated, but potentially neu, K-ras or p53 may be involved.

### 5.3.2 TFIIIC is limiting in ROSE 199-*βgal* cell extracts

PC-C, immunoaffinity purified TFIIIC and DNA-affinity purified TFIIIC were each found to consistently stimulate pol III transcription reconstituted with ROSE 199-*βgal* cell extracts. In contrast, heat-treated PC-C and mock-immunopurified TFIIIC did not stimulate transcription. This suggests that TFIIIC is limiting for transcription in this untransformed ovarian epithelial cell line. Therefore, the increase in TFIIIC2 activity in the transformed cell line ROSE 199-*neu* is likely to contribute, at least to some extent, to the increased pol III transcriptional activity of these cells. Although TFIIIC has been found to be limiting in ROSE 199-*βgal* cells, clearly one cannot make the assumption that this is also true in normal human ovarian epithelial cells. As a spontaneously immortalised cell line, ROSE 199-*βgal* cells will have undergone some changes relative to normal ovarian epithelial cells in vivo. Furthermore, although pol III transcription is well conserved between mammalian species, there are inevitably differences and the limiting factor(s) for pol III transcription in rodent and human ovarian epithelial cells may be very different. In the extract from the

human ovarian tumour cell line A27-80, TFIIIB was found to be the major limiting factor for pol III transcription. In contrast, both TFIIIB and TFIIIC were found to significantly stimulate transcription when titrated into extracts of ROSE 199-*neu* cells. This difference in the limiting factor(s) suggests that the same may also be true for normal human ovarian epithelial cells when compared with ROSE 199-*βgal* cells.

For most class III genes, TFIIIC transcriptional activity is dependent not only on TFIIIC2 but also TFIIIC1. Although ROSE 199-*neu* cells display an increase in TFIIIC2 activity, a concomitant increase in TFIIIC1 activity has not been tested for. Conversely, the finding that TFIIIC is limiting in ROSE 199-*βgal* cell extracts has not been dissected any further. Thus, it is at present unclear whether TFIIIC2, TFIIIC1 or both these components are limiting in ROSE 199-*βgal* cell extracts. It is therefore plausible that the increase in TFIIIC2 activity in ROSE 199-*neu* cells does not contribute to the activation of pol III transcription in these cells because it is TFIIIC1 and not TFIIIC2 that is limiting in the untransformed ROSE 199-*βgal* cells. Although this possibility is considered unlikely, it deserves further investigation.

### 5.3.3 More than one limiting factor

The finding that both TFIIIB and TFIIIC can significantly stimulate transcription when independently titrated into extracts of ROSE 199-*neu* cells indicates that both of these factors are limiting in these extracts. Such a situation may arise where one of the two limiting factors is only slightly in excess of the other limiting factor. For example, suppose TFIIIB is in slight excess of TFIIIC. Despite this, it is unlikely that every single TFIIIC molecule that is promoter-bound at any given time will be associated with TFIIIB. By raising the concentration of TFIIIB the probability of a collision between "free" TFIIIB in solution and promoter-bound TFIIIC may be increased resulting in an increase in the number of active preinitiation complexes. If, however, TFIIIB is in vast excess raising its concentration further is unlikely to significantly alter the probability of these collisions. Indeed, it may actually inhibit transcription, for example by sequestering some pol III from participating in active complex formation.

The limiting factor(s) for pol III transcription in a given cell type can also vary depending on cell cycle position. For example, using extracts of synchronised proliferating HeLa cells it has been shown that in G1 phase extracts TFIIIB is limiting and the addition of partially purified TFIIIC had no effect (571). In contrast, in G2 phase extracts TFIIIC is limiting and the addition of the same partially purified TFIIIC fraction strongly stimulated transcription (571). In this particular study all the extracts have been prepared from asynchronously proliferating cells. It is likely that TFIIIC is only limiting in ROSE 199-*βgal* cells in a restricted window of the cell cycle. Similarly, elevated levels of TFIIIC2 activity observed in asynchronous extracts of ROSE 199-*neu* cells may be restricted to a particular part of the cell cycle. The fact that there can be more than one limiting factor in a given cell type and that the identity of the limiting factor(s) can change with time, for example depending on cell cycle position, may explain why several DNA-tumour viruses target multiple components of the pol III transcriptional apparatus. Thus, the deregulation of TFIIIC2 may only be one of several changes in the pol III transcriptional apparatus of ROSE 199-*neu* cells and human ovarian tumours.

# Chapter 6.

# Modulation of pol III transcription by phosphorylation

## 6.1 Introduction

Protein phosphorylation is arguably the most ubiquitous control mechanism of protein function and certainly the most studied. Primary stimulation or repression of transcription in eukaryotes is largely independent of de novo protein synthesis, suggesting the involvement of posttranslational modifications such as phosphorylation or acetylation (238). The cellular response to extracellular stimuli commonly involves changes in gene expression. This is dependent upon a complex network of intracellular signal transduction pathways of protein kinases that integrate these extracellular signals and transmit them to the nucleus. Thus, protein phosphorylation has a critical role in regulating transcription, whether directly by the phosphorylation of the transcription factors themselves or indirectly, for example by the phosphorylation of upstream activators or repressors.

Little is known as to which kinase signalling pathways impinge on pol III transcription; however, several kinases have been reported to have an effect. The pocket proteins RB, p107 and p130 all repress pol III transcription (93, 314, 503, 504, 579). The activity of the pocket proteins depends on their phosphorylation status; in a hyperphosphorylated state the pocket proteins are inactive (173, 239, 418, 482, 557). The cyclin-dependent kinases that regulate the phosphorylation of the pocket proteins may therefore indirectly exert a regulatory effect on pol III transcription. Another cyclin-dependent kinase, p34cdc2/cyclin B1, is implicated in the mitotic repression of pol III transcription. Mitotic repression of pol III transcription can be reproduced in vitro and is achieved through the phosphorylation and inactivation of TFIIIB (169, 327, 572). p34cdc2/cyclin B1 is the major mitotic kinase and can specifically phosphorylate and inactivate affinity-purified Xenopus TFIIIB (169, 587). Therefore, the downregulation of pol III transcription during

mitosis may be achieved, at least in part, by the phosphorylation of TFIIIB by p34cdc2/cyclin B1.

The hepatitis B virus X protein, which can induce liver cancer in transgenic mice (274), has been shown to substantially elevate pol III transcription in a variety of cell lines (16, 256, 304, 548, 549). This is dependent on Ras signalling (548, 549). Dominant negative Ras or a Ras farnesylation inhibitor both block X-induced activation of pol III transcription (548). Constitutively active Raf, a Ras effector, can overcome this block (548). Moreover, constitutively activated Ras can increase tRNA transcription in cells not infected with X protein (548). Since activating mutations in Ras are a very frequent occurrence in human cancers (344), this may be another mechanism by which pol III transcription is deregulated in transformed and human tumour cells. X protein induces an increase in the abundance of TBP, which may be sufficient to account for the increase in pol III transcription in some cell lines (527, 548, 549). The X protein enhances the transcription of the TBP gene (256). This involves three distinct Ras-activated pathways (256), suggesting that the Ras-Raf-MEK-MAP kinase pathway may not be the only Ras pathway that can affect pol III transcription.

The highly conserved TOR (target of rapamycin) pathway is also implicated in the regulation of pol III transcription. Treatment of budding yeast cells with the antibiotic rapamycin induces the $G_0$ program of molecular events, including the repression of pol III transcription (611). This is TOR-dependent, as extracts from rapamycin-treated yeast strains that lack the rapamycin ligand FKBP12 have comparable pol III transcriptional activity to extracts from untreated cells (611). Direct evidence that TOR signalling can influence pol III transcription is provided by the phenotype of a yeast strain, *tor2*$^{ts}$, harbouring a temperature sensitive mutation in the highly conserved TOR kinase, Tor2. Extracts from the *tor2*$^{ts}$ strain harvested at the permissive temperature have similar pol III transcriptional activity to extracts from cells that are wild-type for Tor2 (611). In contrast, extracts from *tor2*$^{ts}$ cells harvested at the restrictive temperature have much lower pol III transcriptional activity than wild-type cells (611).

In yeast, protein kinase CKII (formerly known as casein kinase II) is required for high levels of basal pol III transcription (216). A yeast strain, *cka2*$^{ts}$, bearing a temperature-sensitive lesion in the catalytic $\alpha'$ subunit of CKII, has significantly

reduced CKII activity compared to wild-type cells, both at the permissive and non-permissive temperature (163, 216). Accordingly, *cka2*$^{ts}$ cells and extracts prepared from this mutant strain display substantially diminished levels of tRNA and 5S rRNA synthesis (163, 216). When *cka2*$^{ts}$ cells are shifted to the restrictive temperature, concomitant with a further decrease in CKII activity, pol III transcription is reduced (216). In contrast, basal pol I- and pol II- transcription were found to be unaffected by the severely reduced CKII activity of the *cka2*$^{ts}$ strain (216). Titration of increasing amounts of purified wild-type CKII into extracts of *cka2*$^{ts}$ cells stimulated pol III transcription in a dose-dependent manner, consistent with a positive role for CKII in yeast pol III transcription (216). Further evidence was provided by the inhibition of tRNA synthesis in wild-type extracts by the commonly used CKII inhibitor, 2,3-diphosphoglycerate (216). The deficiency in pol III transcriptional activity of *cka2*$^{ts}$ cell extracts could also be specifically rescued by TFIIIB purified from wild-type cells, implicating TFIIIB as the CKII-responsive pol III factor (163, 164). Addition of TFIIIC or pol III had no effect (163, 164). TFIIIB is strongly phosphorylated by CKII in vitro; moreover, the dephosphorylation of TFIIIB eliminated its ability to rescue pol III transcription in CKII-deficient extracts (163, 164). Specifically, it is the TBP subunit of TFIIIB that is efficiently phosphorylated by CKII. Furthermore, CKII enhances the ability of recombinant TBP to stimulate pol III transcription in *cka2*$^{ts}$ cell extracts (163, 164). Although this suggests that TBP is at least one of the physiological targets of CKII among the pol III transcription components, it remains to be determined how the phosphorylation of TBP by CKII might stimulate pol III transcription. Nevertheless, the evidence is very convincing for an essential role for CKII in pol III transcription in yeast. In this chapter the role of CKII in mammalian pol III transcription is investigated.

CKII is a highly conserved Ser/Thr kinase (7). It is ubiquitously expressed in eukaryotes and is found both in the cytoplasm and the nucleus (7). Although the exact physiological role of CKII is not known, many lines of evidence suggest that CKII has essential roles in cell proliferation and growth (7). There is elevated CKII activity in rapidly dividing cells, both normal and transformed (243, 387). Indeed, a comparison with the proliferation marker Ki67 suggested that CKII would be a reliable marker protein for proliferation (387). Hence, CKII is elevated in tumour cells but also in normal cells with high mitotic activity, for example colorectal mucosa or embryonic cells during highly proliferative stages (387). Certain

mitogens can stimulate CKII activity, for example insulin, insulin-like growth factor-I or EGF (76, 279, 414, 493). Indeed, CKII is required for cell cycle progression (192, 413). Microinjection of antibodies against the positive-acting regulatory β subunit of CKII into human primary fibroblasts at various times of the cell cycle revealed that CKII is required for transition of $G_0/G_1$, early $G_1$, and $G_1/S$ phases of the cell cycle (413). Disruption of the *CKA1* and *CKA2* genes that encode the α and α′ catalytic subunits respectively of CKII in yeast is lethal, demonstrating that the functions of CKII in the cell are essential (402). Further evidence in support of a role for CKII in growth and proliferation is provided by the identity of many of the proteins that can be phosphorylated by CKII. Many of the protein substrates are involved in gene expression and protein synthesis, for example RNA polymerases I and II, DNA topoisomerases, nucleolin, HMG and ribosomal proteins and a number of translation initiation and elongation factors (7, 419). Additionally, CKII phosphorylates many signal transduction proteins and also transcription factors including nuclear oncoproteins such as myc, myb, fos and jun (7, 419). All of these substrates are consistent with a proliferative or growth promoting function for CKII.

Like many proteins with a positive role in cell proliferation and cell growth, CKII has oncogenic properties. The first hint of this came from the discovery that CKII activity is markedly and specifically elevated in the leukaemia-like cattle disease theileriosis, caused by the parasitic protozoan *Theileria parva* (400, 401). Furthermore, there is no evidence that tyrosine kinases or other signaling pathways are deregulated, suggesting that the upregulation of CKII in theileriosis is highly significant to the development of this disease (400, 401). CKII has since been found to be abnormally active in a variety of human cancers, including leukaemias and solid tumours (141, 160, 387). Direct evidence of the oncogenicity of CKII was provided by transgenic mice overexpressing the catalytic α subunit, that develop lymphomas from six months of age with an incidence of 15-20% per year (474). The latency of onset and monoclonality of the lymphomas indicates that other mutations are required for transformation. However, coexpression of a c-myc transgene with a CKIIα transgene resulted in polyclonal neonatal leukaemia (474, 600). Thus, c-myc and CKIIα can cooperate to transform lymphocytes in a two-step pathway. Deregulation of the lymphoid oncogene tal-1 or loss of the tumour suppressor p53, in

combination with CKIIα overexpression is also sufficient to transform lymphocytes (600).

Since tRNA and 5S rRNA are important determinants of the biosynthetic capacity of the cell, the positive role of CKII in pol III transcription in yeast correlates well with its apparent proliferative and growth-promoting roles. Indeed, the stimulation of basal pol III transcription by CKII may be a critical aspect of its growth-promoting function. In support of this, it has been reported that CKII can phosphorylate the pol I transcription factor UBF in mice, resulting in the stimulation of pol I transcription (540). Thus, CKII may coordinately upregulate pol I- and pol III- transcription ensuring the levels of tRNA and ribosomal RNAs are sufficient to meet the growth demands of the cell.

RNA pol III transcription has been found elevated in a number of human tumours and many transformed cells and is activated by several oncoproteins and repressed by two key tumour suppressors. Since CKII is a putative oncogene it was therefore of particular interest to determine whether CKII has a stimulatory role in mammalian pol III transcription. CKII is remarkably well conserved between yeast and mammals. Furthermore, the pol III factor target of CKII in yeast, TFIIIB, is also required for mammalian pol III transcription and two of its subunits, TBP and BRF, are quite well conserved. However, it has previously been reported that CKII can phosphorylate the human La antigen, which inhibits the reported in vitro ability of La to stimulate recycling of the human pol III transcription complex (140). It remains to be determined though whether inhibition of this activity by CKII is ever responsible for limiting the rate of pol III transcription under physiological conditions. A La homologue has also been found in yeast, but is not required for viability (603).

Preliminary experiments carried out to investigate whether glycogen synthase kinase-3β (GSK-3β) can affect pol III transcription are also described in this chapter. GSK-3β is one of two isoenzymes, α and β, of mammalian GSK-3, each of which is encoded by a separate gene (594). These two isoenzymes are highly related and are thought to perform similar functions; however, the majority of studies on mammalian GSK-3 have been with the β isoform or have not discriminated between the two forms.

GSK-3β is a highly conserved Ser/Thr kinase and is thought to have an important role in translation control (423, 561). GSK-3β phosphorylates the

translation initiation factor eIF2B, which inhibits its guanine nucleotide exchange activity, thus preventing the regeneration of active eIF2 that is required for each new round of translation initiation (423, 562). Following mitogenic stimulation, the phosphatidylinositol 3-kinase (PI3K) pathway switches off GSK-3β, thereby allowing activation of translation (561, 562). The inhibition of translation by GSK-3β may be accounted for solely by its effect on eIF2B; however, it is possible that GSK-3β also targets other essential components of the translational apparatus. Thus, it may be that GSK-3β inhibits pol III transcription. Even if the levels of tRNA and 5S rRNA are not rate-limiting under these circumstances, their reduction may provide a kind of fail-safe mechanism, for example if eIF2 were to become independent of eIF2B regulation. Furthermore, the coordinate downregulation of tRNA and 5S rRNA synthesis with the inhibition of translation is better for the economy of the cell, as the production of these transcripts is energetically costly. In support of a role for GSK-3β in regulating pol III transcription, treatment of serum-stimulated fibroblasts with the PI3K inhibitor wortmannin has been found to inhibit pol III transcription (470). However, PI3K has several other downstream effectors in addition to GSK-3β, and any of these may be responsible for the inhibitory effect of wortmannin on pol III transcription. For example, the p70 S6 kinase lies downstream of PI3K (422); it is required for the serum induction of ribosomal protein synthesis and thus is another strong candidate for a role in regulating pol III transcription.

Another substrate of GSK-3β is cyclin D1 (124). GSK-3β phosphorylates cyclin D1, targeting it for proteolytic degradation by the 26S proteasome (124). The primary function of the D-type cyclins, in combination with their catalytic cdk partner, is the phosphorylation and inactivation of the retinoblastoma protein (214). Therefore, GSK-3β, by negatively regulating cyclin D1 stability, may have a physiological role in ensuring that RB is not inappropriately inactivated. Thus, GSK-3β may negatively regulate pol III transcription indirectly via its effect on the activity of RB.

In addition to its inhibitory effects on cyclin D1 levels and eIF2B, GSK-3β, which is remarkably well conserved between distantly related eukaryotic species, is also involved in essential developmental processes including axis formation in Xenopus, cell fate determination in Dictyostelium and segment polarity in

Drosophila (563). The role of GSK-3β in development is mediated through its involvement in Wnt signalling (69, 412). GSK-3β, facilitated by a multiprotein complex that includes the adenomatous polyposis coli (APC) tumour suppressor, phosphorylates β-catenin targeting it for degradation by the proteasome, as is the case for cyclin D1 (69, 412). Wnt signalling inactivates GSK-3β, enabling the accumulation of β-catenin and its translocation to the nucleus where it can interact with members of the LEF/TCF family of HMG-box containing transcription factors and activate transcription of genes containing LEF/TCF binding sites (69, 412).

Although the primary role of Wnt signalling is in development, in adult tissues it also has a role in regulating cell proliferation (412). Inappropriate activation of Wnt signalling is implicated in a variety of different human cancers (382, 412). The APC tumour suppressor is frequently mutated in colon cancer and the GSK-3β phosphorylation sites of β-catenin are also found to be mutated in human tumours, both of these effects resulting in increased levels of β-catenin and increased transcription of LEF/TCF-responsive genes (382). Many of these genes are involved in development and provide no obvious links to tumorigenesis. Recently, however, a few pro-proliferative targets of Wnt signalling have been identified, such as the c-myc and cyclin D1 genes, both of which are transcriptional targets of the β-catenin/TCF transcription complex (204, 382, 518). In addition to positively regulating cyclin D1 gene expression (518), Wnt signalling also reduces cyclin D1 proteolysis, both of which effects correlate with the inhibition of GSK-3β (435).

Thus, GSK-3β is negatively regulated by at least two distinct signalling pathways, namely the PI-3K pathway and Wnt signalling. These two pathways inhibit GSK-3β through different mechanisms and lead to distinct downstream events (126). However, signalling through both pathways affects cyclin D1 proteolysis (124, 435). The negative regulation of GSK-3β by mitogens and many of its functions are consistent with an anti-proliferative, growth restraining role for GSK-3β. Consistent with this, GSK-3β has also been shown to phosphorylate and inhibit c-jun and can phosphorylate other transcription factors involved in cell proliferation, such as myc, myb and CREB (563). Thus, the inhibition of pol III transcription may be one aspect of a general physiological role of GSK-3β in inhibiting growth and proliferation under certain circumstances.

GSK-3β is also implicated in Alzheimer's disease (254). A key event in the pathogenesis of this disease is the hyperphosphorylation of the microtubule-associated protein tau (254). Normal, underphosphorylated tau has essential functions in microtubule nucleation, organisation and stability (254). Hyperphosphorylation of tau impairs these functions and promotes the formation of paired helical filaments, the building blocks of the neurofibrillary lesions of Alzheimer's disease (254). GSK-3β phosphorylates tau; moreover, GSK-3β has been shown to reduce microtubule abundance and disrupt microtubule organisation when overexpressed with tau, suggesting that the phosphorylation of tau by GSK-3β may be physiologically relevant to the tau phenotype in Alzheimer's disease (254, 328, 343, 542).

As its name suggests, GSK-3β also functions in glycogen metabolism. The phosphorylation of glycogen synthase by GSK-3β inactivates it, thus inhibiting glycogen synthesis (502). Recently, a positive role in cell survival has also been reported for GSK-3β (219). Mice deficient in GSK-3β have been generated and found to die in mid-gestation due to excessive hepatocyte apoptosis (219). This phenotype is consistent with hypersensitivity to TNFα toxicity, as displayed by mice that lack NFκB function (219). Indeed, GSK-3β$^{-/-}$ cells displayed significantly reduced NFκB function implying that GSK-3β has a role in NFκB activation (219). Clearly, GSK-3β performs a variety of functions within the cell. Unlike CKII, a role for GSK-3β in pol III transcription has never previously been reported in any eukaryotic species.

## 6.2 Results

### 6.2.1 The CKII inhibitors 2,3-diphosphoglycerate and quercetin potently inhibit mammalian pol III transcription in vitro

In yeast, CKII is required for high levels of basal pol III transcription (163, 164, 216). To investigate whether CKII has a similar role in pol III transcription in mammals, the effect on transcription of the two well-known CKII inhibitors 2,3-diphosphoglycerate (DPG) and quercetin was tested. As Figure 6.1 shows, specific pol III transcription reconstituted in vitro with HeLa PC-B and PC-C fractions and a human $tRNA^{leu}$ gene was severely repressed both by 2,3-diphosphoglycerate and quercetin. The decrease in transcription was dose-dependent, with little or no tRNA synthesis detectable at the higher dose. The low concentrations of DPG and quercetin used in this experiment have previously been shown to potently and specifically inhibit CKII phosphorylation of established natural substrates, including yeast TFIIIB (216, 364, 530). Similar repression of pol III transcription in the presence of DPG or quercetin was observed when transcription was reconstituted with unfractionated HeLa nuclear extract or the $VA_I$ gene as template (data not shown). Another commonly used CKII inhibitor, the ATP analogue 5,6-dichloro-1-β -D-ribofuranosylbenzimidazole (DRB), was also found to reduce the levels of pol III transcription in a HeLa nuclear extract (257). The fact that three unrelated compounds that function as specific inhibitors of CKII kinase activity have the same effect on pol III transcription suggests that the repression observed is a specific response to a decrease in CKII activity. The effect of quercetin and DRB on pol III transcription in vivo has also been tested. Both CKII inhibitors caused a reduction in the levels of pol III transcripts (470), suggesting that the reduction in mammalian pol III transcription observed in vitro with these CKII inhibitors is of physiological significance.

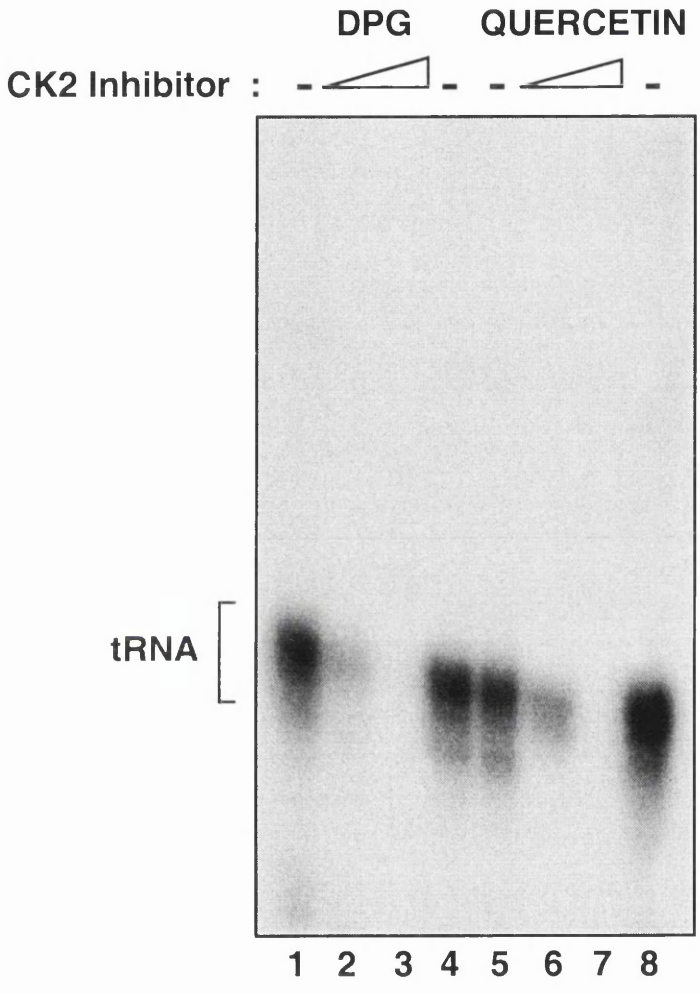
### 6.2.2 CKII activates mammalian pol III transcription in vitro

DPG, DRB and quercetin are all potent inhibitors of CKII, and at the concentrations that were used in these experiments have been shown to have no

**Figure 6.1**

**Pol III transcription decreases in the presence of the CKII inhibitors DPG and quercetin**

Each transcription reaction contained 2µl of HeLa PC-B, 2µl of HeLa PC-C and 250ng of pLeu encoding a $tRNA^{leu}$ gene (all lanes). In addition, lanes 2 and 3 contained 2,3-diphosphoglycerate, 6mM and 12mM respectively. Lane 6 contained 50µM of quercetin and lane 7 contained 100µM of quercetin. Lanes 1, 4, 5 and 8 contained appropriate amounts of control buffer for the two inhibitors. Following a 15 min preincubation at $30^0$C, nucleotides were added to initiate transcription, which was allowed to proceed for 1 h.


