A thesis entitled

Deregulation of RNA polymerase III transcription in response to Polyomavirus transformation

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Abstract

RNA polymerase (pol) III transcription is stimulated in response to a variety of factors. Numerous studies concerning the DNA tumour virus Simian Virus 40 (SV40) have served to identify mechanisms surrounding its ability to elevate pol III transcriptional activity. Polyomavirus, a close relative of SV40, has similarly been shown to induce abnormally elevated levels of pol III transcription; however, the mechanisms involved were not previously established. This study presents an analysis of the mechanisms employed by Polyomavirus, as well as providing further insight into those utilised by SV40. In untransformed fibroblasts, the basal pol III factor TFIIIB is repressed through association with the retinoblastoma protein RB; this restraint is overcome by the large T antigens of Polyomavirus and SV40. Furthermore, cells transformed by these papovaviruses overexpress the B" subunit of TFIIIB, at both the protein and mRNA levels. Despite the overexpression of B", the abundance of other TFIIIB components, TBP and BRF, is unperturbed following papovavirus transformation. In contrast, all five subunits of the basal factor TFIIIC2 are abnormally abundant in fibroblasts transformed by either Polyomavirus or SV40, as demonstrated by the elevated levels of their mRNAs. Thus, both papovaviruses stimulate pol III transcription by boosting production specifically of selected components of the basal machinery.

However, Polyomavirus differs from SV40 in adopting an additional and apparently unique deregulatory mechanism. This study presents the first evidence of a direct increase in pol III itself following viral transformation, as pol III activity and an accompanying elevation in the abundance of pol III subunits are observed following

transformation by Polyomavirus. Another important difference of Polyomavirus is its ability to encode a highly oncogenic middle T antigen that is localised outside the nucleus and activates several signal transduction pathways. Like the large T antigen, the middle T antigen can serve as a potent and specific activator of pol III in transfected cells. This may be mediated through the middle T-induced activation of the MAPK pathway, correlating with an increase in the expression of active ERK. Furthermore, an endogenous interaction between ERK and TFIIIB presents the possibility of a direct role for ERK in the stimulation of pol III transcription. Thus, a striking variety of distinct mechanisms contribute to the dramatically elevated levels of pol III transcription that accompany transformation by Polyomavirus and SV40.

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Abbreviations

Ab	Antibody
Ad	Adenovirus
Ag	Antigen
ATP	Adenosine triphosphate
bp	Base pairs
BRF	TFIIB-related factor
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyltransferase
CDK	Cyclin-dependent kinase
CKII	Casein kinase II
CTP	Cytosine triphosphate
DEPC-H_2O	Diethylpyrocarbonate water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DSE	Distal sequence element
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated protein kinase
GST	Glutathione-S-transferase
GTP	Guanine triphosphate
h	Human
HAT	Histone acetyltransferase
HBV	Hepatitis B virus
HDAC	Histone deacetylase
Hepes	*N*-2-hydroxyethylpiperazine-*N*'-2-ethane-sulphonic acid
HMG	High mobility group
HPV	Human papillomavirus
HTLV-1	Human T-cell leukaemia virus 1
ICR	Intragenic control region

kD	Kilodalton
μg	Micrograms
m	Milligrams
μl	Microlitres
ml	Millilitres
mM	Millimolar
M	Molar
MAPK	Mitogen activated protein kinase
MEK	MAP/ERK kinase
mRNA	Messenger RNA
ng	Nanograms
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PC	Phosphocellulose fraction
PIC	Preinitiation complex
PMSF	Phenylmethylsulfonyl fluoride

Pol	RNA Polymerase
PP2A	Protein phosphatase 2A
PSE	Proximal sequence element
PTF	Proximal sequence element binding transcription factor
rNTPs	Ribonucleotidetriphosphates
RB	Retinoblastoma protein
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SINEs	Short interspersed elements
snRNA	Small nuclear RNA
SV40	Simian virus 40
T	Tumour
TAF	TBP-associated factor
TBP	TATA-binding protein
TBS	Tris buffered saline

TEMED	N, N, N', N'-tetramethylethylenediamine
TF	Transcription factor
Tris	Tris (hydroxymethyl) methylamine
tRNA	Transfer RNA
ts	Temperature sensitive
Tween 20	Polyoxyethylene sorbitan monolaurate
U	Units
UV	Ultraviolet light
UTP	Uridine triphosphate
V	Volts
v/v	Volume/volume
w/v	Weight/volume
y	Yeast

Declaration

All work presented within this thesis was performed entirely by myself and in no way forms part of any other thesis. The work was carried out during my time as a post-graduate student at the Division of Biochemistry and Molecular Biology, Institute of Biological and Life Sciences, University of Glasgow, UK, from October 1998, while under the supervision of Professor Robert J. White.

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Publications

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For my Mum

Chapter 1

Introduction

1.1 Eukaryotic RNA polymerases

While a single RNA polymerase is responsible for the synthesis of all cellular RNA in prokaryotes and archaebacteria, eukaryotes utilise three distinct DNA-dependent RNA polymerases to transcribe nuclear genes (Chambon, 1975). Additionally, mitochondria and chloroplasts each possess their own unique RNA polymerase separable from those found in the nucleus (Chambon, 1975; Sentenac, 1985). The three nuclear eukaryotic RNA polymerases were originally identified through their differential elution on DEAE-Sephadex columns (Roeder and Rutter, 1969). In addition to their distinct chromatographic properties and template preferences, they also vary in salt requirements and display differential sensitivity to the toxin α-amanitin, a cyclin octapeptide produced by the poisonous *Amanita* mushrooms, which was a definitive aspect of their classification (Kedinger et al., 1970; Roeder and Rutter, 1969). In mammals, pol II is the most sensitive to α-amanitin (50% inhibition at 25ng/ml), with pol III displaying intermediate sensitivity (50% inhibition at 20μg/ml) and pol I being completely resistant (Schwartz et al., 1974). Consequently, this property has been gainfully exploited to determine the polymerase responsible for transcription of any given template.

Genes encoding all the subunits of pols I, II and III have been cloned from *Saccharomyces cerevisiae*. These are the best characterised nuclear RNA polymerases, although the situation is believed to be very similar in higher eukaryotes. The three polymerases comprise two large subunits and a series of smaller components. Pols I, II and III share five common subunits, ABC27, 23, 14.5, 10α and 10β, where A denotes a subunit found in pol I, B a subunit of pol II and C a subunit of pol III. An additional two subunits, AC19 and AC40 are found in both pols I and III (Mann et al., 1987). While these subunits are not found in pol II, the B12.5 and B44 pol II subunits are functionally equivalent, respectively (Martindale, 1990). Furthermore, the two largest polypeptides of each are homologous to the equivalent polypeptides of the others (Breant et al., 1983; Buhler et al., 1980) and to the β' and β subunits of prokaryotic RNA polymerases (Allison et al., 1985; Sweetser et al., 1987).

Despite some shared subunits, pols I, II and III differ in their structure and localisation. Consequently, the RNA polymerases do not display functional redundancy and each RNA polymerase is responsible for the transcription of a specific set of genes. The variation in the number of genes transcribed by each polymerase is considerable and, moreover, the size of each set of genes proves highly disproportionate to the contribution to total nuclear transcription. Thus, although pol I synthesises only a single transcript, 45S ribosomal RNA (rRNA), pol I transcription constitutes ~70% of total nuclear transcription in an actively growing cell. Each 45S rRNA molecule is, however, subsequently cleaved to produce 5.8S rRNA, 18S rRNA and 28S rRNA molecules. The essential nature of these RNA components of ribosomes and the requirement of sufficient ribosomes to support cellular protein

synthesis demands may account for the gene encoding the 45S rRNA precursor being highly reiterated in the eukaryotic genome.

In contrast, while pol II alone is responsible for the synthesis of messenger RNA (mRNA) encoding all proteins and also transcribes most small nuclear RNA (snRNA), only ~20% of total nuclear transcription is ascribed to pol II. The remaining 10% of nuclear transcription is carried out by pol III, which transcribes an intermediate sized set of genes encoding a variety of stable RNAs, including 5S rRNA, transfer RNA (tRNA) and U6 snRNA. In view of these distinct groups of templates transcribed by polymerases I, II and III, these sets are commonly termed class I, II and III genes, respectively. In addition to their distinct classes of genes, the polymerases are localised to distinct sites within the nucleus. Pol I transcription occurs at discrete sites called nucleoli, with rRNA being synthesised in the fibrillar centres and subsequently processed and assembled into ribosomes in the surrounding granular regions (Shaw and Jordan, 1995). Pol II also functions at its own spatially separate locations, of which there may be ~8000 per HeLa cell, while pol III transcription is localised to ~2000 sites within the nucleoplasm, with each site containing, on average, five molecules of active pol III (Pombo et al., 1999).