DPG
QUERCETIN
CK2 Inhibitor :
tRNA
1 2 3 4 5 6 7 8

effect on a range of other kinases. However, although these chemical CKII inhibitors are highly specific, none of them can be regarded as totally specific for CKII, especially at higher concentrations. Therefore, it was necessary to verify that CKII does indeed have a positive influence on mammalian pol III transcription in vitro, as suggested by the inhibitory effect of the CKII inhibitors. To test this, in vitro transcription reactions were carried out in the presence of increasing amounts of a peptide substrate containing a consensus CKII phosphorylation site. This CKII peptide was specifically designed based on the sequence preferences of CKII (295, 296). The minimal sequence requirements for a CKII phosphorylation site is Ser/Thr-x-x-Asp/Glu (419). CKII is unusual in that it has a strong preference for acidic residues whereas the specificity determinants of most Ser/Thr kinases are basic (419). A multitude of different peptides containing a CKII phosphoacceptor site have been analysed for their kinetic properties and their specificity as a CKII substrate (419). The CKII peptide substrate used in these experiments is highly specific for CKII and has favourable kinetic properties (295, 296). The presence of CKII peptide in a cell extract provides an alternative substrate for phosphorylation by CKII from the endogenous CKII substrates. Thus, the CKII peptide should compete with endogenous substrates for phosphorylation by CKII. Therefore, the peptide is effectively a competitive inhibitor of the kinase. By being a better substrate or present in much larger amounts, such peptides can divert the kinase for which they contain a phosphoacceptor site from the phosphorylation of its endogenous substrates (540).

Titrating in increasing amounts of CKII peptide into a crude HeLa nuclear extract caused a dose-dependent decrease in pol III transcription of the $VA_I$ gene (Figure 6.2). Transcription was inhibited approximately 90-fold in the presence of 40μg of the CKII peptide (Fig. 6.2, compare lanes 1 and 4). In contrast, the addition of identical amounts of a peptide containing a consensus phosphoacceptor site for protein kinase A (PKA) had no effect on the level of transcription (compare lanes 5, 6 and 7 with lanes 1 and 8). The highly specific inhibitory effect of the CKII peptide is probably caused by the ability of this peptide to reduce the phosphorylation of endogenous substrates by CKII, whereas the PKA peptide is unable to do this. To support this interpretation, the ability of recombinant CKII to phosphorylate the PKA and CKII peptides was tested in an in vitro kinase assay. CKII phosphorylated the CKII peptide approximately 40-fold more efficiently than the PKA peptide or

**Figure 6.2**

**Competitive inhibition of CKII with a peptide containing a consensus CKII phosphoacceptor site specifically represses pol III transcription**

All reactions contained 2μl of HeLa nuclear extract and 250ng of $pVA_I$ (all lanes). In addition, lanes 2-4 contained increasing amounts of a peptide containing a CKII phosphoacceptor site (20μg, 30μg and 40μg, respectively). Lanes 5-7 contained increasing amounts of a peptide containing a PKA phosphoacceptor site (20μg, 30μg and 40μg, respectively). No peptide was added to lanes 1 and 8. Reaction mixtures were preincubated with added peptide for 15 min at $30^0$C prior to the addition of nucleotides to initiate transcription. Transcription was allowed to proceed for 1 h at $30^0$C.

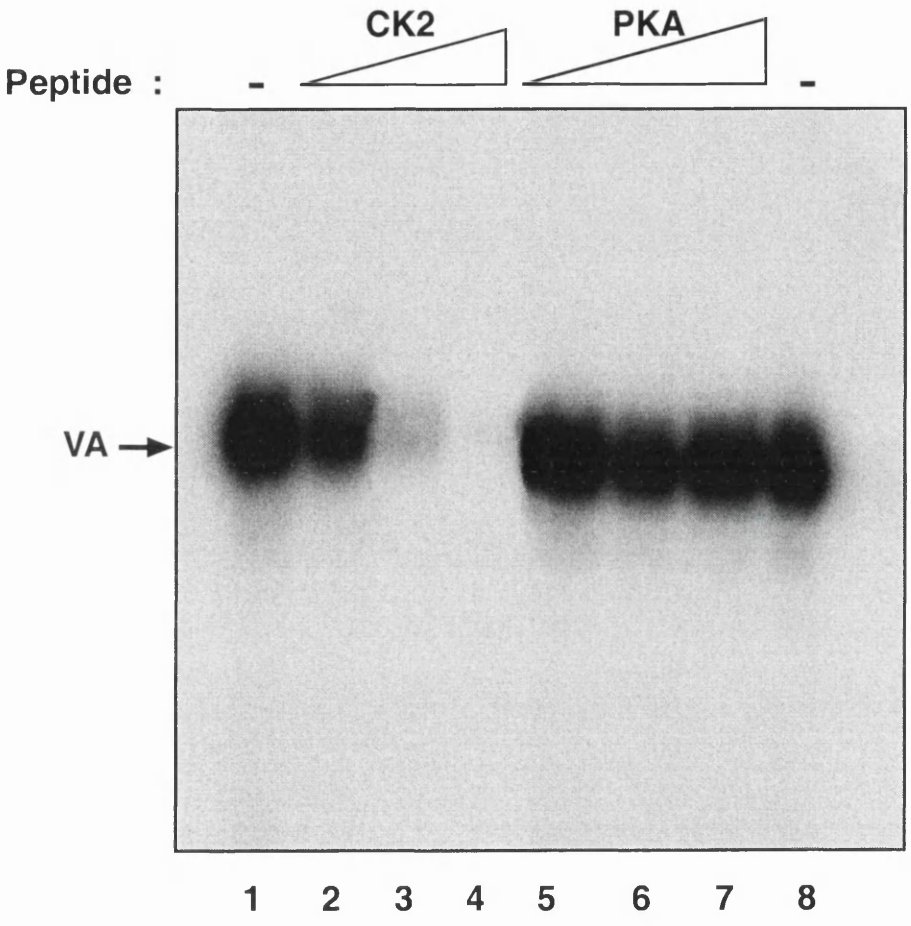
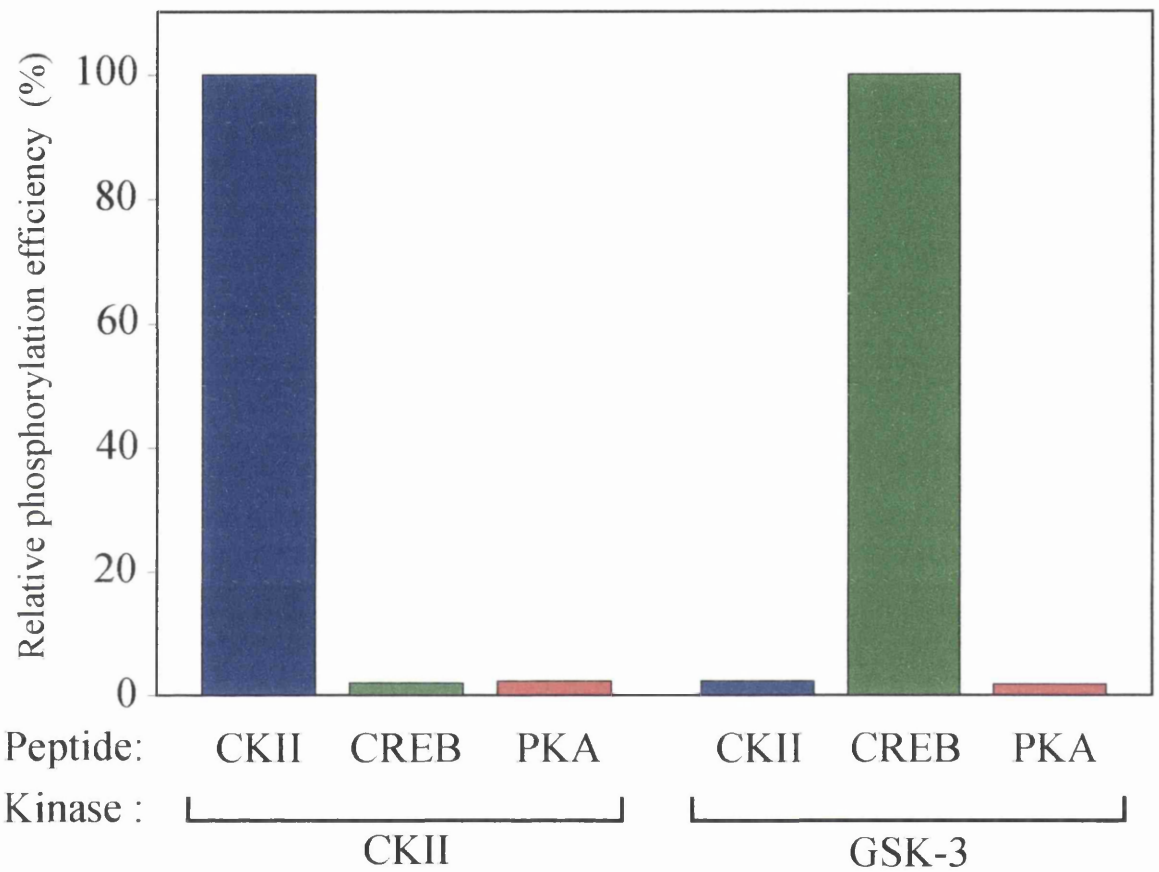

CK2
PKA
Peptide :
-
-
VA
1
2
3
4
5
6
7
8

**Figure 6.3**

**The competitor peptide containing a CKII phosphoacceptor site is specifically and potently phosphorylated by CKII**

In vitro kinase assays were performed using three different peptide substrates, peptide containing a CKII phosphoacceptor site, peptide containing a GSK-3 phosphoacceptor site (CREB) and peptide containing a PKA phosphoacceptor site. Each of these peptide substrates was individually incubated with 0.5 µl of recombinant CKII or GSK-3. 10µCi [$\gamma$-$^{32}$P] ATP was included in each reaction mixture as a source of radiolabelled phosphate donor. In vitro phosphorylation reactions were allowed to proceed for 20 min at $30^0$C. Reactions were then stopped by pipetting samples onto $2cm^2$ phosphocellulose discs (Whatman P81) that the peptides are designed to bind and unincorporated label was removed by extensive washing with 75mM phosphoric acid. The level of phosphorylation of the peptide substrates was then estimated by liquid scintillation counting of the individual discs.


Relative phosphorylation efficiency (%)
100
80
60
40
20
0
Peptide:
CKII
CREB
PKA
CKII
CREB
PKA
Kinase :
CKII
GSK-3

another peptide containing a phosphoacceptor site for GSK-3 (Figure 6.3), confirming that the CKII peptide is likely to be able to strongly compete with endogenous substrates for phosphorylation by CKII, whereas the PKA peptide cannot. Recombinant GSK-3 and p42 MAPK were also tested for their ability to phosphorylate the CKII peptide. Consistent with previous data demonstrating the specificity of the CKII peptide for CKII, the level of phosphorylation of the CKII peptide by these two kinases was negligible (Figure 6.3 and data not shown).

Production of detectable amounts of the appropriately sized transcript in an in vitro transcription assay requires correct initiation, elongation, termination and re-initiation of transcription. The inhibitory effect of the CKII peptide could be caused by a defect in any of these steps. To try and determine at which of these steps the CKII peptide has its inhibitory effect, primer extension analysis of the transcripts produced in the presence or absence of CKII peptide in an in vitro pol III transcription assay, was performed. Whereas all the transcripts produced in a standard pol III run-off assay are detected and can be distinguished only on the basis of size, primer extension analysis will only detect a subset of the transcripts; cDNAs will only be produced from RNA species that contain a specific sequence that can hybridize to the radiolabelled primer added. Therefore, most of the non-specific transcripts produced will not be detected. In addition to primer extension analysis reducing background noise, the size of the cDNAs can be used to map the 5′ terminus of the RNAs and thus determine if transcription initiated at the correct site.

Figure 6.4 shows the results of primer extension analysis of RNA from in vitro pol III transcription of the $VA_I$ gene in HeLa nuclear extract in the presence or absence of CKII peptide. No cDNA of any size could be detected following primer extension of RNA from a transcription reaction that contained CKII peptide (Fig. 6.4, compare lane 3 with lanes 1, 2, 4 and 5), suggesting that the inhibitory effect of the CKII peptide may occur at the level of transcription initiation. The presence of PKA peptide resulted in a cDNA of the same size as in the absence of any added peptide, demonstrating the specificity of the effect of the CKII peptide.

The effect of the CKII peptide was also tested in a more purified transcription system using fractionated factors rather than a crude extract and a tRNA gene as the pol III template, rather than $VA_I$ (Figure 6.5). As for $VA_I$ transcription, tRNA synthesis was inhibited in a dose-dependent manner and was almost completely

abolished by the higher doses of CKII peptide. In contrast, neither the PKA peptide nor a third peptide containing a consensus phosphoacceptor site for p34cdc2 had any effect on the level of tRNA synthesis demonstrating the sequence-specificity of the effect of the CKII peptide on transcription.

The consistent reduction in pol III transcription when CKII activity is specifically inhibited, whether by a chemical CKII inhibitor or by peptide containing a CKII phosphorylation site, suggests that CKII has a positive influence on pol III transcription. Indeed, transcription is almost completely abolished by doses of inhibitor commonly used to specifically inhibit CKII kinase activity, suggesting that CKII may be essential for high levels of mammalian pol III transcription, at least in vitro.

The influence of CKII on mammalian pol III transcription in vivo has also been tested further. An antisense approach was adopted, as has previously successfully been used to deplete cells of endogenous CKII activity (414, 474). Primary human fibroblasts (IMR-90) were exposed to oligodeoxynucleotides complementary to the start region of mRNAs coding for the positively-acting regulatory β subunit of CKII. This provoked a significant reduction in the abundance of endogenous CKIIβ protein, severely impairing the CKII activity of the cells (257). RNA was harvested from these cells and analysed by RT-PCR using intron-specific primers against two different tRNA species, thus providing an indication of the rate of ongoing pol III transcription (104, 105). Exposure of cells to antisense CKIIβ oligonucleotides substantially reduced the levels of tRNA synthesis in vivo relative to that of untreated cells or cells treated with sense CKIIβ oligonucleotides (257). In contrast, there was no change in the abundance of mRNA encoding glyceraldehyde phosphate dehydrogenase (257), demonstrating the specificity of the effect of the antisense CKIIβ oligonucleotides on tRNA synthesis.

The inhibitory effect of depleting cells of CKIIβ protein on mammalian pol III transcription in vivo could be very indirect. Although the exact functions of CKII in cell growth and proliferation have yet to be elucidated, there is considerable evidence that it has essential roles in these two processes. Indeed, decreasing endogenous CKII activity using antibodies against CKIIβ can inhibit progression through particular stages of the cell cycle (413). Depletion of CKII activity with antisense CKIIβ oligonucleotides from IMR-90 fibroblasts has previously been

**Figure 6.4**

**Primer extension analysis of transcripts produced by in vitro transcription of the $VA_I$ gene in the presence of CKII peptide or PKA peptide**

In vitro transcription reactions were reconstituted with 2μl of HeLa nuclear extract (all lanes), 250ng of $pVA_I$ (all lanes) and 40μg of peptide containing a consensus phosphoacceptor site for CKII (lane 3) or PKA (lane 5). After a 15 min preincubation at $30^0$C, transcription was initiated by the addition of equimolar concentrations of rATP, rCTP, rGTP and rUTP, none of which were radiolabelled. DNA was removed from transcription reaction products by treating samples with RNase-free DNase. 2.5ng of [$\gamma$-$^{32}$P]-labelled oligonucleotide primer specific for a coding region of the $VA_I$ gene was added to each RNA sample for primer extension analysis. After heating the samples at $80^0$C for 10 min to denature the RNA, the samples were incubated for ~2h at $50^0$C to allow the slow annealing of the primer to the complementary sequence present in RNA species. Reverse transcription was then allowed to proceed for 1h at $42^0$C. cDNA products were analysed on a 7M urea 7% polyacrylamide sequencing gel.

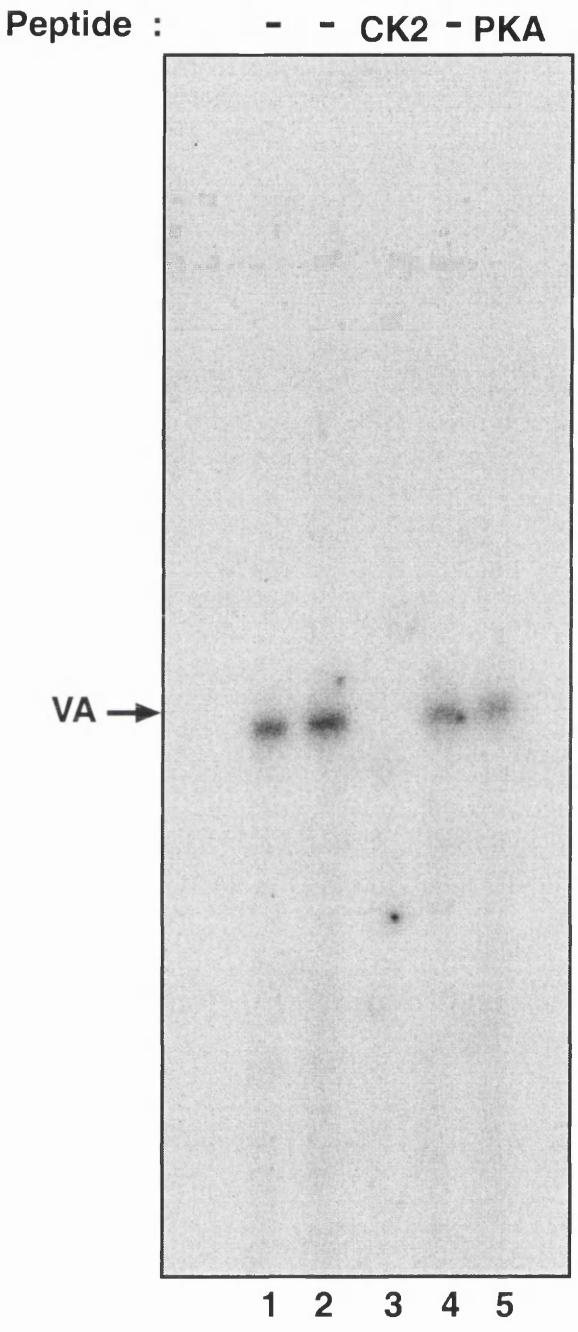
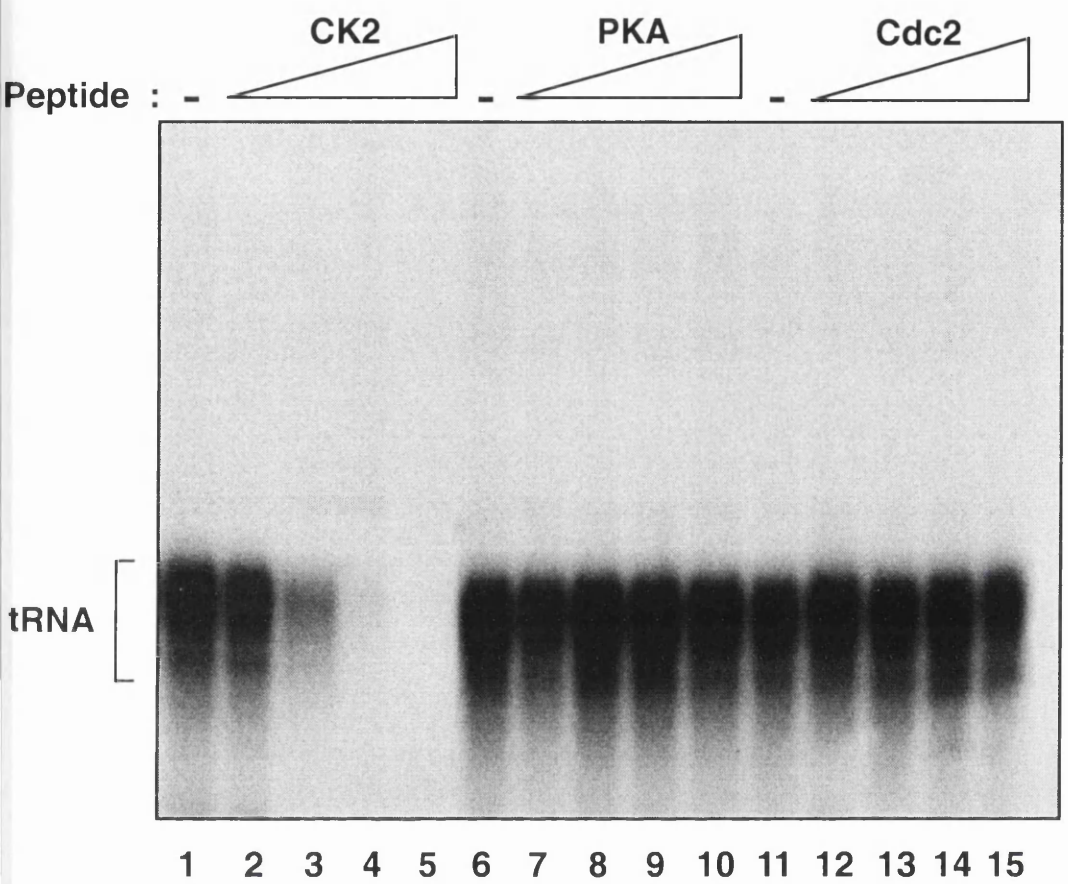

Peptide :
-
-
CK2
-
PKA
VA
1
2
3
4
5

**Figure 6.5**

**tRNA synthesis is inhibited specifically by a competitor peptide with a consensus CKII phosphoacceptor site**

Pol III transcription was reconstituted in vitro with HeLa PC-B (2μl) and PC-C (2μl) and a $tRNA^{leu}$ gene as the specific pol III template (250ng of pLeu) (all lanes). Additionally, lanes 2-5 contained increasing amounts of a peptide containing a CKII phosphoacceptor site (10μg, 20μg, 30μg and 40μg, respectively). Lanes 7-10 contained increasing amounts of a peptide containing a PKA phosphoacceptor site (10μg, 20μg, 30μg and 40μg, respectively). A peptide containing a $p34^{cdc2}$ phosphoacceptor site was added to lanes 12-15 (10μg, 20μg, 30μg and 40μg, respectively). No peptide was added to reactions 1, 6 and 11. Reaction mixtures were preincubated with added peptide for 15 min at $30^0$C prior to the addition of nucleotides to initiate transcription. Transcription was allowed to proceed for 1 h at $30^0$C.


CK2
PKA
Cdc2
Peptide : - - -
tRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

shown to transiently inhibit cell growth stimulation by EGF (414). Since pol III transcription is also subject to cell cycle and growth control (96, 255, 363, 525, 569, 571), it may be that the decrease in tRNA synthesis is an indirect response to a change in the growth or proliferative rate of the cell caused by the reduction in the abundance of CKIIβ protein. Alternatively, perhaps CKII regulates the activity of a transcription factor that can modulate the transcription of genes encoding components of the pol III transcription apparatus. Such indirect mechanisms might be responsible for the pol III transcriptional response to CKII in vivo. However, the potent inhibitory effect of reducing endogenous CKII activity on pol III transcription in vitro demonstrates that CKII can directly influence pol III transcription independently of changes in gene expression of pol III components or the proliferation status of the cell.