That transcription in eukaryotes is divided between the three nuclear RNA polymerases may reflect the greater complexity of most eukaryotic genomes and of precise regulatory control mechanisms for gene expression, as compared with bacteria.

1.2 Pol III transcripts

The class III genes transcribed by pol III encode small RNA molecules that serve vital functions in cellular metabolism. At approximately 120 nucleotides long, 5S rRNA is the smallest of the ribosomal RNAs and the only one transcribed by pol III (White, 1998a). It is found associated with the large subunit of ribosomes in all eukaryotic organisms and has a critical role in translation. The haploid human genome contains 200 to 300 5S genes, many of which occur in clusters of tandem repeats; however, some are dispersed as single copies (Sorensen and Frederiksen, 1991). Other class III genes that are similarly essential in translation are the tRNA genes. tRNAs range between 70 to 90 nucleotides in length and serve as adaptor molecules, translating the genetic information contained within mRNA into the specific order of amino acid residues of the protein it encodes. The three residue anticodon sequence of a given tRNA is specific for a particular amino acid. Consequently, base-pairing of the tRNA anticodon with the complementary codon of the mRNA ensures the accurate synthesis of the polypeptide chain encoded by the mRNA nucleotide sequence. Eukaryotic cells possess 50 to 100 distinct tRNA species (Sharp et al., 1984), although the proportions of different tRNAs vary significantly between cell types (Garel, 1976). The human genome contains in the region of 500 tRNA genes giving rise to 60 to 90 different tRNA species (Hatlen and Attardi, 1971). The considerable redundancy displayed among tRNA genes results in an average copy number of 10-20 genes for an amino acid tRNA adaptor.

U6 snRNA genes also fall into the class III gene family. U6 is the smallest of five snRNA species that comprise a ribonucleoprotein (RNP) complex termed a

spliceosome (Kunkel et al., 1986; Moenne et al., 1990). Spliceosomes function in post-transcriptional processing of pre-mRNA (Maniatis and Reed, 1987), removing introns to generate mature mRNA. The 106 nucleotide U6 transcript is the most highly conserved of the spliceosomal RNAs (Brow and Guthrie, 1988) and the only one not transcribed by pol II (Kunkel et al., 1986; Moenne et al., 1990).

Pol III is also responsible for the transcription of other components of ribonucleoprotein complexes, including 7SL, H1, MRP and 7SK. There are four 7SL genes in the human genome, encoding a highly conserved 300 nucleotide transcript (Ullu and Tschudi, 1984; Ullu and Weiner, 1984). 7SL RNA forms the scaffold of the signal recognition particle (SRP), which plays an essential role in intracellular localisation of proteins through its involvement in the co-translational insertion of nascent polypeptides into the endoplasmic reticulum (Walter and Blobel, 1982).

H1 is a 369 nucleotide RNA which forms part of RNase P, an endoribonclease involved in processing the 5'-termini of pre-tRNA (Bartkiewicz et al., 1989; Lee and Engelke, 1989; Morrissey and Tollervey, 1995) and which exhibits several blocks of sequence homology to MRP RNA (Gold et al., 1989). MRP is a 265 nucleotide RNA forming part of RNase MRP, another endoribonuclease, which serves an important role in the endonucleolytic processing of pre-rRNA (Morrissey and Tollervey, 1995; Schmitt and Clayton, 1993). That some essential transcripts for post-transcriptional processing are encoded by class III genes, suggests the influence of pol III transcription on protein synthesis may not be restricted to the confines of translational components.

The 330 nucleotide 7SK RNA associates with eight proteins to form a 12S RNP (Murphy et al., 1986) with an unknown role, that is likely important, as implied by its considerable evolutionary conservation (Ullu et al., 1982).