The sensitivity of mammalian pol III transcription in vivo to a decrease in CKII activity is very important as it demonstrates that CKII can influence mammalian pol III transcription in a physiological context as well as in vitro. The in vivo pol III response mimics that in vitro, suggesting an activating function for CKII in mammalian pol III transcription. The evidence for this is derived from the effect on transcription of reducing the endogenous CKII activity. The converse experiments were also performed in which the abundance of CKII is artificially raised above endogenous levels. The overexpression of CKII in human osteosarcoma cells had no effect on pol III transcription (257). Similarly, titrating in increasing amounts of recombinant CKII into in vitro transcription reactions reconstituted with HeLa nuclear extract or HeLa PC-B and PC-C fractions failed to stimulate transcription (data not shown). The lack of a stimulatory effect of exogenous CKII can be reconciled with the decrease in pol III transcription when endogenous CKII levels are reduced if endogenous CKII is normally in relative excess. In support of this possibility, endogenous CKII is saturating for pol III transcription in wild type yeast cell extracts (216).

In HeLa extracts containing the highly specific CKII peptide, endogenous CKII is competitively inhibited from phosphorylating its natural substrates and is thought to become limiting for its stimulatory effect on mammalian pol III transcription, hence the decrease in pol III transcription observed. If the inhibitory effect on pol III transcription of the CKII peptide truly is caused by a reduction in the CKII phosphorylation of endogenous substrates, then the impaired pol III

transcription of extracts or fractionated factors containing CKII peptide should be rescued by the addition of recombinant CKII. As Figure 6.6 shows, the addition of recombinant CKII to a HeLa extract containing CKII peptide strongly stimulated transcription. This provides direct evidence that CKII has an activating function in mammalian pol III transcription in vitro. The recombinant CKII substantially rescued transcription that was almost completely abolished by the presence of CKII peptide in these extracts (Figure 6.6, compare lanes 4 and 5). In contrast, addition of the same amount of recombinant CKII had no stimulatory effect in HeLa extracts containing PKA peptide rather than CKII peptide (Fig. 6.6, compare lanes 7 and 8). Addition of CKII to extracts lacking peptide had little stimulatory effect on transcription, as previously observed (Fig 6.6, compare lane 2 with lanes 1 & 3). Similarly, transcription of a $tRNA^{leu}$ gene using HeLa PC-B and PC-C, repressed by the addition of CKII peptide, could also be rescued by recombinant CKII (data not shown). These results demonstrate that CKII contributes significantly to the level of mammalian pol III transcription in vitro but also that it is in excess for pol III transcription in unfractionated HeLa extracts and HeLa PC-B or PC-C fractions.

### 6.2.3 CKII and TFIIIB interact stably

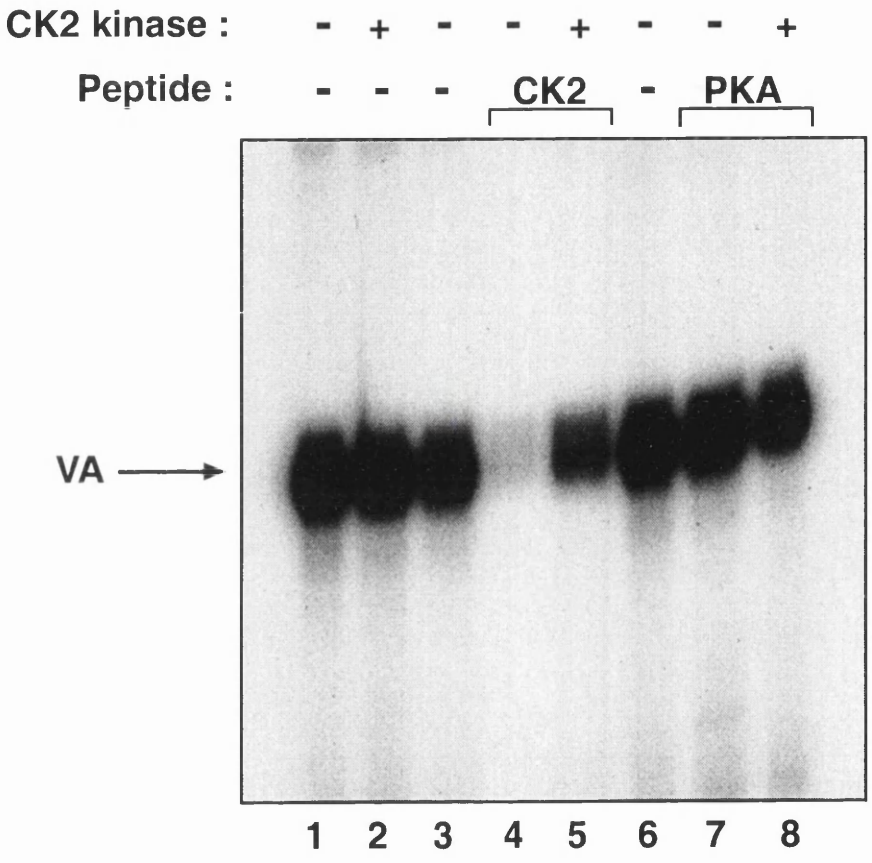
The responsiveness of pol III transcription reconstituted with PC-B and PC-C to CKII peptide or chemical CKII inhibitors and the inability of CKII to stimulate such transcription except in the presence of peptide or inhibitor, suggests that some endogenous CKII has copurified with the pol III transcription components in PC-B or PC-C. This copurification on phosphocellulose might be because CKII physically interacts with a component of the pol III transcription apparatus or it may be entirely fortuitous. These phosphocellulose fractions are relatively crude and contain many proteins. The possibility that CKII may stably associate with a component of the pol III transcription apparatus was further investigated by seeing if CKII is present in highly purified fractions of the different pol III transcription components.

As a sensitive means of assaying for the amount of CKII activity in these fractions, in vitro kinase assays were performed using peptide containing a CKII phosphoacceptor site as substrate. Immunoaffinity-purified TFIIIB fractions were found to contain between 2- and 4- fold as much CKII activity as mock-

**Figure 6.6**

**Recombinant CKII rescues pol III transcription reconstituted in the presence of peptide containing a consensus phosphoacceptor site for CKII**

All reactions contained 2μl of HeLa nuclear extract and 250ng of $pVA_I$ (all lanes). In addition, reactions 4 and 5 contained 30μg of peptide containing a phosphoacceptor site for CKII. Reactions 7 and 8 contained 30μg of PKA peptide. 1μl of recombinant CKII was also added to reactions 2, 5 and 8. Following a 15 min preincubation at $30^0$C, nucleotides were added to initiate transcription, which was allowed to proceed for 1 h at $30^0$C.


CK2 kinase : - + - - + - - +
Peptide : - - - CK2 - PKA
VA
1 2 3 4 5 6 7 8

immunopurified TFIIIB fractions generated using columns carrying preimmune serum (data not shown). In contrast, immunoaffinity-purified TFIIIC fractions contained only low levels of CKII activity similar to the levels found in mock-immunopurified TFIIIC fractions (data not shown). An immunoaffinity-purified TFIIIB fraction and a mock-immunopurified TFIIIB fraction were also assayed for PKA activity. This was found to be similar in the two fractions, demonstrating the specificity of the higher levels of CKII activity found in the immunoaffinity-purified TFIIIB fraction (data not shown).

These results suggest that CKII and TFIIIB may stably interact. It was therefore investigated whether they cofractionate during gradient chromatography. As Figure 6.7 shows, the TFIIIB activity and CKII activity of eluted fractions were found to closely coincide following gradient chromatography of a PC-B fraction on hydroxyapatite. PKA activity was also assayed and found to fractionate differently (data not shown). Similar cofractionation of TFIIIB and CKII was observed following gradient chromatography of a PC-B fraction on heparin-Sepharose (data not shown). Eluted fractions from Mono Q gradient chromatography of a PC-B fraction were also analysed. A Mono Q gradient resolves TFIIIB into two essential components, B′ and B″, that elute at 0.38M KCl and 0.48M KCl, respectively (374). B′ consists minimally of TBP and BRF, whereas the composition of B″ is uncertain but is thought to contain a human homologue of yeast B″ (374). Two significant peaks of CKII activity were found to elute from Mono Q. Comparison with the elution profile of TFIIIB revealed that the CKII peak that eluted slightly later in the gradient coincides with the peak of B′ activity (Figure 6.8). In contrast, peak B″ fractions contained hardly any CKII activity. The specificity of this cofractionation of a sizeable proportion of the CKII in a PC-B fraction with B′ was further demonstrated by the fractionation of DNA-dependent protein kinase (DNA-PK) and GSK-3. DNA-PK eluted early in the gradient, well before B′ or B″, whereas the bulk of GSK-3 activity eluted in the flowthrough (data not shown).

The criteria by which heparin, hydroxyapatite and Mono Q separate proteins are distinct. The cofractionation of TFIIIB and CKII during gradient chromatography on each of these three distinct adsorbent types suggests that TFIIIB and CKII stably interact. The specific cofractionation of CKII with B′ and not B″ on

**Figure 6.7**

**TFIIIB and CKII cofractionate during gradient chromatography of PC-B on hydroxyapatite**

Gradient-eluted fractions were assayed for TFIIIB by in vitro transcription reconstituted with 2μl of PC-C, 250 ng of $pVA_I$ and 4μl of eluted fraction. CKII activity of individual fractions was assayed by in vitro phosphorylation reaction using peptide containing a consensus CKII phosphoacceptor site as the substrate. Fraction numbers are indicated. FT, flowthrough.

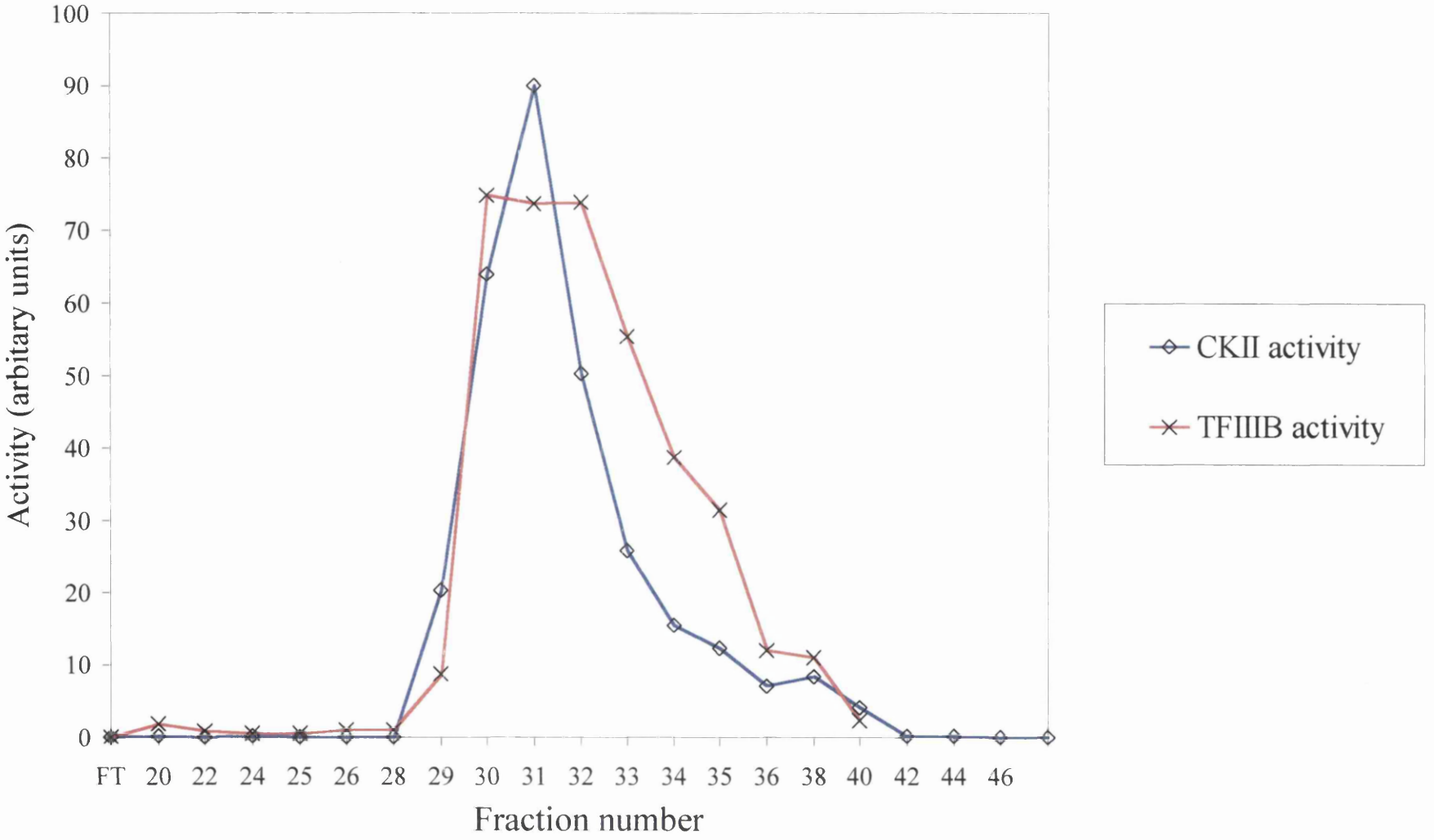

100
90
80
70
60
50
40
30
20
10
0
Activity (arbitary units)
FT 20 22 24 25 26 28 29 30 31 32 33 34 35 36 38 40 42 44 46
Fraction number
CKII activity
TFIIIB activity

**Figure 6.8**

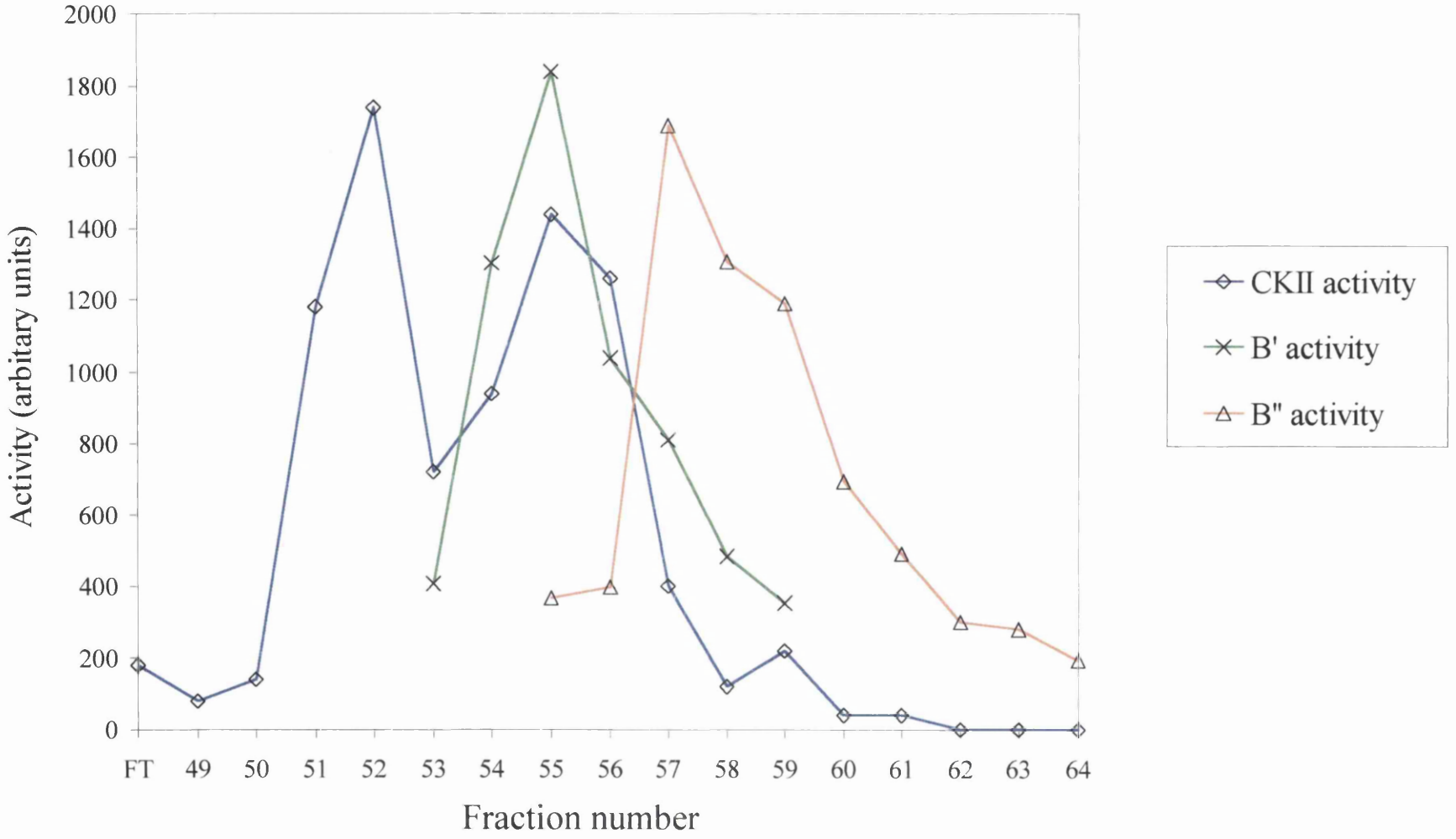
**A subpopulation of CKII molecules closely cofractionates with TFIIIB B′ activity during gradient chromatography of PC-B on Mono Q**

The TFIIIB B′ activity of individual fractions was assayed by in vitro transcription reaction reconstituted with the $VA_I$ gene as the specific pol III template (250ng), TBP-immunodepleted nuclear extract (4μl) and the individual Mono Q fraction (2μl). The levels of $VA_I$ transcript produced were quantitated by phosphoimager analysis*.

To assay for B″ activity, transcription reactions were reconstituted with 250ng of $pVA_I$, 2μl of PC-C, 2μl of the Mono Q fraction of peak B′ activity (fraction number 55) and 2μl of the individual Mono Q fraction. As for B′ activity, the levels of $VA_I$ transcript produced were quantitated by phosphoimager analysis*.

CKII activity of individual fractions was assayed by in vitro phosphorylation reaction using peptide containing a consensus CKII phosphoacceptor site as the substrate. Fraction numbers are indicated. FT, flowthrough.

*Determination of B′ and B″ activity of Mono Q fractions by in vitro transcription assay and phosphoimager quantitation of transcript levels were performed by Robert J. White.


2000
1800
1600
1400
1200
1000
800
600
400
200
0
Activity (arbitary units)
FT
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
Fraction number
CKII activity
B' activity
B" activity

Mono Q suggests that CKII may directly interact with either the TBP or BRF component of TFIIIB.

As an independent test of a physical association of endogenous TFIIIB and CKII, immunoprecipitation experiments were performed. Antiserum against the BRF subunit of TFIIIB was consistently found to coimmunoprecipitate CKII activity from HeLa nuclear extract or HeLa PC-B (Figure 6.9 and data not shown). A low level of CKII activity was also detected in immunoprecipitates of the pre-immune serum, presumably due to non-specific interactions; however, this was always significantly less than the amount of CKII activity immunoprecipitated with the BRF antiserum. On average, approximately three times as much CKII activity is detected in immunoprecipitates obtained using the BRF antiserum as in those obtained with the appropriate pre-immune serum (Fig. 6.9 and data not shown). The converse experiment was also performed, in which CKII was specifically immunoprecipitated from HeLa nuclear extract using an antiserum raised against the catalytic α subunit of CKII and the presence of TFIIIB in the precipitated material was assayed for by Western blotting using an antiserum raised against BRF. The typical results of such an immunoprecipitation are shown in Figure 6.10. As positive controls, antisera against BRF itself and RB, which has previously been shown to bind to TFIIIB (93, 314, 315, 503, 504), were used and as expected precipitated significant amounts of BRF (Fig. 6.10, lanes 1 and 2). The CKIIα antiserum was also found to immunoprecipitate BRF; indeed, comparable levels of BRF coprecipitated with CKII as found in immunoprecipitates obtained using the RB antiserum (Fig. 6.10, compare lanes 2 and 3). In contrast, an antiserum against the $TAF_I48$ subunit of the pol I-specific transcription factor SL1 was unable to immunoprecipitate BRF (lane 4), demonstrating the specificity of the presence of BRF in immunoprecipitates obtained with the CKIIα antiserum. This specific coimmunoprecipitation of BRF with an antiserum raised against the catalytic α subunit of CKII was confirmed using a second antiserum raised against a different region of BRF (data not shown). This coimmunoprecipitation data, along with the consistent cofractionation of CKII and TFIIIB through a variety of different column types, suggests that a subpopulation of endogenous CKII molecules stably and specifically associate with endogenous TFIIIB.

**Figure 6.9**

**Coimmunoprecipitation of CKII activity with endogenous TFIIIB**

Immunoprecipitation reactions were carried out using equivalent amounts of 330 antiserum raised against BRF or the corresponding pre-immune serum, prebound to protein A-Sepharose, and 20μl of PC-B. Following 3h incubation at $4^0$C on an orbital shaker, samples were pelleted, supernatants were removed and the immunoprecipitated material was washed five times with 250μl of LDB. Immunoprecipitates were then assayed for CKII activity by in vitro phosphorylation using peptide containing a consensus phosphoacceptor site for CKII as substrate. [γ-$^{32}$P] -ATP (10μCi) was included in each reaction mixture as a source of radiolabelled phosphate donor, enabling the levels of phosphorylation of CKII peptide substrate to be estimated by liquid scintillation counting. Results shown are the averages and standard errors from four independent immunoprecipitations for both of the two sera.

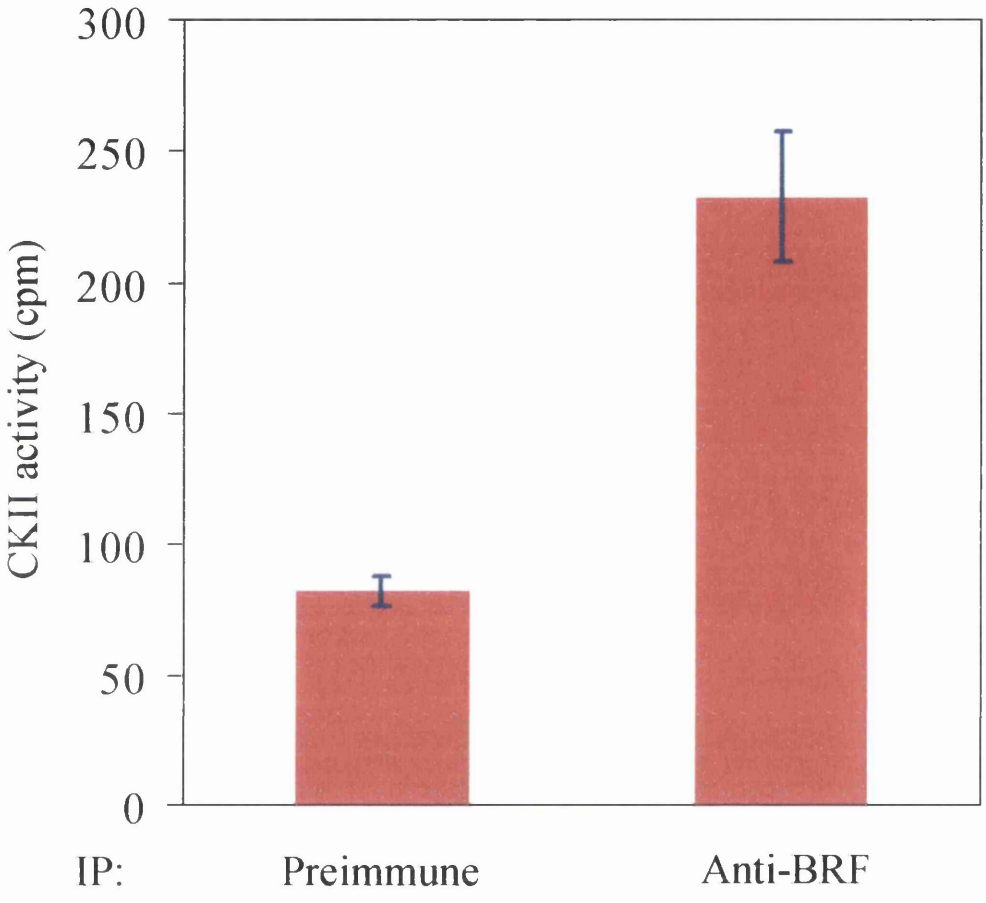
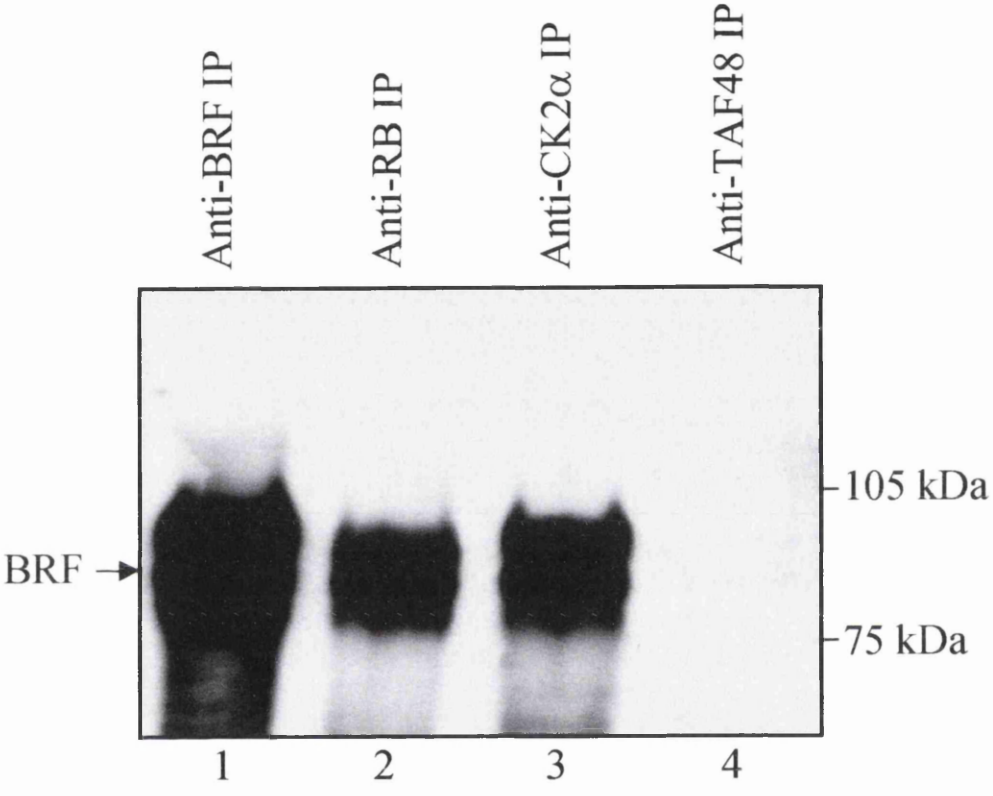

300
250
200
150
100
50
0
CKII activity (cpm)
IP:
Preimmune
Anti-BRF

**Figure 6.10**

**Endogenous CKII interacts with TFIIIB**

HeLa nuclear extract (150μg) was immunoprecipitated using anti-BRF antiserum 128 (lane 1), anti-RB antibody C-15 (lane 2), anti-CKIIα antibody H-286 (lane 3) or anti-$TAF_I48$ antibody M-19 (lane 4). Precipitated material was resolved on a SDS-7.8% polyacrylamide gel and then analysed by western blotting with anti-BRF antiserum 330.


Anti-BRF IP
Anti-RB IP
Anti-CK2α IP
Anti-TAF48 IP
BRF
105 kDa
75 kDa
1
2
3
4

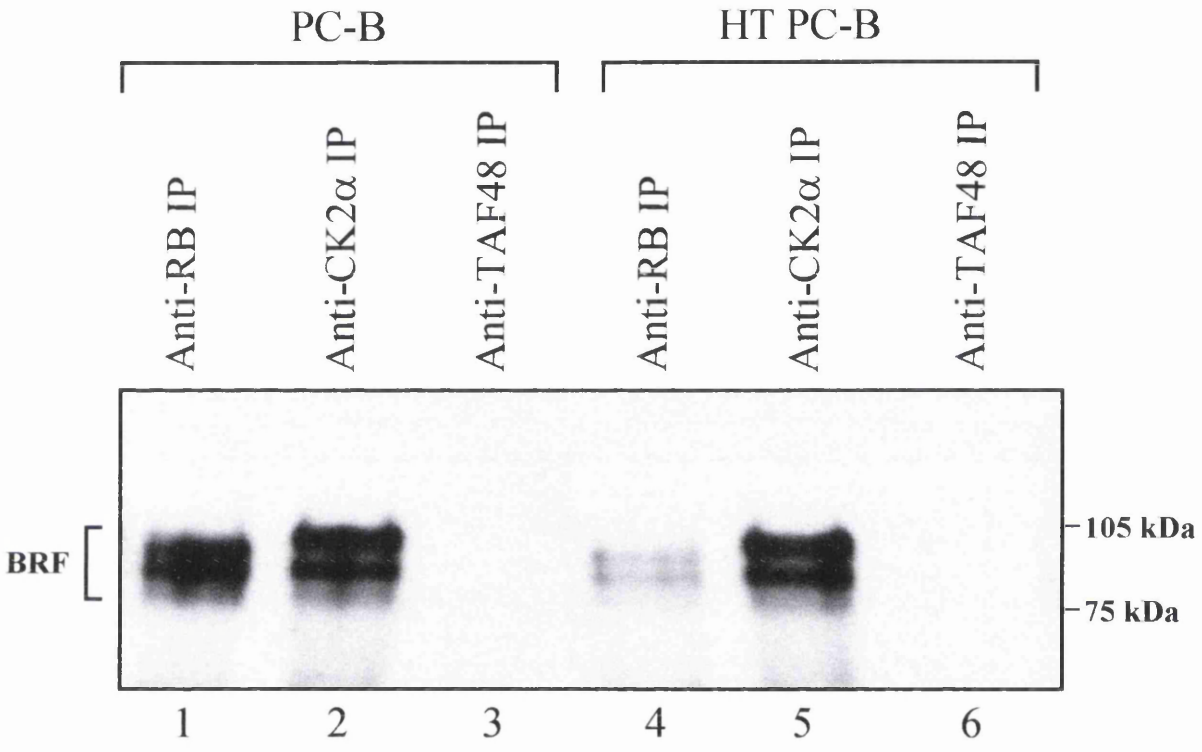
TFIIIB is sufficient to restore pol III transcription to wild-type levels in cell extracts of the yeast *cka2*$^{ts}$ strain (163, 164). Transcriptional rescue by TFIIIB is abolished if TFIIIB is pretreated with phosphatase (164). These observations suggest that TFIIIB is the CKII-responsive component of the pol III transcription apparatus in yeast. TBP, an essential component of TFIIIB both in yeast and mammals, is the preferred substrate of yeast TFIIIB for phosphorylation by CKII (163, 164). Phosphorylation by CKII enhances the ability of recombinant TBP to stimulate pol III transcription in *cka2*$^{ts}$ extracts (163, 164). These data suggest that phosphorylation of TBP by CKII may mediate the stimulation of pol III transcription in yeast by CKII. Since TBP is likely to be the physiological target of CKII in yeast it was therefore of considerable interest as to whether an interaction between human TFIIIB and CKII is dependent on TBP. This was investigated by heat inactivating TBP in a PC-B fraction and assaying whether TFIIIB and CKII still coimmunoprecipitate. As Figure 6.11 shows, the heat inactivation of TBP had little or no detectable effect on the ability of an antiserum raised against the catalytic α subunit of CKII to specifically immunoprecipitate BRF (compare lanes 2 and 5). An in vitro transcription assay confirmed that the TBP in the heat treated PC-B used in this immunoprecipitation experiment had been inactivated, at least in its ability to support transcription (data not shown). As a positive control an antiserum against RB was used. Surprisingly, the coimmunoprecipitation of BRF with RB was diminished when heat treated PC-B was used, suggesting that TBP might facilitate the interaction between RB and BRF. This possibility warrants further investigation, although the effect of heat inactivating TBP on the levels of BRF in RB immunoprecipitates was much less in other immunoprecipitation experiments conducted (data not shown). These experiments also suggested that heat inactivated TBP can still interact with BRF (data not shown), causing questions to be raised as to the exact nature by which TBP is heat inactivated and the molecular basis for the loss of its ability to support pol III transcription. Therefore, although the heat inactivation of TBP was consistently observed to have no effect on the level of BRF that coimmunoprecipitated with CKII, it can not be concluded from this that an interaction between CKII and TFIIIB is independent of TBP.

In Figure 6.11, several bands of slightly different mobility about the size of BRF are detected with the BRF antiserum used. Confirmation that these bands correspond to BRF and are not non-specific proteins recognised by the antiserum has

**Figure 6.11**

**Heat inactivation of TBP has no effect on the endogenous association of BRF and CKII**

20μl of untreated PC-B (lanes 1-3) or HT PC-B (lanes 4-6) was immunoprecipitated using anti-Rb antibody C-15 (lanes 1 and 4), anti-CKIIα antibody H-286 (lanes 2 and 5) or anti-$TAF_I48$ antibody M-19 (lanes 3 and 6). After extensive washing with LDB buffer, precipitated material was resolved on a SDS-7.8% polyacrylamide gel and analysed by western blotting with anti-BRF antiserum 330.


PC-B
HT PC-B
Anti-RB IP
Anti-CK2α IP
Anti-TAF48 IP
Anti-RB IP
Anti-CK2α IP
Anti-TAF48 IP
BRF
105 kDa
75 kDa
1
2
3
4
5
6

been obtained using a second antiserum that was raised against a different region of BRF (data not shown). This phenomenon of multiple BRF bands has previously been observed using these BRF antisera (315, 504). These different forms of BRF may represent splice variants of BRF, of which several have recently been identified and cloned (365). Alternatively, the different bands may correspond to differentially phosphorylated forms of a single BRF species.

Many protein kinases can stably interact with their substrates. Therefore, the identification of proteins that associate with a particular kinase can sometimes help identify putative substrates. The consistent cofractionation and coimmunoprecipitation of endogenous human TFIIIB and CKII, suggestive of their physical association, raises the possibility that TFIIIB may be a physiological substrate for phosphorylation by CKII. The amino acid sequence of human TBP, hBRF and hB″ were searched for potential CKII phosphoacceptor sites based on the minimal sequence requirements for this kinase. The sequence of hTBP has two potential CKII phosphoacceptor sites, the sequence of hBRF has a total of twelve potential CKII phosphoacceptor sites and the recently cloned hB″ contains a remarkable 46 potential CKII phosphorylation sites (data not shown). Thus, hTBP, hBRF and hB″ could all be phosphorylated by CKII, depending on the surface accessibility of these potential CKII phosphoacceptor sites. Preliminary data suggest that human BRF and hB″ are phosphorylated by CKII in vitro (257).

### 6.2.4 CKII kinase activity promotes the interaction between TFIIIB and TFIIIC2

Assuming that CKII phosphorylates TFIIIB in vivo and that this phosphorylation is responsible for the stimulatory effect of CKII on mammalian pol III transcription, the question then arises as to how this phosphorylation stimulates transcription. The primary function of TFIIIB in pol III transcription at most class III genes is to act as a bridging factor between promoter-bound TFIIIC and RNA polymerase III, thus allowing the recruitment of pol III to the appropriate promoters. It was therefore investigated whether CKII kinase activity can influence the essential interactions of TFIIIB with either TFIIIC or pol III. At present there is no evidence

to suggest that CKII has any influence on the interaction between TFIIIB and pol III, however, coimmunoprecipitation experiments suggest that CKII activity does affect the interaction of TFIIIB with TFIIIC.

The monoclonal antibody MTBP6, which specifically recognises a sequence in the N-terminus of TBP, efficiently immunoprecipitates the TBP-containing pol III factor TFIIIB from HeLa extracts. Furthermore, TFIIIC2 is specifically coimmunoprecipitated with TFIIIB (data not shown). This antibody therefore enabled the effect of CKII kinase activity on the interaction between TFIIIB and TFIIIC2 to be investigated. The effect of the CKII inhibitor quercetin on the ability of the MTBP6 antibody to coprecipitate TFIIIC2 was tested. As Figure 6.12 **A** shows, the inclusion of quercetin in immunoprecipitation reactions, to specifically inhibit CKII kinase activity, reduced the levels of TFIIIC2 that coprecipitated. The amount of TFIIIB that was immunoprecipitated did not change (Fig 6.21 **A**, lower panel and data not shown), however, suggesting that the inhibition of CKII kinase activity impairs the interaction between TFIIIB and TFIIIC; hence the reduced levels of TFIIIC2 in the immunoprecipitates. The levels of TBP in the precipitated material were unaffected by the inclusion of quercetin in the reactions (Figure 6.12 **A**, lower panel). Similarly, the levels of BRF in the immunoprecipitates remained the same (data not shown); demonstrating the specificity of the decrease in the levels of coprecipitated TFIIIC2 in response to inhibiting CKII activity with quercetin. Additionally, similar doses of quercetin have been shown to have no effect on the coprecipitation of BRF with CKII (257).

A similar reduction in the levels of TFIIIC2 that are coprecipitated with TFIIIB was observed in the presence of the unrelated CKII inhibitor, DRB (Figure 6.12 **B**, lanes 6 and 7). Immunoprecipitation experiments have also been carried out with HeLa nuclear extract supplemented with radiolabelled in vitro translated BRF and the 4286 antiserum that is specific for the TFIIICβ subunit of TFIIIC2. The 4286 antiserum is able to coprecipitate BRF with TFIIIC2 (503). However, the presence of quercetin or DRB was found to abolish the coprecipitation of BRF (257), providing supporting evidence for the observed inhibitory effect of reducing CKII activity on the interaction of endogenous TFIIIB and TFIIIC2. Together, these results suggest that CKII kinase activity can promote the interaction of TFIIIB and TFIIIC2. A model is proposed in which the phosphorylation of TFIIIB by CKII promotes the interaction between TFIIIB and TFIIIC (Figure 6.13). It remains

**Figure 6.12**

**The CKII inhibitors quercetin and DRB compromise the interaction of endogenous TFIIIB and TFIIIC**

**A)** HeLa nuclear extract (150μg) was immunoprecipitated using no antibody (lane 1) or anti-TBP monoclonal antibody MTBP6 (lanes 2-6). Lanes 3 and 4 contained 100μM or 500μM quercetin, respectively. Lanes 1, 2, 5 and 6 contained appropriate amounts of control buffer. After extensive washing with LDB buffer, precipitated material was resolved on a SDS-7.8% polyacrylamide gel and analysed by western blotting with anti-TFIIICβ antiserum 4286 (upper panel), or with anti-TBP antibody SL30 (lower panel).

**B)** HeLa nuclear extract (150μg) (lane 1 and lanes 3-7) was immunoprecipitated using no antibody (lane 1) or anti-TBP monoclonal antibody MTBP6 (lanes 2-7). Lanes 4 and 5 contained 150μM or 500μM quercetin, respectively. Lanes 1-3 contained appropriate amounts of control buffer. Lane 7 contained 120μM DRB and lane 6 contained 1.2 % ethanol as a buffer control for the DRB. After extensive washing with LDB buffer, precipitated material was resolved on a SDS-7.8% polyacrylamide gel and analysed by western blotting with anti-TFIIICβ antiserum 4286.

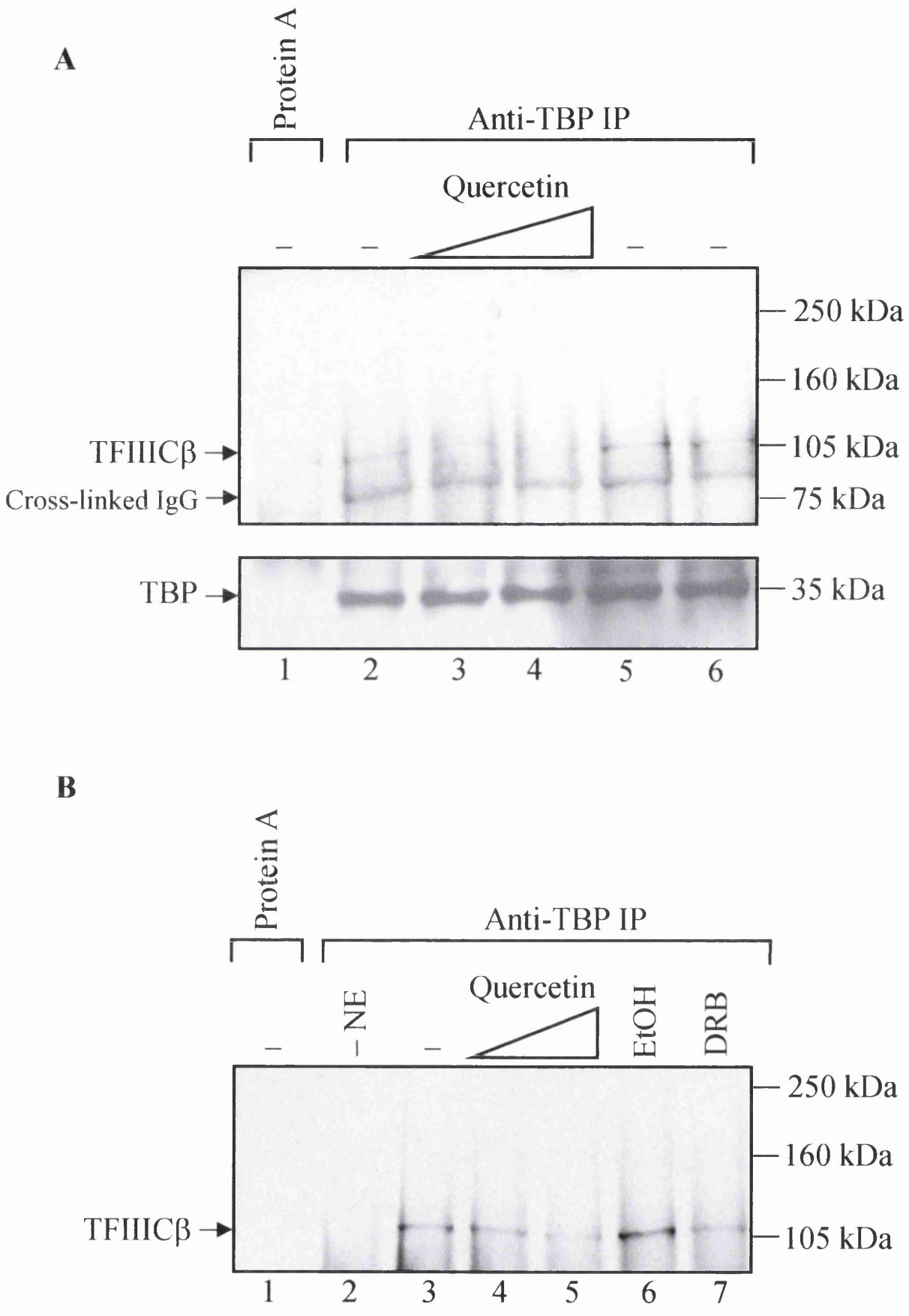
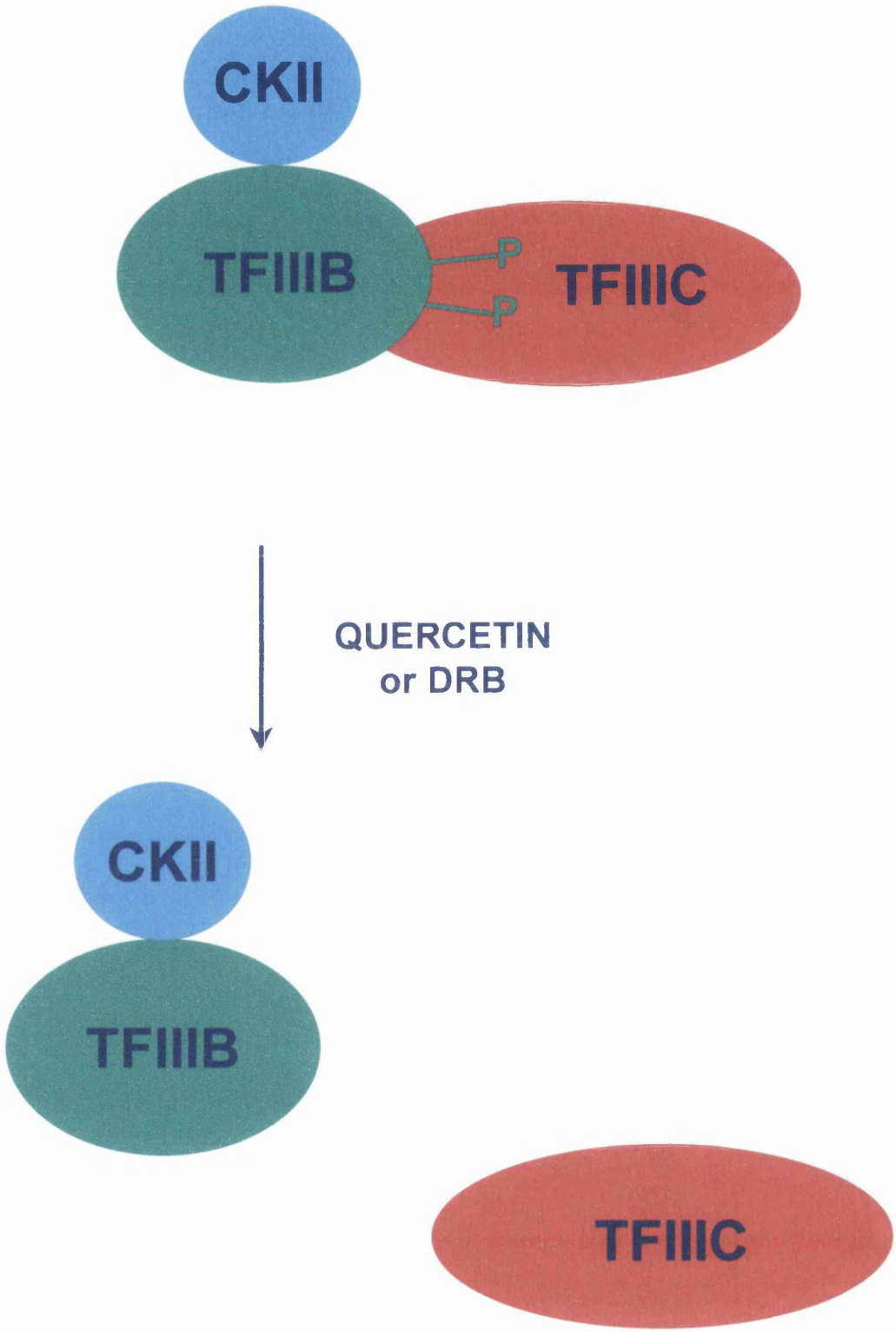

A
Protein A
Anti-TBP IP
Quercetin
–
–
–
–
250 kDa
160 kDa
105 kDa
75 kDa
TFIIICβ
Cross-linked IgG
TBP
35 kDa
1
2
3
4
5
6
B
Protein A
Anti-TBP IP
Quercetin
– NE
–
EtOH
DRB
250 kDa
160 kDa
105 kDa
TFIIICβ
1
2
3
4
5
6
7

**Figure 6.13**

**Model proposing that CKII may promote the interaction between TFIIIB and TFIIIC by the direct phosphorylation of TFIIIB**

The specific inhibition of CKII kinase activity by quercetin or DRB causes a reduction in the levels of TFIIIB and TFIIIC that are associated. Since CKII stably interacts with TFIIIB and the TFIIIB component BRF can be phosphorylated by CKII in vitro this suggested that CKII might facilitate the interaction between TFIIIB and TFIIIC by the phosphorylation of TFIIIB.


CKII
TFIIIB
P
P
TFIIIC
QUERCETIN
or DRB
CKII
TFIIIB
TFIIIC

uncertain as to whether the stimulatory effect of CKII on the interaction between TFIIIB and TFIIIC is sufficient to account for the activating function of CKII on mammalian pol III transcription or whether CKII may influence pol III transcription by additional mechanisms. The interaction of TFIIIB and TFIIIC is essential for transcription initiation of most class III genes, which makes it an excellent candidate for transcriptional control. Whereas CKII stimulates the interaction of TFIIIB and TFIIIC2 and in doing so may positively regulate pol III transcription, the tumour suppressor RB disrupts the interaction between TFIIIB and TFIIIC2 (see Chapter 4 and (503)). The targeting of the interaction of TFIIIB and TFIIIC by both a tumour suppressor and a putative oncogene product suggests that this interaction can be rate-limiting for pol III transcription and is tightly regulated. It remains to be determined whether the antagonistic effects of CKII and RB on this interaction are related or whether they occur by distinct mechanisms.

### 6.2.5 Recombinant GSK-3β inhibits pol III transcription in vitro

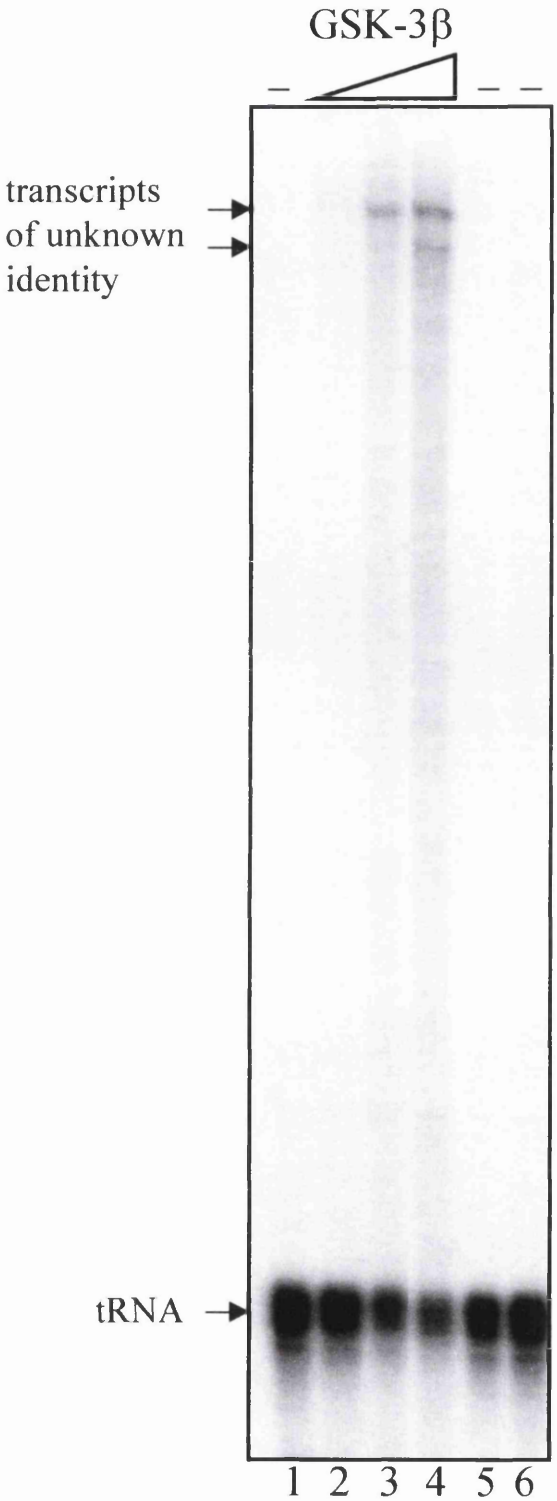
To begin to investigate whether GSK-3β has a role in regulating pol III transcription, the effect was tested of adding bacterially expressed recombinant GSK-3β to pol III transcription reactions reconstituted in vitro. As Figure 6.14 shows, titrating in increasing amounts of recombinant GSK-3β caused a dose-dependent decrease in the levels of pol III transcription. The specificity of this decrease in the levels of correctly sized tRNA transcript with increasing doses of GSK-3β is demonstrated by the levels of two very large transcripts of unknown identity that are produced in the reactions. Whereas these large transcripts were undetectable in reactions that lacked GSK-3β, the reactions with the higher doses of GSK-3β and reduced levels of tRNA synthesis generally displayed higher levels of these two transcripts. This may be caused by increased availability of polymerase for non-specific transcription or perhaps GSK-3β inhibits transcription termination and these transcripts are derived from read-through transcription of the tRNA gene.

This clear inhibition of pol III transcription in vitro in response to increasing amounts of recombinant GSK-3β suggests that GSK-3β may negatively regulate pol III transcription, consistent with its inhibitory effect on eIF2B and a role for GSK-3β

**Figure 6.14**

**Glycogen synthase kinase-3β represses pol III transcription**

pLeu template (250 ng) was transcribed using 2μl of PC-B and 2μl of PC-C in the presence of 0.5μl, 1μl or 4μl of recombinant GSK-3β (New England Biolabs) (lanes 2, 3, and 4, respectively). Lanes 1, 5 and 6 contained appropriate amounts of control buffer. Prior to the addition of nucleotides to initiate transcription, reaction mixtures were preincubated for 10 min at $30^0$C. Transcription was allowed to proceed for 1 h. Ethanol-precipitated transcription products were separated on a 7M urea 7% polyacrylamide sequencing gel and were visualised by autoradiography.


GSK-3β
transcripts
of unknown
identity
tRNA
1 2 3 4 5 6

in restraining cellular growth (423, 561, 562). The extent of the inhibition is relatively small, however, compared to that observed in the presence of recombinant RB or when CKII activity was inhibited, for example. It may be that the recombinant GSK-3β is of low activity compared to that of recombinant RB, maybe lacking an activating post-translational modification or perhaps GSK-3β is only a weak repressor of pol III transcription. The relatively mild inhibitory effect of GSK-3β may also reflect the fact that, for many substrates, the minimal sequence requirements for phosphorylation by GSK-3β are a phosphorylated serine residue in the + 4 position (563). Therefore, for GSK-3β to phosphorylate its endogenous substrates this commonly requires a priming phosphorylation event by another kinase (563). A limiting amount of priming kinase will restrict any effect of increased levels of GSK-3β.

### 6.2.6 Lithium, a potent and specific inhibitor of GSK-3, stimulates pol III transcription in vitro

Since the addition of recombinant GSK-3β caused a decrease in the levels of pol III transcription, it was predicted that inhibiting endogenous GSK-3β activity should increase the levels of pol III transcription. To test this, CREB phosphopeptide, a synthetic peptide that contains a consensus phosphoacceptor site for GSK-3 (147, 550), was titrated into transcription reactions. This peptide was predicted to compete with endogenous substrates for phosphorylation by GSK-3, in an analogous manner to the earlier described use of a peptide containing a CKII phosphoacceptor site as a competitive inhibitor of CKII. Unexpectedly and in apparent contradiction to the transcriptional inhibition observed using recombinant GSK-3β, the CREB phosphopeptide also caused a dose-dependent decrease in the levels of pol III transcription, although the decreases were only small relative to those observed using CKII peptide when it was assayed in parallel (data not shown). Perhaps GSK-3α and GSK-3β, both of which can phosphorylate the CREB phosphopeptide (147), have opposing effects on pol III transcription and the inhibitory effect predominates. Although possible, this is deemed unlikely as the available evidence suggests there is substantial functional redundancy between the

two GSK-3 isoforms (219). An alternative plausible explanation is that the CREB phosphopeptide is not totally specific for GSK-3.

The CREB phosphopeptide is derived from the GSK-3 phosphoacceptor site at $Ser^{129}$ of the cAMP response element binding protein (CREB) and is commonly used as the substrate for assaying GSK-3 activity in crude and fractionated extracts (147). It is phosphorylated ~ 40 fold more efficiently by recombinant GSK-3β than by recombinant CKII in an in vitro kinase assay (Figure 6.3). However, in the relatively crude fractions that are used to reconstitute pol III transcription in vitro there are likely to be a whole host of different kinases, one of which may be able to phosphorylate the CREB peptide reasonably efficiently. Indeed, problems of high levels of background phosphorylation of GSK-3 peptide substrates in crude cell extracts have previously been reported (454). Thus, the inhibitory effect of the CREB peptide on pol III transcription may result from the competitive inhibition of a kinase other than GSK-3 that has an activating role in pol III transcription. The likelihood of the non-specific phosphorylation of the CREB peptide is increased by the presence of two serine residues in this short peptide (147). Although this peptide has been synthetically phosphorylated on the + 4 Ser, it may become dephosphorylated by endogenous phosphatases in crude extracts. This would create an additional potential phosphorylation site and would prevent the phosphorylation of this peptide by GSK-3 without prior phosphorylation at the + 4 position by another kinase.

To distinguish between GSK-3-specific phosphorylation of the CREB peptide in crude cell extracts and non-specific background phosphorylation of this peptide, lithium ions ($Li^+$) have been used (454). Lithium ions are a potent and specific inhibitor of GSK-3, both in vitro and in vivo (207, 280, 454, 498). Doses of lithium ions that were found to almost completely abolish GSK-3 activity in vitro had negligible effect on a range of other protein kinases tested that included CKII, PKA, JNK and MAPK, demonstrating the specificity of the kinase inhibitory effect of lithium ions for GSK-3 (280, 498). To date no other lithium-sensitive kinases have been identified, suggesting that the sensitivity of GSK-3 to lithium may be a unique feature of this kinase (454). The inhibitory effect of lithium is also highly conserved, forms of GSK-3 from invertebrates such as Dictyostelium and Drosophila as well as those from vertebrates such as Xenopus and mammals all displaying sensitivity to lithium (207, 280, 454, 498). GSK-3 is not the sole target of lithium action,

however. Lithium is also a potent inhibitor of inositol monophosphatase (IMPase) and inositol polyphosphate 1-phosphatase (IPPase), key enzymes required for the synthesis and recycling of inositol (34, 186).

The physiological effects of lithium are diverse. Lithium has profound effects on development in numerous organisms (260, 280, 348, 497, 536) and is one of the most effective drugs used for the treatment of bipolar disorder (582). Several hypotheses have been proposed as to how lithium might exert these effects (34, 280, 582). The inositol depletion hypothesis is based on the inhibitory effect of lithium on the inositol phosphatases, IMPase and IPPase (34). Repression of these enzymes by lithium is proposed to reduce the free pool of inositol which ultimately will lower the intracellular concentration of the second messenger inositol (1,4,5)-triphosphate ($IP_3$) (34). This in turn will have severe consequences for phosphoinositide signalling, providing a potential molecular mechanism by which at least some of the physiological effects of lithium may be achieved (34). Lithium-induced blockage of cell cycle transitions in sea urchin embryos is prevented by the microinjection of myo-inositol, suggesting that the inhibition of IMPase or IPPase may be involved in mediating this effect of lithium (28). Similarly, in Xenopus, duplication of the dorsal axis induced by lithium treatment was prevented by exogenous myo-inositol, implicating the involvement of IMPase or IPPase (68). Recently, a class of compounds that are ~ 1000 fold more potent than lithium in inhibiting IMPase have become available, providing a powerful tool for dissecting which physiological effects of lithium are mediated through the inhibition of IMPase (15). These novel inhibitors of IMPase were found to have no discernible effect on the morphogenesis of Xenopus embryos in contrast to the dorsalisation induced by lithium, so clearly the inhibition of IMPase is not sufficient to account for this effect of lithium (280).

The discovery that GSK-3 is also potently repressed by lithium identified a highly appealing alternative target to IMPase or IPPase by which the physiological effects of lithium may be mediated, the role of GSK-3 in cell fate determination in many eukaryotic species (563) correlating well with the effects of lithium on development. In support of the candidacy of GSK-3 as the dominant endogenous target of lithium action and the use of lithium as an inhibitor of GSK-3 in vivo, lithium treatment actually phenocopies loss of GSK-3 function (207, 219, 280, 502). For example, insulin signalling inactivates GSK-3, stimulating glycogen synthesis; lithium also stimulates glycogen synthesis (89, 502). Expression of a dominant

negative mutant of GSK3β dorsalises Xenopus embryos (205); lithium also causes dorsalisation (259, 260, 280). In Dictyostelium, the disruption of GSK-3 alters cell fate (197); lithium mimics this effect (348, 536). There is also considerable evidence of lithium antagonising GSK-3 function in vivo. Thus, lithium treatment induces β-catenin accumulation in Drosophila and mammalian cells, mimicking the effects of wnt signalling (498). GSK-3, which is inactivated by wnt signalling, phosphorylates β-catenin, targeting it for degradation (69, 382, 412, 450). In addition to the effects of lithium on body axis formation in Xenopus embryos, $Li^+$ has also been shown to result in the activation of an AP-1 luciferase reporter (207), consistent with previous observations that GSK-3β inhibits c-jun activity (46). The overexpression of GSK-3 in cultured human neurons induces an Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau (190). Lithium reduces tau phosphorylation by GSK-3 (498) and can counteract the inhibitory effect of GSK-3 on tau-induced processes outgrowth (224, 328). Normal hypophosphorylated tau promotes microtubule assembly and nucleation (224, 254, 328). The inhibition of these functions of tau by GSK-3-dependent phosphorylation (328, 343, 542) suggests that the efficacy of lithium in treating depression and other disorders of the nervous system is likely to be mediated at least in part through the inhibition of GSK-3. Thus, GSK-3 seems to be a major physiological target of lithium action.

Lithium ions are widely and routinely used to investigate GSK-3 function (333, 449, 502). The effects of lithium seem to be considered a reliable indication of the possible involvement of GSK-3 in a particular process (333, 449). The striking correlation between the physiological effects of lithium in evolutionarily distant species and the consequences of disrupting GSK-3 function, suggests that there is some justification in regarding the effects of lithium on a particular process as a good indication of whether or not GSK-3 is involved.

The effect of lithium ions on pol III transcription reconstituted in vitro was therefore tested. As a source of lithium ions, lithium chloride was used. Chloride ions have previously been shown to have little effect on GSK-3 activity (280). To control for the addition of lithium chloride, the effect of adding an equal concentration of sodium chloride was assayed in parallel. As Figure 6.15 shows, millimolar concentrations of LiCl, commonly used to specifically inhibit GSK-3 (333), stimulated tRNA synthesis relative to the levels obtained with an equal

concentration of NaCl. Little or no stimulation was observed in the presence of 20mM LiCl (data not shown), possibly because at this concentration the inhibition of endogenous GSK-3 was too slight to have any effect on pol III transcription. At a concentration of 100mM LiCl, or higher, transcription was less than with NaCl, probably because of non-specific inhibitory effects of lithium (data not shown). Pol III transcription reconstituted in vitro is very sensitive to changes in ionic strength, hence the variation in the levels of pol III transcription between the different doses of sodium chloride. Nonetheless, there is a clear stimulatory effect of lithium ions on the levels of tRNA synthesis. Stimulation of pol III transcription by lithium ions was also observed using the $VA_I$ gene as template, but to a much lesser extent (data not shown). This differential sensitivity to lithium may reflect the difference in promoter strength of the two genes. The $VA_I$ promoter is stronger than the promoters of tRNA genes and thus is likely to be less sensitive to changes in the levels or activity of repressors or activators.

### 6.2.7 Lithium ions increase the pol III transcriptional activity of cells

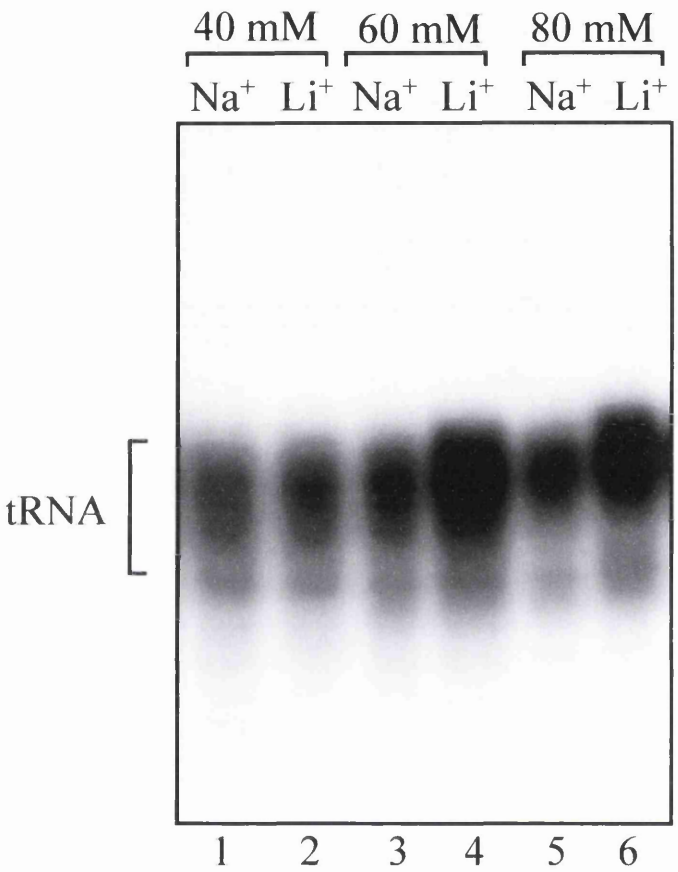
The stimulation of pol III transcription in vitro by doses of lithium ions that are sufficient to inhibit endogenous GSK-3 activity together with the inhibition of pol III transcription by recombinant GSK-3β suggests that GSK-3β may be a repressor of pol III transcription, at least in vitro. To investigate this possibility further, the effect on pol III transcription of inhibiting GSK-3 activity in vivo using lithium was tested. The concentrations of lithium chloride used to inhibit GSK-3 in vivo and the length of time cells are incubated in media to which LiCl has been added that are reported in the literature vary considerably. Therefore the effect on pol III transcription of a range of different concentrations of LiCl and incubation times were tested.

Asynchronous populations of actively proliferating Balb/c 3T3 (A31) murine fibroblasts were cultured for 12 h in media supplemented with a range of different concentrations of LiCl, or NaCl as a control. Cell extracts were then prepared, and transcriptional activity was assessed by in vitro transcription assay. Extracts from $Li^+$-treated cells generally had elevated pol III transcriptional activity compared to

**Figure 6.15**

**Stimulation of pol III transcription by lithium ions**

pLeu template (250 ng) was transcribed using 2μl of PC-B and 2μl of PC-C in the presence of 40mM, 60mM or 80mM NaCl (lanes 1, 3 and 5, respectively) or LiCl (lanes 2, 4 and 6, respectively). Following a 15 min preincubation at $30^0$C, nucleotides were added to initiate transcription which was allowed to proceed for 1 h at $30^0$C.


40 mM
60 mM
80 mM
Na+ Li+ Na+ Li+ Na+ Li+
tRNA
1 2 3 4 5 6

control extracts, as shown in Figure 6.16, using a $tRNA^{Arg}$ gene as template. Extracts from cells that were incubated in media containing 12.5 mM LiCl were of similar transcriptional activity to their NaCl-treated counterparts. However, extracts prepared from cells cultured in media containing 25 mM LiCl, 50 mM LiCl or 75mM LiCl all supported higher levels of tRNA synthesis than equal amounts of extracts from cells treated with the same concentrations of NaCl. Extracts prepared from A31 cells that were treated with 100mM LiCl had lower levels of transcriptional activity than the corresponding extracts prepared in parallel from cells treated with 100mM NaCl, suggesting that at this concentration lithium may have non-specific inhibitory effects.

A time course in which A31 cells were incubated in media containing 25 mM LiCl or 25 mM NaCl for different lengths of time before harvesting was also conducted. As Figure 6.17 shows, all the extracts prepared from $Li^+$-treated cells had elevated pol III transcriptional activity compared to the corresponding extracts from $Na^+$-treated cells, apart from the pair of extracts from the cells that were treated for the shortest period of time. The reproducibility of this specific increase in the transcriptional activity of extracts from cells treated with low concentrations of lithium ions was tested by independently preparing several extracts from different batches of A31 cells treated with 25 mM LiCl or 25mM NaCl for 12 h. Extracts prepared from $Li^+$-treated cells consistently displayed substantially higher levels of pol III transcriptional activity than the corresponding extracts from $Na^+$-treated cells (data not shown). The concentration of LiCl in the culture media that was required to specifically raise the pol III transcriptional activity of cell extracts was quite low. Furthermore, the intracellular concentration of lithium ions after 12 h incubation is likely to be lower than that in the media in which the cells were cultured. It has previously been reported that after a 4 h incubation of Xenopus embryos in LiCl the intracellular concentration of lithium ions did not exceed 5% of the extracellular concentration (50). The low concentration of lithium chloride required to increase pol III transcriptional activity increases the likelihood that this is a specific response to the inhibition of GSK-3 rather than some non-specific effect of lithium ions.

The consistently higher pol III transcriptional activity of extracts prepared from $Li^+$-treated cells was observed by in vitro transcription assay using a tRNA gene as the specific pol III template. Since the earlier in vitro studies suggested that the sensitivity to lithium, and thus potentially to repression by GSK-3, may differ for

**Figure 6.16**

**Extracts from cells treated with lithium ion display increased pol III transcriptional activity**

BALB/c3T3 A31 cells were grown in DMEM supplemented with 10% fetal calf serum, 100U/ml penicillin, and 100μg/ml streptomycin to a confluency of ~ 80 %. Cells were then incubated for 12 h in fresh media supplemented with varying concentrations of NaCl or LiCl. After 12 h, cells were harvested and whole cell extracts were prepared by the freeze-thaw method. The $tRNA^{Arg}$ gene (250ng of pArg) was transcribed using 10μg of cell extract from cells treated with 12.5mM NaCl or LiCl (lanes 1 and 2, respectively), 25mM NaCl or LiCl (lanes 3 and 4, respectively), 50mM NaCl or LiCl (lanes 5 and 6, respectively), 75mM NaCl or LiCl (lanes 7 and 8, respectively), or 100mM NaCl or LiCl (lanes 9 and 10, respectively).

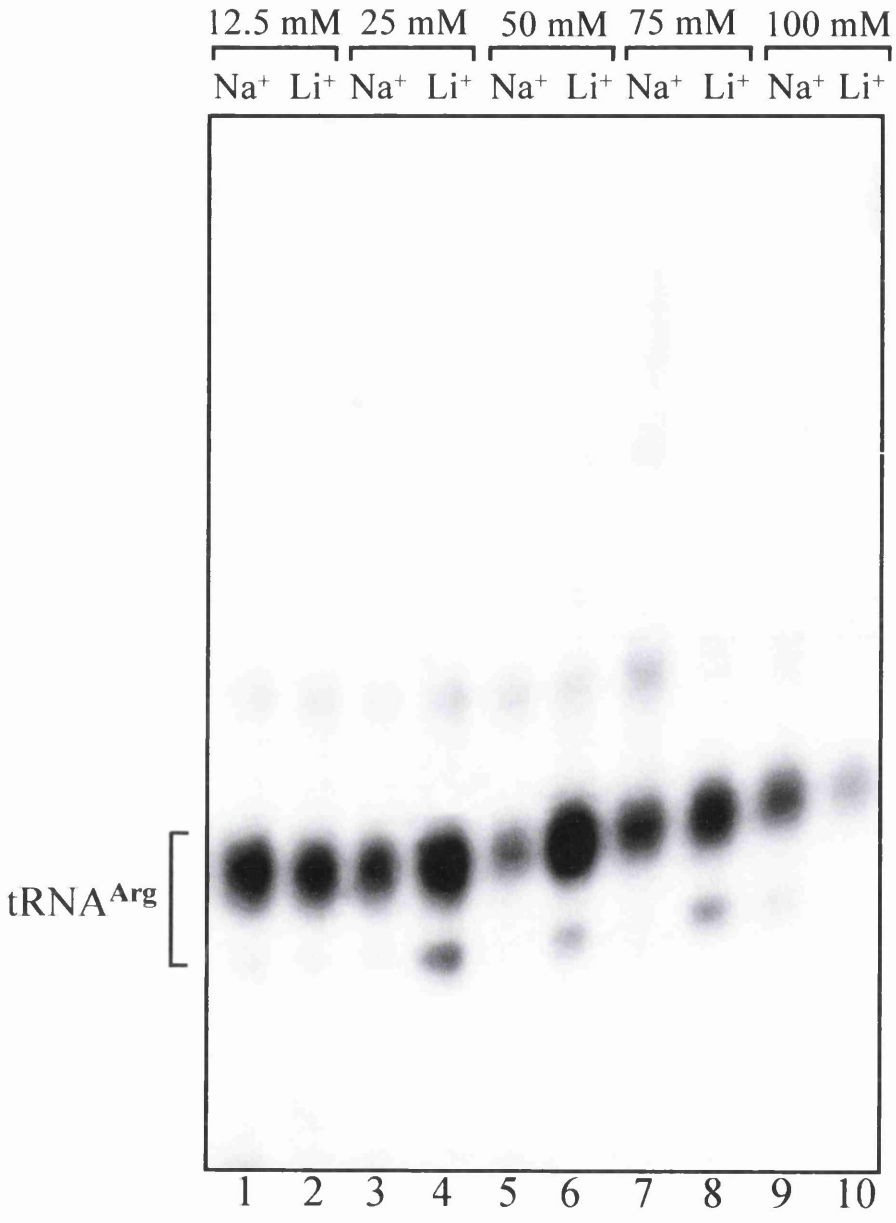
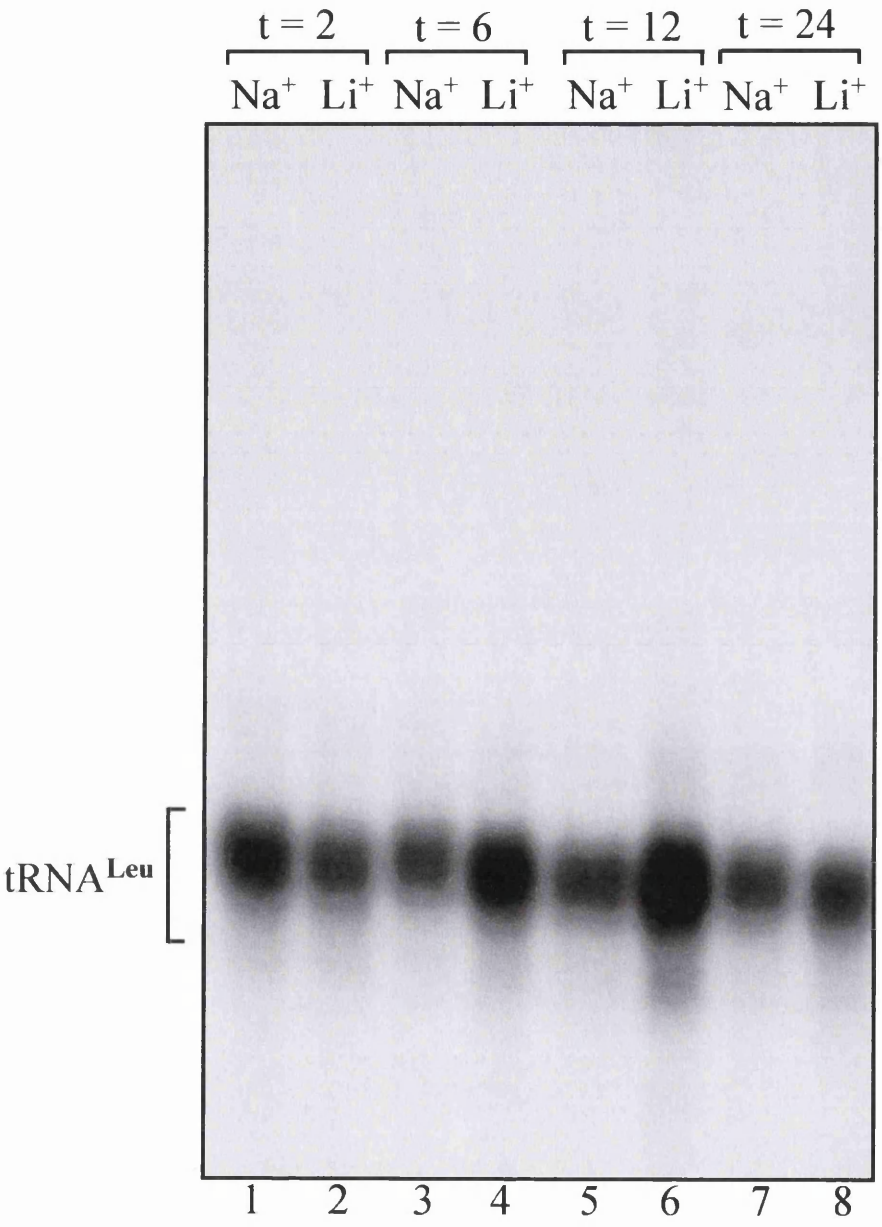

12.5 mM
25 mM
50 mM
75 mM
100 mM
Na+ Li+ Na+ Li+ Na+ Li+ Na+ Li+ Na+ Li+
tRNA^Arg
1 2 3 4 5 6 7 8 9 10

**Figure 6.17**

**The pol III transcriptional activity of cells increases in response to $Li^+$**

Exponentially growing BALB/c3T3 A31 cells were incubated for varying lengths of time in DMEM supplemented with 10% fetal calf serum, 100U/ml penicillin, 100μg/ml streptomycin, and 25mM NaCl or LiCl. At the appropriate times, cells were harvested and whole cell extracts were prepared. pLeu (250ng) was transcribed using 10μg of cell extract from cells incubated in media supplemented with 25mM NaCl or LiCl for 2 h (lanes 1 and 2, respectively), 6 h (lane 3: NaCl, lane 4: LiCl), 12 h (lane 5: NaCl, lane 6: LiCl), or 24 h (lane 7: NaCl, lane 8: LiCl). Transcription products were resolved on a 7M urea 7% polyacrylamide sequencing gel and were visualised by autoradiography.


t = 2
t = 6
t = 12
t = 24
Na+ Li+ Na+ Li+ Na+ Li+ Na+ Li+
tRNALeu
1 2 3 4 5 6 7 8

different class III genes this was tested using corresponding pairs of extracts from $Na^+$- and $Li^+$-treated cells. Figure 6.18 shows the results of pol III transcription reactions using the same pair of extracts with three different class III genes as template. As expected, the extract from the $Li^+$-treated cells supported higher levels of transcription of a $tRNA^{leu}$ gene than the same amount of the corresponding extract from $Na^+$-treated cells. 5S rRNA gene transcription reconstituted with the same pair of extracts was substantially elevated in the extract from the $Li^+$-treated cells compared to that obtained with the extract from the $Na^+$-treated cells, indeed to a significantly greater extent than observed using a tRNA gene as template. In contrast, when the same pair of extracts were assayed for their ability to support $VA_I$ transcription, the levels of $VA_I$ transcript produced were slightly higher in extract from the $Na^+$-treated cells. This differential effect of treating cells with lithium ions on the pol III transcriptional activity of extracts when reconstituted with different class III genes is consistent with the effects of lithium on pol III transcription in vivo being specific.

### 6.2.8 Pol III transcripts are specifically elevated in $Li^+$-treated cells

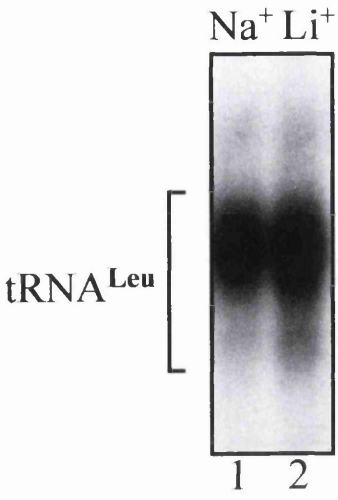
It is conceivable that the increased pol III transcriptional activity of extracts from $Li^+$-treated cells relative to extracts from $Na^+$-treated cells could result from the two treatments having differential effects on the recovery of pol III transcription components or their susceptibility to inactivation during extract preparation. Therefore, as an independent test of the stimulatory effect of lithium ions on pol III transcription in vivo, total RNA was isolated from $Li^+$- and $Na^+$-treated A31 cells and the levels of B2 RNA, a murine-specific pol III transcript, were compared by Northern blot analysis. B2 transcripts were found to be substantially more abundant in $Li^+$-treated cells relative to $Na^+$-treated cells (Figure 6.19, upper panel). This effect is specific, because $Na^+$- and $Li^+$-treated cells express similar levels of a pol II transcript encoding acidic ribosomal phosphoprotein P0 (ARPP P0) (Figure 6.19, lower panel). This specific increase in the abundance of B2 transcripts in $Li^+$-treated cells relative to $Na^+$-treated cells is consistently observed in different RNA preparations from $Na^+$- and $Li^+$-treated cells (Fig 6.19 and data not shown). Thus, lithium ions can increase the levels of pol III transcription both in vitro and in vivo.

**Figure 6.18**

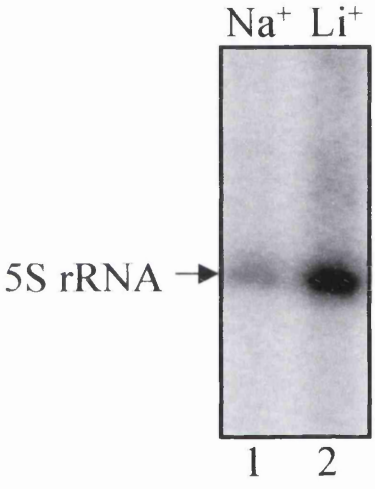
**Differential effect of lithium ions on pol III transcription of different class III genes**

The same pair of extracts from $Na^+$- and $Li^+$- treated A31 cells were tested for their ability to support pol III transcription of three different class III genes, $tRNA^{Leu}$ gene **(A)**, 5S rRNA gene **(B)**, and $VA_I$ gene **(C)**. Transcription was reconstituted with 10μg of extract (for **(A)**, **(B)**, and **(C)**, lane 1: extract from $Na^+$-treated cells, lane 2: extract from $Li^+$-treated cells) and 250ng of pol III template. Nucleotides were added to initiate transcription, which was allowed to proceed for 1 h at $30^0$C. Transcription products were ethanol-precipitated and analysed on a 7M urea 7% polyacrylamide sequencing gel.

A




B




C

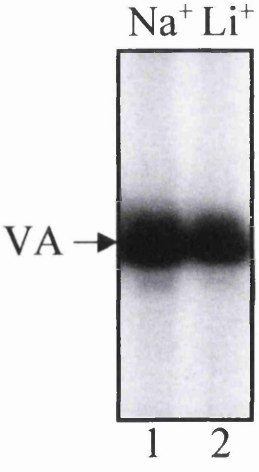
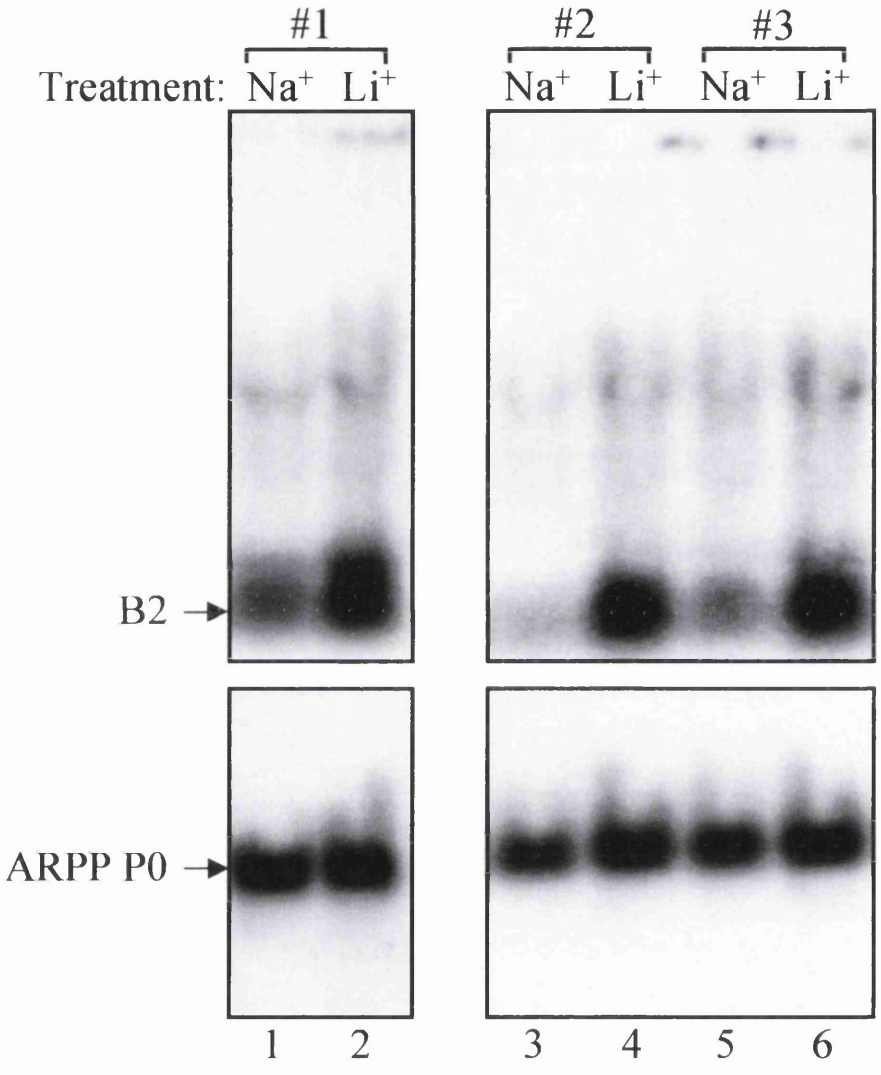


**Figure 6.19**

**B2 RNA levels are specifically increased in A31 cells that have been cultured in the presence of millimolar concentrations of lithium ions**

Northern blot analysis of total RNA (10μg) prepared from three independent sets of $Na^+$- or $Li^+$- treated A31 cells (odd-numbered lanes: $Na^+$-treated; even-numbered lanes: $Li^+$-treated). Cells were incubated in media supplemented with 12.5 mM NaCl or LiCl (lanes 3 and 4, respectively) or 25mM NaCl or LiCl (lanes 1 & 5, and lanes 2 & 6, respectively) for 9 h (lanes 1 and 2) or 12 h (lanes 3-6) and then harvested. The upper panel shows the blot probed with a B2 gene and the lower panel shows the same blot that has been stripped and reprobed with the acidic ribosomal phosphoprotein P0 (ARPP P0).


#1
#2
#3
Treatment: Na+ Li+ Na+ Li+ Na+ Li+
B2
ARPP P0
1 2 3 4 5 6

The lack of a change in the levels of the pol II transcript ARPP P0 in $Li^+$-treated cells in contrast to the elevated expression of B2 transcripts demonstrates that the stimulation of pol III transcription in vivo by lithium ions is highly specific and not due to a general increase in nuclear transcription.

Although these results are supportive of a postulated function for GSK-3 in the repression of pol III transcription it is clearly possible that this specific increase in pol III transcription in vivo in response to lithium treatment is mediated by a different mechanism to the specific repression of GSK-3 by lithium ions. Therefore, as a more direct investigation of the possible involvement of GSK-3β in regulating pol III transcription in vivo, attempts were made to overexpress GSK-3β in cells by carrying out transient transfections using an expression vector encoding GSK-3β. Primer extension analysis was used to determine the levels of transcription of a cotransfected $VA_I$ gene that was used as a Pol III reporter. To enable the levels of $VA_I$ transcription to be normalised for variation in transfection efficiency, cells were also transfected with a control plasmid that contained the CAT gene under the control of the constitutively active cytomegalovirus (CMV) promoter.

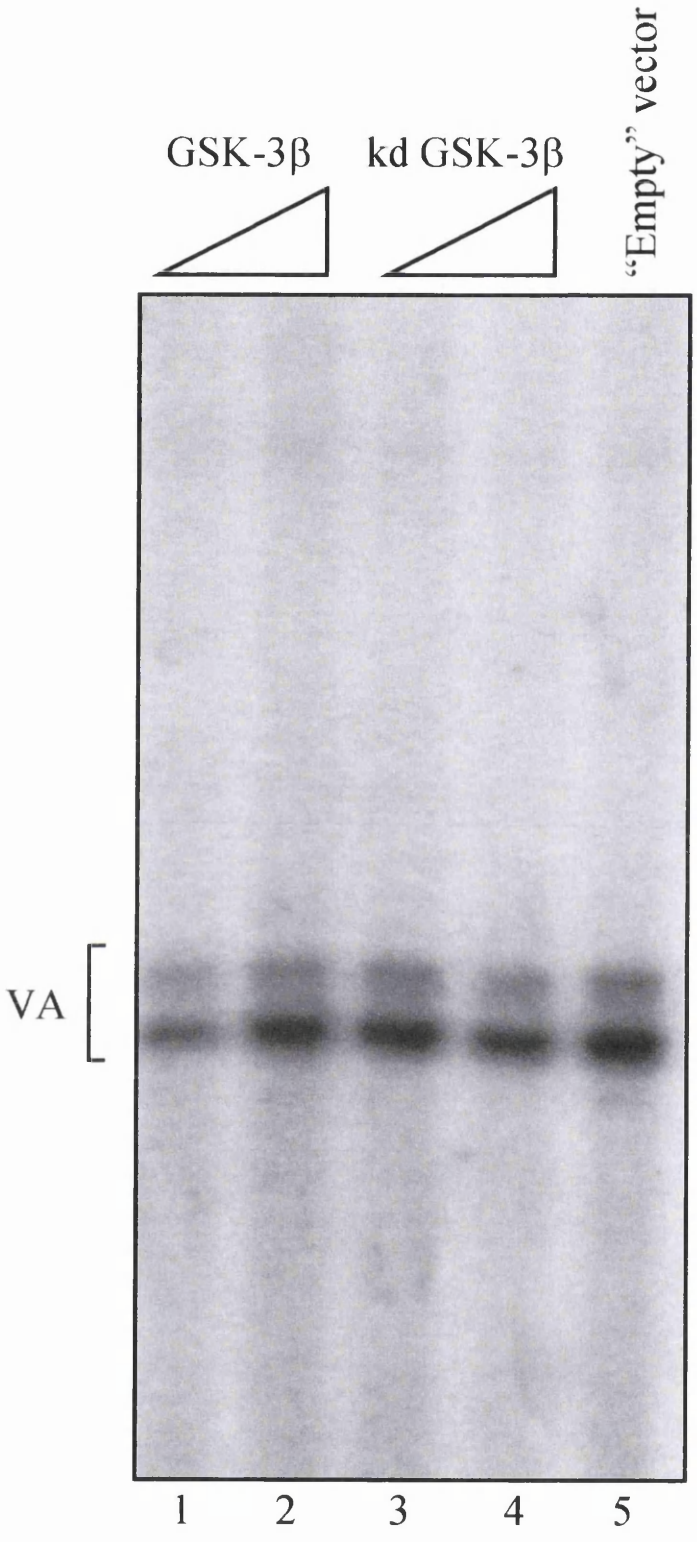
Similar levels of $VA_I$ transcript were produced in cells transfected with "empty" expression vector as in cells transfected with the same amount of expression vector encoding GSK-3β (Fig 6.20, compare lanes 2 and 5). The expected dose-dependent decrease in the levels of $VA_I$ transcription in cells transfected with expression vector encoding GSK-3β was not observed. The slightly reduced level of $VA_I$ transcription observed in cells transfected with the lower dose of GSK-3β may result from slightly reduced transfection efficiency, although there were no quantifiable differences in the levels of CAT RNA for the different transfections (data not shown). In repeated transfections, the expression vector encoding GSK-3β reproducibly had little or no effect on the levels of $VA_I$ transcription relative to cells transfected with a kinase-inactive GSK-3β form or "empty" expression vector (data not shown). These results suggest that GSK-3β has little or no influence on pol III transcription in vivo, in contrast to the repression of pol III transcription observed in vitro with the addition of recombinant GSK-3β.

Recently, it has been shown that GSK-3β regulates cyclin D1, targeting it for degradation by the 26S proteasome (124). This GSK-3β-induced reduction in cyclin D1 levels is predicted to cause a decrease in the activity of the cyclin D1-dependent

**Figure 6.20**

**Primer extension analysis of $VA_I$ transcript levels following transient transfection of NIH 3T3 fibroblasts with expression vector encoding GSK-3β**

NIH 3T3 fibroblasts (~6 x $10^5$ cells) were transiently transfected with 4μg of $pVA_I$, 4μg of pCAT and 10μg or 20μg of pJ3M-GSK-3β, encoding wild-type GSK-3β (lanes 1 and 2, respectively), 10μg or 20μg of pJ3M-GSK-3βkd, encoding kinase inactive GSK-3β (lanes 3 and 4, respectively), or 20μg of "empty" pJ3M vector that lacks an insert (lane 5). Cells transfected with 10μg of pJ3M-GSK-3β or pJ3M-GSK-3βkd (lanes 1 and 3) were also transfected with 10μg of "empty" pJ3M vector, thereby ensuring that the amount of plasmid DNA transfected was the same for each reaction. $VA_I$ primer extension products are shown.


GSK-3β
kd GSK-3β
"Empty" vector
VA
1
2
3
4
5

kinases, cdk4 and cdk6, whose primary function is the phosphorylation and inactivation of RB. Therefore, GSK-3β, through the regulation of cyclin D1, may be able to regulate the activity of RB; hence the lack of any effect of GSK-3β on $VA_I$ transcript levels in vivo was very surprising. Even if GSK-3β does not influence pol III transcription directly, GSK-3β was expected to have an indirect effect as a result of its ability to regulate cyclin D1 proteolysis, since RB is an established repressor of pol III transcription. Furthermore, it has previously been shown that cells transfected with an expression vector encoding cyclin D1 display elevated levels of pol III transcription, consistent with the inactivation of RB (C.A. Cairns, unpublished observations).

One possible explanation for the lack of any effect on pol III transcription of transfecting cells with an expression vector encoding GSK-3β is that the GSK-3β is poorly expressed. Therefore, duplicate transfections were performed in parallel to enable analysis of both RNA levels by primer extension and protein expression by western blotting. The transfected GSK-3β protein has an N-terminal myc-tag enabling its detection with the myc epitope-specific 9E10 monoclonal antibody and it to be distinguished from endogenous GSK-3β. Figure 6.21 **A** shows the level of expression of myc-tagged GSK-3β protein for the duplicate transient transfection for which the primer extension analysis is shown in Figure 6.20. Expression of transfected GSK-3β protein was very low. The kinase-inactive GSK-3β form was expressed at much higher levels (Fig 6.21 **A**, compare lanes 2 and 3 with lanes 4 and 5). Although transfecting cells with a larger amount of expression vector encoding GSK-3β increased the levels of expression, this was still very poor relative to the levels of kinase-inactive GSK-3β and had no detectable effect on the levels of $VA_I$ transcription of transfected cells. Since the levels of expression of transfected proteins can vary between cell types, transfections were also attempted in HeLa cells and NIH 3T3 fibroblasts, in case the poor level of expression of GSK-3β was specific to Balb/c 3T3 A31 cells. Several different preparations of high quality DNA of the GSK-3β expression vector were also tested, as the quality of the DNA is critical to the efficiency of transfection. However, none of these changes made any significant difference, the GSK-3β protein was consistently expressed only at low levels (data not shown). Figure 6.21 **B** and **C** shows the results of Western blotting of protein extracts from transfected HeLa cells. Expression of GSK-3β protein was

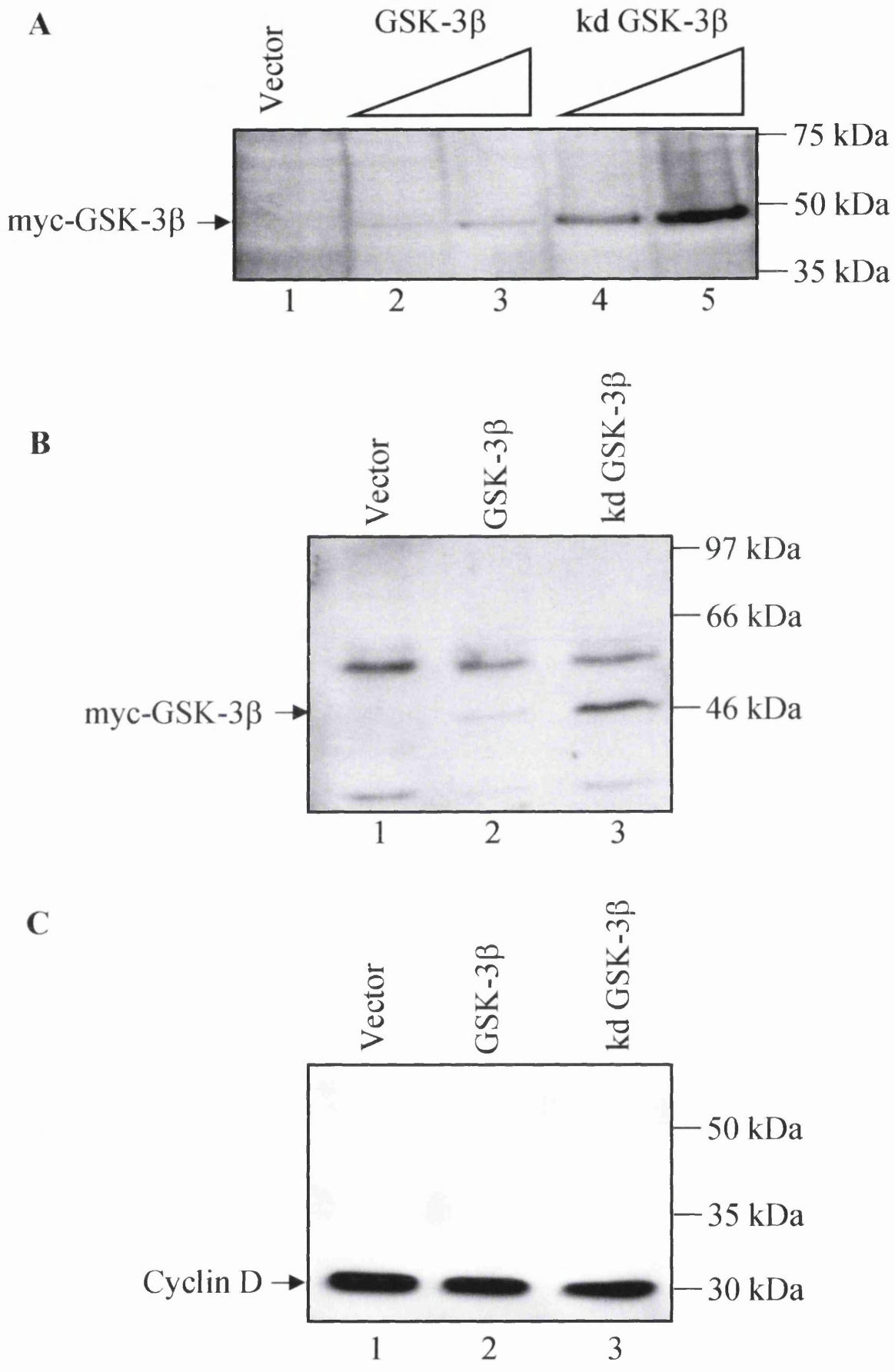
**Figure 6.21**

**GSK-3β is poorly expressed in transfected cells and has no effect on cyclin D protein levels**

**(A)** Equal amounts of total protein (50μg) prepared from transiently transfected NIH 3T3 fibroblasts (~6 x $10^5$ cells) were resolved by SDS-7.8% PAGE and analysed by western blotting for the expression levels of myc-tagged GSK-3β by probing with anti-myc antibody 9E10. Lanes 2 and 3: protein extract from cells transiently transfected with 10μg or 20μg of pJ3M-GSK-3β, respectively. Lanes 4 and 5: protein extract from cells transiently transfected with 10μg or 20μg of pJ3M-GSK-3βkd, respectively. Lane 1: protein extract from cells transiently transfected with 20μg of "empty" pJ3M vector.

**(B)** Protein extracts (50μg) from transiently transfected HeLa cells were similarly resolved by SDS-7.8% PAGE and analysed by western blotting using the anti-myc antibody 9E10. Lane 1: 50μg extract from cells transfected with pJ3M (20μg). Lane 2: 50μg extract from cells transfected with GSK-3β expression vector (20μg pJ3M-GSK-3β). Lane 3: 50μg extract from cells transfected with GSK-3βkd expression vector (20μg pJ3M-GSK-3βkd).

**(C)** 50μg of the same HeLa protein extracts analysed for myc-GSK-3β in **(B)** were independently analysed for endogenous cyclin D1 protein levels by western blotting with the anti-cyclin D1 antibody R-124.


A
Vector
GSK-3β
kd GSK-3β
75 kDa
50 kDa
35 kDa
myc-GSK-3β
1
2
3
4
5
B
Vector
GSK-3β
kd GSK-3β
97 kDa
66 kDa
46 kDa
myc-GSK-3β
1
2
3
C
Vector
GSK-3β
kd GSK-3β
50 kDa
35 kDa
30 kDa
Cyclin D
1
2
3

very poor (Figure 6.21 **B**). The low levels of GSK-3β protein that were expressed had no detectable effect on endogenous cyclin D1 levels (Figure 6.21 **C**). Thus, the lack of an effect on the levels of $VA_I$ transcript can be accounted for by the very poor expression of GSK-3β. The use of the $VA_I$ gene as the Pol III reporter for these transient transfections compounded the problems of poor GSK-3β expression, subsequent comparative experiments in vivo using lithium suggesting that $VA_I$ gene transcription in vivo may be much less sensitive to any repressive effect of GSK-3β than tRNA genes or the 5S rRNA gene. The available evidence in support of a role for GSK-3β in regulating pol III transcription in vivo is therefore only conjectural, as it is derived from the stimulatory effect of lithium ions on pol III transcription in vivo and is based on the assumption that it is the specific inhibition of GSK-3 by $Li^+$ that is responsible for the observed stimulation.

Any effect GSK-3β might have on pol III transcription in vivo could be very indirect, such as through modulating cyclin D1 levels. However, the ability of recombinant GSK-3β to repress pol III transcription in vitro that was reconstituted with fractionated HeLa factors suggests that GSK-3β can influence pol III transcription in a much more direct manner. Indeed, this result suggests that GSK-3β may actually target one of the components of the basal pol III transcriptional apparatus. The primary amino acid sequence of cloned components of the pol III transcriptional apparatus were searched for the GSK-3 consensus phosphoacceptor site Ser-x-x-x-Ser(P) (where x represents any amino acid), to see if any of the pol III transcription components are potential substrates for GSK-3. TFIIICβ has eleven Ser-x-x-x-Ser motifs; TFIIICα has nine such motifs, while TFIIICδ and TFIIICγ both have three. Additionally, there are four Ser-x-x-x-Ser motifs in the primary sequence of BRF and one in TBP. The surface accessibility of these phosphoacceptor sites is not known, nor whether the serine residue in the + 4 position is phosphorylated in a physiological environment, which is required to generate a phosphoacceptor site for GSK-3. However, the number of potential GSK-3 phosphoacceptor sites suggests that at least one component of the pol III transcriptional apparatus may be phosphorylated by GSK-3.

Some kinases stably interact with their substrates. It was therefore investigated whether GSK-3 stably interacts with TFIIIB or TFIIIC, which would be strongly supportive of a role for GSK-3 in the regulation of pol III transcription.

Since the consistent cofractionation of two proteins is highly suggestive of a stable association, it was tested whether GSK-3β is enriched in any of the immunopurified TFIIIB or TFIIIC fractions, or DNA affinity-purified TFIIIC fractions. However, GSK-3β was not detectable by Western blotting in any of these fractions (data not shown). As a more sensitive assay, these fractions were tested for GSK-3 activity in an in vitro kinase assay using CREB phosphopeptide as substrate. Only very low levels of phosphorylation were detected, which were similar for the different fractions (data not shown). Western blot analysis of equal amounts of HeLa PC-B and PC-C for GSK-3β protein indicated a significant enrichment of GSK-3β protein in PC-B relative to PC-C or unfractionated HeLa nuclear extract (data not shown). Since PC-B is enriched in TFIIIB and lacks TFIIIC, this suggested that GSK-3β might associate with TFIIIB. However, PC-B and PC-C are extremely crude, increasing the likelihood of this cofractionation of GSK-3β with TFIIIB being entirely fortuitous. In support of this possibility, GSK-3 did not cofractionate with TFIIIB during gradient chromatography of a PC-B fraction on Mono Q (data not shown). Immunoprecipitations were also carried out to see if endogenous GSK-3 associates with endogenous TFIIIB or TFIIIC2. However, GSK-3 was not found to specifically coimmunoprecipitate with either of these factors (data not shown). Together, these immunoprecipitation experiments and analyses of fractionated factors provided little evidence in support of a stable association of GSK-3 with a component of the pol III transcriptional apparatus. However, this possibility cannot be excluded at present as an interaction with TFIIIC1 or pol has yet to be tested.

It was also investigated whether recombinant GSK-3β or CKII have any effect on TFIIIC B-block DNA-binding activity. Electrophoretic mobility shift assays were carried out using B-block oligonucleotide as the specific probe and unfractionated extract or PC-C in the presence of exogenous GSK-3β or CKII and a suitable phosphate donor. However, neither kinase had any significant effect either on the intensity or the mobility of TFIIIC-specific shifts (data not shown). These results suggest that neither recombinant GSK-3β nor CKII have any influence on TFIIIC B-block binding activity, nor can they bind or phosphorylate the B-block binding components of TFIIIC as this would be predicted to cause a change in the mobility of the shifts. The CKII inhibitor quercetin was also found to have no effect on TFIIIC B-block binding activity (data not shown), providing further evidence of

the specificity of the inhibitory effect of quercetin on the interaction between TFIIIB and TFIIIC.

## 6.3 Discussion

### 6.3.1 Protein kinase CKII activates mammalian pol III transcription and specifically interacts with TFIIIB

A combined biochemical and genetic approach by Schultz and colleagues had earlier shown that the ubiquitous Ser/Thr kinase CKII activates basal pol III transcription in the yeast Saccharomyces cerevisiae by phosphorylating TFIIIB (163, 164, 216). Since CKII and TFIIIB are both highly conserved between yeast and mammals, this suggested that CKII might have a similar stimulatory effect on pol III transcription in mammals as it does in yeast. The data presented here showing that CKII is required for efficient mammalian pol III transcription was therefore not entirely unexpected. Thus, three unrelated CKII inhibitors, 2,3-DPG, quercetin and DRB, each potently repressed pol III transcription reconstituted in vitro with HeLa cell extract or partially purified fractions. Peptide containing a consensus phosphoacceptor site for CKII also significantly reduced the levels of pol III transcription. This inhibition was substantially relieved by the addition of recombinant CKII demonstrating the specificity of the inhibitory effect of CKII peptide and the activating influence of CKII upon mammalian pol III transcription. As in yeast, recombinant CKII was unable to completely restore pol III transcription to the levels observed in the absence of added peptide, however. Perhaps some of the endogenous CKII is peptide-inhibited but still remains stably associated with its pol III substrate, thus preventing recombinant CKII from functionally substituting for endogenous enzyme effectively.

The addition of recombinant CKII to HeLa cell extracts in the absence of peptide or other CKII inhibitor had no effect on the levels of pol III transcription in vitro. Similarly, the overexpression of CKII in vivo by transfection caused no change in the levels of pol III transcription in actively proliferating human osteosarcoma cells (257). However, in support of the repression of pol III transcription in vitro in response to inhibiting endogenous CKII activity, the depletion of endogenous CKII activity in vivo using antisense oligonucleotides inhibited pol III transcription (257). Together, these results suggested that CKII has an activating function in mammalian pol III transcription but is in relative excess in actively proliferating HeLa and osteosarcoma cells. Since CKII is activated in

response to serum and certain mitogens (76, 279, 493), it may be that in resting cells CKII is limiting for pol III transcription and exposure to exogenous CKII will stimulate pol III transcription.

The consistent cofractionation and coimmunoprecipitation of endogenous human CKII and TFIIIB suggests that they associate stably. Since many kinases interact with their substrates and yeast TFIIIB is phosphorylated by CKII, this suggested that CKII might phosphorylate mammalian TFIIIB. Indeed, very recent preliminary data indicates that purified recombinant human BRF and the recently cloned human B″ factor are both phosphorylated by CKII in vitro (257). Human BRF has also very recently been shown to be phosphorylated in vivo, moreover, the CKII inhibitors quercetin and DRB have been found to reduce the phosphorylation of BRF (470). In contrast to the observed phosphorylation of recombinant human BRF and B″, recombinant CKII specifically and efficiently phosphorylates the TBP subunit of yeast TFIIIB, but at present there is no evidence that yBRF or yB″ are phosphorylated by CKII (163, 164). Perhaps the CKII phosphorylation sites of hBRF and hB″ that can be phosphorylated by recombinant CKII in vitro are poorly conserved in the homologous yeast proteins. In yeast TBP there are four consensus CKII phosphorylation sites (S/TxxD/E). However, only one of these two sites, S183, is absolutely conserved in human TBP and molecular modelling of yeast TBP indicates that this serine is buried in the crystal structure of TBP and therefore highly unlikely to be accessible for phosphorylation by CKII (81). However, it remains possible that human TBP might be phosphorylated by CKII. The human factor contains another consensus CKII phosphorylation site that is absent from yeast TBP.

In support of BRF being a physiological target of CKII in mammals, the heat inactivation of TBP in a HeLa PC-B fraction had no effect on the levels of endogenous BRF that CKII was able to coimmunoprecipitate. This suggested the interaction between BRF and CKII may be direct or at least not mediated by TBP. Surprisingly, however, the heat-inactivated TBP, verified by its inability to support pol III transcription, was found to be able to still interact with BRF, so the possibility that TBP may be involved in mediating the interaction between BRF and CKII could not be excluded. GST-BRF has recently been successfully expressed and purified in our laboratory for the first time, providing the opportunity to test whether CKII and

BRF can interact directly or whether this association is dependent upon TBP or some other factor.

Having ascertained which components of the pol III transcriptional apparatus are phosphorylated by CKII, the sites of phosphorylation within these components can then be mapped by phosphopeptide analysis. Many protein substrates that are phosphorylated in vitro by a particular kinase are not physiological substrates for this kinase. Therefore, it will be essential to determine whether these mapped sites are phosphorylated by CKII in vivo. This can be achieved by in vivo metabolic labelling with [$^{32}$P]-orthophosphate carried out in the presence or absence of CKII peptide inhibitor or antisense oligonucleotide, followed by phosphopeptide mapping of immunoprecipitated, gel-purified pol III components. Having identified particular serine or threonine residues that CKII phosphorylates in a particular pol III component in vivo as well as in vitro, the functional significance of phosphorylation of these residues can then be investigated by site-directed mutagenesis. Thus, the effect on pol III transcription of substituting a Ser or Thr phosphorylated in vivo by CKII with an alanine residue can be tested by transiently transfecting cells with expression vector encoding the wild-type subunit or the non-phosphorylatable mutant and carrying out primer extension analysis of the RNA levels of a cotransfected $VA_I$ gene. Alternatively, the phosphorylatable residue can be substituted with an aspartate residue to see if this mimics the phosphorylated state and results in constitutive activation of pol III transcription.

### 6.3.2 CKII stimulates the interaction between TFIIIB and TFIIIC2

The activation of pol III transcription by CKII in yeast is thought to be achieved by the phosphorylation of TBP (163, 164). However, the molecular mechanism by which this may stimulate pol III transcription has yet to be elucidated. In contrast, in mammals it was found that the inhibition of CKII activity in cell extracts specifically reduced the levels of TFIIIC2 that coimmunoprecipitated with TFIIIB. This suggests that CKII activity may stimulate the interaction between TFIIIB and TFIIIC2, providing a potential mechanism by which CKII activates pol III transcription in mammals. However, the effect of inhibiting CKII activity on the levels of coimmunoprecipitating TFIIIC2 was quite small compared to the potent

inhibitory effect of similar doses of CKII inhibitor on pol III transcription in vitro, suggesting that there may be additional mechanisms by which CKII exerts its stimulatory effect on mammalian pol III transcription. Nonetheless, the interaction between TFIIIB and TFIIIC2 is essential for pol III transcription of most class III genes, so the effect of CKII activity on this interaction may be sufficient to account for its stimulatory effect, depending on whether this interaction is usually limiting for pol III transcription. This is very likely since RB, an established physiological repressor of pol III transcription, also targets this interaction (503). The targeting of this interaction by both RB and CKII indicates the importance of the binding of TFIIIB to TFIIIC2 for pol III transcription and suggests that the regulation of this interaction may be crucial for the physiological control of pol III transcription.

### 6.3.3 CKII – upregulation versus constitutive enhancement of pol III transcription

Although the data indicate a stimulatory role for CKII in pol III transcription in mammals, as previously shown for Saccharomyces cerevisiae, it is less clear to what extent CKII is able to regulate pol III transcription, either in yeast or in mammals. This is largely because of uncertainty as to the extent to which CKII itself is regulated (7, 419). In the past, there was controversy as to whether the expression and activity of CKII is regulated or whether CKII is a constitutively active kinase that is permanently expressed at constant levels from so-called housekeeping genes (7). Today, all researchers in the field agree that CKII is subject to some sort of regulation, although its control remains poorly understood (7). CKII does not seem to be regulated by any known second messenger and no major signal transduction pathway has convincingly been demonstrated to regulate CKII (7). Nonetheless, the protein levels and specific activity of CKII have been found to be elevated in rapidly proliferating cells and a range of tumours and in response to certain mitogens (76, 243, 279, 387, 493).

Several proteins have recently been identified that are able to modulate CKII activity and thus may represent upstream regulators of CKII. Thus, both p53 and $p21^{WAF1}$ can bind to the regulatory β subunit of CKII, inhibiting CKII activity (170,

171). Very recently, several stress stimuli have been shown to stimulate CKII activity; this could be blocked by the p38 MAP kinase inhibitor SB203580, implying that p38 MAP kinase may regulate CKII (457). Indeed, phosphorylation-activated p38α MAP kinase can directly bind the regulatory β subunit of CKII causing an increase in CKII kinase activity (457). Perhaps MAP kinase signalling is involved in the physiological regulation of CKII. CKII is also likely to be regulated at the level of gene expression. The promoter for the CKIIα gene contains many features characteristic of housekeeping genes, but it also contains binding sites for Ets1and NF-κB (294). These two transcription factors can both bind to the CKIIα promoter and are major determinants of promoter activity (294). The stimulation of pol III transcription by CKII may also be regulated by the modulation of its intracellular location. Thus, in dividing cells CKII is found mainly in the nucleus, whereas in quiescent cells it is located mainly in the cytosol (6, 511). CKII may also be regulated by the promiscuous interactions of the catalytic (α and α′) and regulatory subunits (β) with other proteins that prevents their binding to each other and the formation of the CKII holoenzyme (6). Thus, the protein kinases Mos, RafA and $p90^{Rsk}$ can each specifically bind to CKIIβ (6, 41, 86). However, this binding prevents CKIIβ from interacting with and thus activating CKIIα (6). Similarly, CKIIα can interact with the catalytic subunit of protein phosphatase 2A (PP2A) but cannot simultaneously bind CKIIβ (212). The binding of several kinases to CKIIβ suggests that CKII may be regulated by phosphorylation. This has not been shown to be the case for Mos, RafA or $p90^{Rsk}$, but the tyrosine kinase c-Abl has been reported to phosphorylate CKIIα in vitro and inhibit its activity (211). CKII may also be regulated by autophosphorylation (419). Clearly, there are a lot of potential mechanisms by which CKII may be regulated; however, the physiological relevance of these and their relative contributions to the overall regulation of CKII have yet to be fully elucidated. Nevertheless, the accumulating evidence is strongly supportive of CKII being regulated but suggest that its control may be complicated.

Another reason for uncertainty as to whether CKII can regulate pol III transcription is that CKII was found to be saturating for pol III transcription in actively proliferating human osteosarcoma cells and asynchronous HeLa extracts. Although there is evidence that CKII activity and levels of expression vary, for example between quiescent and actively proliferating cells, the variation is not

dramatic. It may be that the range within which CKII activity is altered under different physiological conditions is insufficient for it to become limiting for pol III transcription. Hence, although CKII seems to have a stimulatory role in pol III transcription, both in yeast and in mammals, it is possible that it exerts no regulatory influence. However, it should be noted that we have only overexpressed CKII in two tumour cell lines; osteosarcoma cells in vivo and in extracts from HeLa cells in vitro. Since CKII activity is known to be higher in many tumour cells, it is very possible that if we overexpressed CKII in untransformed cells pol III transcription would be stimulated.

Regardless, the demonstration that CKII has an activating influence on pol III transcription in mammals is highly significant since only a handful of kinases have so far been shown to modulate the levels of pol III transcription (568). This is surprising since pol III transcription is tightly regulated and protein phosphorylation is a major control mechanism of protein function. However, although it is well established that pol III transcription is strongly regulated, the molecular mechanisms involved largely have still to be fully elucidated. Pol III transcription is regulated in response to a variety of extracellular stimuli (568), which suggests several protein kinase signalling cascades may impinge on the pol III transcription apparatus. It is likely that protein phosphorylation has a fundamental role in the control of pol III transcription. In support of this, the pocket proteins RB, p107 and p130, which are strongly implicated in the regulation of pol III transcription in response to growth conditions and the cell cycle, are negatively regulated by phosphorylation by the cyclin-dependent kinases. In addition to the regulation of pol III transcription by the phosphorylation of upstream regulators, transcription is also regulated by the direct phosphorylation of the pol III transcriptional apparatus (163, 164, 169, 217, 572). Metabolic labelling with $^{32}$P-orthophosphate has previously shown that TFIIIC and pol III are phosphorylated in vivo (481, 568), providing further support for the contention that direct phosphorylation of pol III factors has an important role in the physiological regulation of pol III activity. Recently, the BRF subunit of hTFIIIB has also been shown to be phosphorylated in vivo (470). Significantly, the CKII inhibitors quercetin and DRB were found to reduce the in vivo phosphorylation of BRF (470), consistent with in vitro evidence suggesting that CKII can phosphorylate BRF and the contention that TFIIIB is the CKII-responsive target. However, clearly there are multiple kinases in addition to CKII that may be responsible for the in vivo

phosphorylation of TFIIIB. It also remains to be demonstrated that the phosphorylation of TFIIIB by CKII stimulates TFIIIB transcriptional activity.

### 6.3.4 CKII, pol III transcription and cancer

Recent data show that pol III products are overexpressed in a variety of human and rodent tumours (87, 88, 586), consistent with the negative regulation of pol III transcription by two key tumour suppressors, p53 and RB, that are frequently dysregulated in mammalian tumours (70, 579). Additionally, Ras, which is frequently mutationally activated in human tumours (344), has been shown to increase TFIIIB activity in Drosophila (548). Furthermore, several viral oncoproteins have been shown to activate pol III transcription and pol III transcription is elevated in a broad range of transformed cell types (569). The demonstration that CKII, a putative oncogene (600), can activate mammalian pol III transcription suggests a novel mechanism by which pol III transcription may be dysregulated in cancers.

### 6.3.5 GSK-3β may negatively regulate pol III transcription

The titration of increasing amounts of recombinant GSK-3β into pol III transcription reactions reconstituted in vitro caused a dose-dependent decrease in the levels of pol III transcript produced, suggesting that pol III transcription may be negatively regulated by GSK-3β. To investigate this further the effect of inhibiting endogenous GSK-3β on pol III transcription was tested. As a specific and potent inhibitor of GSK-3, low doses of lithium ions were used. In support of an inhibitory role for GSK-3β in pol III transcription, lithium ions were found to specifically stimulate pol III transcription both in vitro and in vivo. However, although GSK-3 seems to be the dominant physiological target of lithium ions, similar doses of lithium ions to those that were used have previously also been shown to potently inhibit the inositol phosphatases IMPase and IPPase (34). It is also plausible that there may be other unidentified targets of lithium ions that could be responsible for

the observed stimulatory effect of lithium ions on pol III transcription. Thus, the data demonstrate that low doses of lithium ions can specifically activate pol III transcription both in vitro and in vivo. The mechanism responsible clearly warrants investigation. The demonstration that the stimulation is due to the inhibition of GSK-3 would strongly indicate a physiological inhibitory role for GSK-3 in pol III transcription; as it is, the stimulatory effect of lithium ions can only be regarded as weakly suggestive of an in vivo role for GSK-3 in pol III transcription. On the other hand, the discovery that a GSK-3 independent mechanism is responsible for the stimulation may actually help identify a novel regulator of pol III transcription.

As a more direct investigation as to whether GSK-3 can influence pol III transcription in vivo, attempts were made to overexpress GSK-3β by transiently transfecting cells with an expression vector encoding GSK-3β. However, expression from the transfected gene was very poor and no effect on pol III transcription was observed. Therefore, at present the only in vivo evidence in support of a role for GSK-3 in modulating the levels of pol III transcription is circumstantial, being based on the stimulatory effect of lithium ions. As an alternative approach to investigating whether GSK-3β has any influence on pol III transcription in vivo, the effect of depleting endogenous GSK-3β using antisense oligonucleotides could be investigated with more time available. The levels of pol III transcription in GSK-3β$^{-/-}$ murine fibroblasts and corresponding wild-type cells could also be compared; GSK-3β-knockout cells have recently successfully been generated by Hoeflich et al. and used to demonstrate a novel function for GSK-3β in cell survival (219). Since the only genetic difference between GSK-3β$^{+/+}$ and GSK-3β$^{-/-}$ fibroblasts is the presence of the GSK-3β gene, the levels of pol III transcription in these cells should provide a definitive answer as to whether endogenous GSK-3β truly does have a role in the repression of pol III transcription. However, it is possible that the effect of the loss of GSK-3β may be functionally compensated for by GSK-3α, which may explain the lack of any notable effect of loss of GSK-3β on Wnt signalling, cyclin D1 levels or β-catenin accumulation observed by Hoefflich et al. (219). Thus, the generation of GSK-3α and GSK-3β double knockouts may be necessary to investigate the physiological role of GSK-3β in pol III transcription by this approach. The GSK-3β$^{-/-}$ fibroblasts would also enable it to be directly tested whether the stimulatory effect of low doses of lithium ions on pol III transcription in vivo is

dependent on GSK-3β. Both the knockout and antisense approaches reduce endogenous levels of GSK-3β and thus represent more physiological tests of the in vivo role of GSK-3β than overexpression by transient transfection, since the overexpression of proteins can sometimes induce artefactual interactions. One disadvantage of these approaches is that they provide no information as to whether the endogenous protein is usually saturating, as seems to be the case for CKII, at least in some cells. However, the inhibitory effect on pol III transcription in vitro of artifically raising the levels of GSK-3β in cell extracts using recombinant GSK-3β suggests that endogenous GSK-3β is not usually in excess, consistent with its inactivation by mitogenic signalling.

Although the evidence for a physiological role for GSK-3β is currently weak, it is expected that GSK-3β can inhibit pol III transcription in vivo, since it negatively regulates cyclin D1 protein levels (124, 435). However, the demonstration that recombinant GSK-3β inhibits pol III transcription reconstituted in vitro with fractionated factors suggests a more direct influence of GSK-3β upon pol III transcription. GSK-3β does not seem to stably associate either with TFIIIB or TFIIIC2; however, it has yet to be tested whether it can interact with pol III or TFIIIC1. Potential GSK-3 phosphoacceptor sites have been identified in numerous components of the pol III transcriptional apparatus including four of the five subunits of TFIIIC2, BRF and TBP. It is possible that GSK-3β transiently associates with and phosphorylates one or more of these components. Alternatively, perhaps GSK-3β phosphorylates an upstream regulator of pol III transcription, altering its ability to transrepress or transactivate pol III transcription. The inhibitory effect of recombinant GSK-3β on pol III transcription in vitro is supported by the stimulatory effect of inhibiting endogenous GSK-3β using low doses of lithium ions. Confirmation that reducing the levels of endogenous GSK-3β increases pol III transcription in vitro awaits the development of novel GSK-3β inhibitors or a competitor peptide that is substantially more specific for GSK-3 than the CREB phosphopeptide.

## Chapter 7.

# Summary and perspective

Mammalian pol III transcription is subject to considerable physiological regulation. The two basal factors TFIIIB and TFIIIC have both been shown to be limiting for pol III transcription at different stages of the cell cycle (571) and are thus obvious targets for exerting regulatory control upon the transcriptional process. Despite this and the essential roles of TFIIIB and TFIIIC in mammalian pol III transcription, the composition of both factors have yet to be fully elucidated. Nevertheless, several components of both factors have been cloned. This provided me with the opportunity to develop effective immunoaffinity steps for the separate purification of TFIIIB and TFIIIC. In our laboratory, we have tended to rely on TFIIIB and TFIIIC purified by conventional chromatography. However, affinity chromatography offers the potential to achieve a much higher level of purity in fewer steps (468). Unfortunately, this potential was not fully realised because of problems of loss of activity that were encountered when attempting to wash the immunoisolated complexes with salt concentrations above 100mM NaCl. Nonetheless, the extent of purification of active TFIIIB and TFIIIC was still significantly greater than that of TFIIIB and TFIIIC commonly used in regulatory studies. There are a number of possible causes for the loss of activity that accompanied more stringent washing of the immunoisolated complexes and these require further investigation. The most likely explanation is that the TFIIIB and TFIIIC complexes are disrupted. The TFIIIC1 and TFIIIC2 components of hTFIIIC are relatively easily dissociated (118, 607). Recent work from the laboratory of Hernandez suggests that mammalian TFIIIB resembles the yeast factor, with B″ only very loosely associated with TBP and BRF (463).

Using this immunoaffinity approach, a complex containing TFIIIB, TFIIIC and pol III was isolated, lending support to the possible existence of a pol III holoenzyme in vivo. This complex was used to show that recombinant RB is capable of disrupting the interaction between TFIIIB and TFIIIC2, providing a possible explanation as to how RB represses transcription of TFIIIC2-dependent class III genes. Using an immunoprecipitation approach, recombinant RB has also been

shown to reduce the association between pol III and TFIIIB, suggesting that multiple mechanisms may contribute to the repressive effects of RB (503). However, in both approaches RB was overexpressed. It is clearly important to determine whether RB can also impair these interactions when present at physiological concentrations. This could be tested by carrying out immunoprecipitations using extracts from $Rb^{-/-}$ and $Rb^{+/+}$ fibroblasts and comparing the level of TFIIIB that can be coimmunoprecipitated with TFIIIC or pol III in the presence or absence of endogenous RB.

The pocket proteins p107 and p130, which share ~30-35% identity with RB, also repress pol III transcription, both in vitro and in vivo (504). As previously shown for RB, I have found that a population of endogenous p107 and p130 molecules consistently cofractionate and coimmunoprecipitate with endogenous TFIIIB, demonstrating that all three pocket proteins can associate stably with TFIIIB at physiological concentrations. Since the three pocket proteins are structurally so similar and they target the same pol III factor, TFIIIB, this suggests that the mechanism(s) of repression of pol III transcription by p107 and p130 may be the same as for RB. However, it remains to be tested whether p107 and p130 are able to diminish the interaction of TFIIIB with TFIIIC2 or pol III, as has been demonstrated for RB. Alternatively, p107 and p130 may inhibit pol III transcription by different mechanisms to RB. All three pocket proteins have previously been found to associate with histone deacetylase activity and this appears to be actively involved in the repression by the pocket proteins of some E2F-responsive genes transcribed by pol II (53, 144, 346, 349). The histone deacetylase inhibitor trichostatin A (TSA) was found to have no effect on RB-mediated repression of pol III transcription. However, TSA has been shown to cause an increase in the levels of B2 RNA in vivo in a RB-independent manner (503). Perhaps p107- or p130- mediated repression of pol III activity involves histone deacetylase activity.

The stimulatory effect of TSA upon B2 RNA levels provides the first in vivo evidence that histone acetylation or deacetylation can affect the expression levels of pol III products. This is consistent with recent reports from the Roeder laboratory that human TFIIIC has histone acetyltransferase activity (230, 298). However, I have been unable to verify that this is actually the case. Although the B-block affinity-purified TFIIIC was enriched in HAT activity, immunoaffinity-purified TFIIIC showed no increase in HAT activity compared with mock immunoaffinity-

purified protein. Since the reported HAT activity of human TFIIIC is so weak, either of these results can be readily explained by the effect of small quantities of contaminants, in the one case producing a false positive and in the other case obscuring a real but weak activity. With more time available, to begin to resolve this, in-gel HAT assays could be performed to see if the HAT activity detected corresponds with polypeptides of approximately the same size as known subunits of human TFIIIC. Further purification of TFIIIC might also be required, because silver staining reveals that immuno- and DNA- affinity purified TFIIIC fractions contain a complex mixture of polypeptides.

The acetylation of histones at promoters is associated with the formation of a less compact chromatin structure and increased gene activity and has been shown to facilitate transcription factor access to chromatinised promoter sequences (321). Consistent with the reported HAT activity of hTFIIIC, increased concentrations of the factor have been shown to relieve chromatin-mediated repression of a tRNA gene assembled into chromatin in vitro (298). Yeast TFIIIC has also been shown to play a dominant role in relieving the repressive effects of chromatin, both in vitro and in vivo (67). However, preliminary results from the laboratory of Sentenac suggest that yeast TFIIIC lacks HAT activity (83). TFIIIC is poorly conserved between yeast and humans and none of the three subunits of human TFIIIC reported to possess HAT activity share any significant sequence homology with any of the yeast τ subunits.

For pol II-transcribed genes, chromatin remodelling is an important mechanism of regulating gene expression. In recent years, a variety of transcriptional coactivators have been found to possess HAT activity and for several of these proteins the intrinsic HAT activity has been shown to be essential for their ability to function as a coactivator (303, 359, 500). Conversely, a number of transcriptional corepressors have been found associated with histone deacetylases (5, 308). In addition, several ATP-dependent nucleosome remodelling factors have been shown to closely associate with particular pol II transcriptional coactivators, thereby targeting their activity to certain genes. Changes in chromatin structure have also been implicated in various aspects of the physiological regulation of pol III transcription, for example in the developmental regulation of 5S gene expression in Xenopus (568). However, it has yet to be studied in detail to what extent changes in chromatin structure are involved in regulating pol III transcription or how chromatin

remodelling is achieved. The stimulatory effect of TSA in vivo and the reported HAT activity of hTFIIIC suggest that histone acetylation and deacetylation may be important. Perhaps some of the histone acetyltransferases and histone deacetylases involved in pol II transcription can also influence expression of class III genes, akin to the repressive effects of the pocket proteins upon both pol II and pol III transcription. The recruitment of particular chromatin remodelling factors, such as p300 for example, to class III genes could easily be tested by carrying out chromatin immunoprecipitations with antibodies to these components.

One area of pol III research that has been largely unexplored is its regulation by phosphorylation. The regulation of pol III transcription in response to a variety of different environmental stimuli suggests that multiple kinase signalling pathways may target the pol III machinery. All five subunits of TFIIIC2 have been shown to be phosphorylated in vivo, as well as several pol III subunits (83, 481). This has also recently extended to include the BRF subunit of TFIIIB (470). However, so far only a few kinases have been shown to affect the levels of pol III transcription. The identification of kinases that can modulate pol III activity is made more difficult by the vast number of different kinases that there are in a cell.

In this particular study, I have investigated the possible involvement of two different kinases, CKII and GSK-3β, that I considered particularly strong candidates for a role in regulating pol III transcription. One of these, the ubiquitously expressed serine/threonine kinase CKII, had previously been shown to function in yeast pol III transcription (163, 164, 216). Since CKII has growth-promoting and oncogenic properties it was of particular interest to see whether it is also involved in mammalian pol III transcription. The competitive inhibition of endogenous CKII kinase activity in mammalian cell extracts and protein fractions potently inhibited pol III transcription, suggesting a stimulatory role for CKII as previously demonstrated in yeast. This was confirmed by the addition of recombinant CKII, which was able to restore pol III transcription to depleted extracts. The reduction of endogenous CKII levels in human fibroblasts using antisense oligonucleotides also caused a significant decrease in pol III transcription, demonstrating that CKII is required for efficient transcription in a physiological context (257).

The inhibitory effect of CKII inhibitors upon mammalian pol III transcription reconstituted with fractionated factors suggested that CKII might directly associate

with one of the components of the basal pol III transcriptional machinery. Further investigation revealed that endogenous CKII and TFIIIB stably interact, as shown by their consistent cofractionation and coimmunoprecipitation. Very recently, CKII has also been shown to phosphorylate recombinant human BRF and B″ in vitro (257). Furthermore, in vivo phosphorylation of BRF is reduced by chemical CKII inhibitors (470). A mechanism by which the phosphorylation of human TFIIIB by CKII stimulates pol III transcription has also been proposed. Coimmunoprecipitation experiments showed that the level of TFIIIC2 that can be coimmunoprecipitated with TFIIIB is specifically reduced in the presence of chemical CKII inhibitors. It is therefore proposed that the phosphorylation of TFIIIB by CKII promotes its association with TFIIIC2. Significantly, the tumour suppressor RB disrupts this very same interaction. Perhaps the phosphorylation of TFIIIB by CKII inhibits RB from binding TFIIIB or disrupting the interaction between TFIIIB and TFIIIC2. Alternatively, the opposing effects of CKII and RB on this crucial interaction may be independent of each other. This could easily be tested by seeing if CKII kinase activity promotes the interaction between TFIIIB and TFIIIC2 in a RB-negative background. As for RB, there may be additional mechanisms yet to be discovered that also contribute to the stimulatory effect of CKII on pol III transcription.

The regulation of CKII remains something of an enigma, but CKII activity is found to be specifically elevated in a variety of transformed and tumour cells and rapidly proliferating cell types, suggesting that it may contribute to the increase in pol III transcript levels frequently observed in response to transformation (141, 160, 387). In addition, CKII is a candidate for the cell cycle regulation of pol III transcription because CKII has been shown to be required for transition of particular stages of the cell cycle (413). CKII has yet to be shown to be limiting for pol III transcription except when its endogenous levels were artificially reduced. However, the lack of a stimulatory effect when overexpressed in HeLa extracts or human osteosarcoma cells is not so surprising since CKII is often abnormally elevated in tumour cells. Clearly, it is important to determine under what physiological conditions CKII can stimulate pol III transcription when overexpressed.

GSK-3β negatively regulates protein synthesis by phosphorylating and inhibiting the translation initiation factor eIF2B (423, 562). Since tRNA and 5S rRNA are also required for protein synthesis, this raised the possibility that GSK-3β

may also inhibit pol III transcription as part of its repressive effects upon the translational machinery. Recently, GSK-3β has also been shown to induce cyclin D1 proteolysis (124). Cyclin D1 levels vary periodically and are the principal determinant as to the timing of RB inactivation each cell cycle. This suggested that GSK-3β might repress pol III transcription indirectly through its effects on RB activity. Although some weak evidence was obtained to suggest that GSK-3β might inhibit pol III transcription, most of this relied on the use of low doses of lithium ions as a specific inhibitor of GSK-3 activity. Although lithium is routinely used to investigate GSK-3β function and is considered a highly specific inhibitor of GSK-3, the possibility remains that the stimulation of pol III transcription by lithium that was consistently observed both in vitro and in vivo was not due to the inhibition of GSK-3β (280). The molecular basis for the stimulatory effects of lithium ions upon pol III transcription requires further investigation to see if the inhibition of GSK-3 is responsible. In support of this and a role for GSK-3β in regulating mammalian pol III transcription, the addition of recombinant GSK-3β to in vitro transcription reactions caused a dose-dependent decrease in the levels of pol III transcript produced.

The bulk of a cell's dry mass is protein, which is why the rate of protein synthesis is such a critical determinant of the rate of cellular growth. tRNA and 5S rRNA, as essential components of the translational apparatus, can therefore serve to restrain cellular growth when their levels are limiting. This might explain why pol III products are overexpressed in many transformed and tumour cell types; this may be necessary in order to sustain elevated levels of growth. The increase in pol III transcript levels in response to transformation has recently been extended to include actual tumours (87, 88, 586). Two key repressors of pol III transcription RB and p53 are frequently inactivated in human tumours, suggesting two potential mechanisms by which pol III transcript levels may become abnormally elevated in cancers. Indeed, several naturally occurring mutations in RB found in tumours have been shown to prevent RB from repressing pol III transcription. Recent work from our laboratory has shown that pol III products are abnormally elevated in nine out of nine human ovarian tumour samples analysed, relative to normal healthy tissue from the same patients (586). Further analysis revealed that the specific DNA-binding activity of TFIIIC2 is specifically elevated in each of the tumour samples (586).

Using purified factors, I show that TFIIIC can be limiting in normal ovarian cell extracts, suggesting that the increase in TFIIIC2 activity in ovarian tumours is at least partially responsible for the overexpression of pol III transcripts in these tumours.

The most common genetic alterations detected in human ovarian cancers are mutations in *Ki-ras*, *p53* and *erbB2/neu* (159), suggesting that a change in the protein product of one of these genes may be responsible for the increase in TFIIIC2 activity in ovarian tumours. Roles for p53 (70, 90) and Ras (548) in pol III transcription have previously been reported. p53 specifically inhibits TFIIIB and Ras can cause an increase in the abundance of TBP. The *erbB2/neu* gene encodes a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTKs) and is activated in approximately one-third of human ovarian cancers (30, 159, 241, 399, 491). The possibility that erbB2/neu might influence pol III transcription and be responsible for the increase in TFIIIC2 activity was investigated using a rodent ovarian epithelial cell line transformed by an activated *neu* oncogene (ROSE 199-*neu*) (115). ROSE 199-*neu* cells can induce tumorigenesis in vivo and are considered a good model of certain aspects of human ovarian cancer (115). Comparison with untransformed control cells, ROSE 199 cells retrovirally transduced with the β-galactosidase gene, revealed that extracts from ROSE 199-*neu* cells support higher levels of pol III transcription. This provides the first evidence that erbB2/neu has a role in pol III transcription. Electrophoretic mobility shift assays were also performed and showed that extracts from the ROSE 199-*neu* cells display increased B-block binding activity. This strongly suggests that erbB2/neu may be at least partially responsible for the increase in TFIIIC2 activity observed in human ovarian tumours. However, the neu status of these tumours was not examined and it is possible that the increase in TFIIIC2 activity was achieved by a different mechanism.

Neu (erbB2) is the first cell surface receptor for which a role in pol III transcription has been demonstrated. The mitogenic stimulation of pol III transcription is well established, but the identities of the mitogens that are capable of activating this response have not been deduced. Serum has invariably been used to induce the mitogenic response and contains a complex mix of mitogens, which can vary depending on the serum batch. The ability of activated neu to induce an

increase in pol III transcription suggests that in a normal healthy cell, epidermal growth factor (EGF) and other ligands utilised by the EGFR family of RTKs can stimulate pol III transcription. Although a direct ligand for erbB2/neu has not been identified, it can dimerise with every other member of the EGFR family on their binding of ligand and can therefore be induced by a whole host of ligands (115, 399). Activated neu receptor has multiple downstream effectors and can influence a variety of intracellular signalling pathways (241, 323, 399). The mechanism by which the activation of neu tyrosine kinase activity at the cell surface results in an increase in TFIIIC2 B-block-binding activity has yet to be elucidated but likely involves a cascade of phosphorylation events that transmit the stimulatory signal from neu to TFIIIC2. Cell growth stimulation by EGF can be inhibited with CKII antisense oligonucleotides (414) suggesting that CKII might be a downstream effector of neu and might therefore be responsible for the increase in TFIIIC2 activity. Protein kinase B (PKB), which can phosphorylate and inactivate GSK-3β, is also implicated as a downstream target of erbB2/neu signalling (194). However, the preincubation of cell extracts with recombinant CKII or GSK-3β in the presence of a suitable phosphate donor had no effect on TFIIIC2 B-block binding activity when assayed in an electrophoretic mobility shift assay. Perhaps a protein that has not yet been shown to have any role in the modulation of pol III activity is responsible for the stimulation of TFIIIC2 activity induced by neu. The direct cause of the increase in TFIIIC2 activity also remains to be determined and might provide valuable clues as to the identity of the factor(s) directly responsible. For example, the human ovarian tumour samples were found to express elevated levels of mRNAs encoding the five subunits of TFIIIC2, suggesting that the rise in TFIIIC2 B-block binding activity was caused by an increase in the expression of TFIIIC2 (586). Assuming that the rise in mRNA levels was due to increased transcription and not because of a change in the stability of mRNAs, this in turn suggests that a change in a transcription factor or a chromatin-remodelling factor might have been responsible for the increase in TFIIIC2 activity. Alternatively, a change in the phosphorylation pattern of TFIIIC2 in response to activated neu would imply that a kinase or phosphatase directly targets TFIIIC2 and could be responsible for the change in activity.

Neu/erbB2 signalling may target multiple components of the basal pol III transcriptional apparatus. Cyclin D1 levels are also induced by neu signalling (323)

suggesting that TFIIIB activity may also be increased in ROSE 199-*neu* cells because of the inactivation of RB by cyclin D1-cdk4/6. Moreover, TFIIIB was found to be weakly limiting for transcription in extracts from untransformed control cells (ROSE 199-*βgal*) suggesting that an increase in TFIIIB activity in response to neu would also contribute to the increase in pol III transcription, albeit probably to a lesser extent than TFIIIC2.

The molecular basis for the physiological regulation of mammalian pol III transcription is slowly being elucidated. The list of proteins that are capable of modulating the levels of pol III transcription and are therefore potential regulators of the transcriptional process is steadily being extended. Here, I have identified a novel role in mammalian pol III transcription for two cellular proteins, CKII and neu, both of which have been shown for the first time to be capable of stimulating mammalian pol III activity. The mechanisms by which proteins exert their modulatory effects upon pol III transcription are also gradually being determined. I have shown that recombinant RB can disrupt the interaction between TFIIIB and TFIIIC2 whereas CKII kinase activity can promote this interaction. Activated neu was shown to stimulate TFIIIC2 activity, and p107 and p130 were both found to stably interact with TFIIIB, however, the mechanistic bases of the effects of these three proteins upon pol III transcriptional activity have not been further examined at present. For many of the proteins for which a role in modulating pol III transcriptional activity has been demonstrated, it remains to be determined under what physiological conditions they actually contribute to the regulation of pol III transcription. In some cases, this can be inferred from knowledge of the other functions of the protein and their own physiological regulation. However, it is clearly important to establish whether or not these inferences are indeed correct and to determine the relative contributions of proteins to the regulation of pol III transcription under particular physiological conditions.

The level of pol III transcription is tightly linked to the rate of cellular growth. It has been suggested that under certain physiological conditions the level of pol III transcription, by restricting the production of 5S rRNA and tRNA, can restrain cell growth and that this may constitute an important preventive barrier to tumorigenesis (313, 567). In support of this contention, the unrelated tumour suppressors RB and p53 both repress pol III transcription (70, 90, 93, 579). The

basal pol III transcriptional apparatus is also targeted for activation by several cellular and viral oncoproteins (569). Furthermore, pol III products are frequently overexpressed in many transformed and tumour cells. Together, these results suggest that elevated levels of pol III transcription may actually contribute towards tumour development. Direct evidence for this has recently been provided by two rarely studied pol III transcripts that are encoded by the genome of the Epstein-Barr virus (EBV). These two transcripts, EBER1 and EBER2, have been shown to induce tumorigenicity in EBV- negative Burkitt lymphoma cells (278). This provides the first direct evidence that a pol III transcript can be oncogenic. The functions of EBER1 and EBER2 have yet to be fully defined; however, they can activate interleukin (IL)-10 expression in Burkitt's lymphoma (278). The IL-10 induced acts as an autocrine growth factor allowing the cells to grow in low serum conditions (278). However, transfection of EBV-negative Burkitt lymphoma cells with IL-10 reveal that this is not sufficient to account for the oncogenicity of EBER1 and EBER2 (EBERs) (278). The EBERs may also be involved in the subversion of the host cell's translational apparatus, as has been reported for the adenovirus VA RNAs (35). Other pol III transcripts may also be capable of inducing tumorigenicity. There are quite a large number of pol III transcripts of unknown function. Among these are BC1 and BC200 RNA that are highly induced in a range of rodent and human tumours respectively and therefore particularly good candidates for being oncogenic (87, 88).

It remains to be determined whether tRNA and 5S rRNA are actually ever limiting for protein synthesis in mammalian cells and thus whether their overexpression can contribute to tumorigenesis by releasing a constraint imposed upon cell growth. However, a further indication that this might be the case is provided by pol I transcription. The output of pol I transcription is devoted solely to the production of large rRNA, which is required for protein synthesis. Significantly, like pol III, pol I transcription is repressed by RB and a range of transformed cells display an enlarged nucleolus suggesting that pol I activity is elevated in these cells (78). These observations suggest that large rRNA can be limiting for protein synthesis. Furthermore, there appears to be some coordination of transcription by pols I and III, with the activities of both polymerases increasing in parallel following serum stimulation and fluctuating together during passage through the cell cycle. Thus, tRNA and 5S rRNA may also become limiting when large rRNA does.

In this particular study, two cellular oncoproteins CKII and neu have been identified as novel modulatory, and potentially regulatory, proteins of mammalian pol III transcription. The frequent overexpression of pol III products in response to transformation and evidence that a pol III transcript can actually promote tumour development suggests that other oncoproteins and tumour suppressors may also target the pol III transcriptional machinery. Regardless, in light of the extensive physiological regulation of mammalian pol III transcription it is likely that additional proteins to those already identified also contribute to regulation of pol III activity.

The subunit compositions of mammalian TFIIIB and TFIIIC have still to be fully defined. A lack of knowledge as to the precise composition of components of the basal pol III machinery and a lack of molecular reagents against individual subunits of these components severely restricts the extent to which the regulation of pol III transcription can be dissected at the molecular level. This is further complicated by recent evidence suggesting that a different form of TFIIIB functions at TATA-less and TATA-containing promoters (365, 463, 515). The relationship between these different forms of TFIIIB, which appear extremely labile (463), and the possibility of interchange of common subunits between the different forms and coregulation is rather unclear at present. The majority of regulatory studies have examined only a single TFIIIB activity and in many cases it will be necessary to readdress whether the particular modulatory protein or stimulus studied affects one or both forms of TFIIIB, and if the latter is true, whether the mechanistic basis is the same. For this study, I focused entirely on the regulation of pol III transcription of class III genes with TATA-less promoters, since these constitute the majority of class III templates, including the essential 5S rRNA and tRNA genes, and more was already known about their regulation. TFIIIB and TFIIIC were both purified on the basis of their ability to support transcription of the adenovirus $VA_I$ gene or a tRNA gene, both of which lack TATA boxes in their promoters. In the case of TFIIIB, the factor was immunopurified using antibody 128 or 330, raised against residues 533-547 and 664-677 respectively of BRF1. BRF1 appears to be specific to the form of TFIIIB utilised by TATA-less promoters; moreover, the regions of BRF1 that 128 and 330 recognise are both absent from the homologous BRF2 and BRFU proteins reportedly involved in transcription from TATA-containing promoters (365, 463, 515). The immunopurified TFIIIB is therefore likely to be specific for transcription of TATA-less class III promoters. I also obtained some evidence in support of the

existence of a pol III holoenzyme as previously reported by the Roeder laboratory (551). The assembly of components into a functional complex prior to recruitment to the promoter has severe implications for the regulation of pol III transcription at a molecular level and this is a possibility that deserves further investigation. Clearly, a lot of unanswered questions remain concerning the regulation of the mammalian pol III transcriptional machinery and this regulation may be considerably more complex than was originally thought.

# Chapter 8.

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