Similarly, the function of vaults, which are large cytoplasmic RNPs containing pol III-transcribed vault RNA (Rome et al., 1991), remains elusive, as does the role of the 69 to 112 nucleotide Y RNAs that associate with the Ro autoantigen (Wolin and Steitz, 1983). Likewise, the BC1 and BC200 transcripts of rodents and primates, respectively, that are restricted to a specific subset of neurons in the central and peripheral nervous systems (DeChiara and Brosius, 1987; Tiedge et al., 1991) have no known role.

The various gene families of repetitive short interspersed elements (SINEs) constitute quantitatively important classes of pol III template in higher organisms (Jelinek and Schmid, 1982; Singer, 1982), with SINE DNA accounting for a substantial proportion of mammalian genomes. The principal SINE in primates is the Alu family, of which there are in the region of one million copies in the haploid human genome (Britten, 1994; Jelinek et al., 1980; Rubin et al., 1980), totalling ~10% of the total genome. Alu genes consist of two imperfect repeats separated by an 18bp spacer (Deininger et al., 1981; Rubin et al., 1980) with a functional pol III promoter located in the upstream repeat (Paolella et al., 1983).

Of the various SINE families found in rodent species, B1 and B2 genes are the most abundant, with ~100 000 and 80 000 copies per haploid mouse genome, respectively (Bennett et al., 1984; Krayev et al., 1980; Rogers, 1985). B1 genes show approximately 80% homology with human Alu genes, while the B2 family is specific to rodents and alone constitutes ~0.7% of total mouse genomic DNA. SINEs are

frequently clustered and Alu or B1 genes are found immediately downstream of 7SK, H1 and MRP genes (Baer et al., 1990; Chang and Clayton, 1989; Murphy et al., 1986).

Retrotransposition, where pol III transcripts are reverse transcribed into DNA and subsequently integrated into new genomic sites, is thought to allow the dispersal and amplification of SINEs (Weiner et al., 1986). The high rate of transpostition of SINEs relative to other retroposons may be accounted for by the presence of internal promoters found in many class III genes. Consequently, promoters are included in the transcripts resulting in their duplication during retroposition. Thus, each gene copy has the potential to be transcribed and generate additional copies, removing the requirement of fortuitous insertion into DNA at an active promoter, which is a limiting event when external promoters are necessary (White, 1998a).

A clearly defined functional role has not been established for a SINE family (Howard and Sakamoto, 1990). The principal SINE families appear to be derived from class III genes of known physiological significance. tRNA genes seem the likely evolutionary source of SINE families such as B2 and ID (Daniels and Deininger, 1985), while the B1 and Alu families are believed to have evolved from the 7SL gene (Ullu and Tschudi, 1984). The possibility exists that SINEs represent large numbers of pseudogenes of no functional significance, however, during the course of evolution certain SINE transcripts may have acquired roles. Proposed functions for particular SINEs include roles in regulating expression of adjacent genes (Britten and Davidson, 1969), splicing (Krayev et al., 1982), translation (Chang et al., 1994), DNA replication (Anachkova et al., 1985; Anachkova et al., 1984; Ariga, 1984), cell stress response (Fornace and Mitchell, 1986; Liu et al., 1995) and regulation of growth

(Sakamoto et al., 1991) or the turnover of specific mRNAs (Clemens, 1987). However, an argument against roles in gene regulation or RNA processing is presented by the relatively recent multiplication of repetitive families. Thus, SINE functions would have to be subsidiary or highly responsive to evolution.

The insertion of SINEs into new genomic locations will have a significant impact on the structure and evolution of the genome, irrespective of possessing a functional role. Indeed, as a major source of genetic variability and fluidity of genomes through increased recombination events, SINEs have a huge capacity to disrupt sequences at the site of integration, conferring a mutagenic potential, which may account, at least in part, for their extremely low overall expression level.

1.3 Class III gene promoters

Within the class III gene family exists three further categories, types I, II and III, which relate to promoter structure. A significant and unusual property of the promoters of most class III genes, namely those of types I and II, is their requirement for sequence elements downstream of the transcription start site. They include discontinuous intragenic control regions (ICRs) that are composed of essential sequence blocks separated by non-essential nucleotides, a characteristic which sets them apart from pol I and pol II promoters and from the third pol III promoter type. In contrast, type III promoters, lacking intragenic elements, are similar to pol I and pol II promoters and rely on 5' flanking sequences to direct transcription (Murphy et al., 1987). The class III promoter types are presented schematically in figure 1.1.

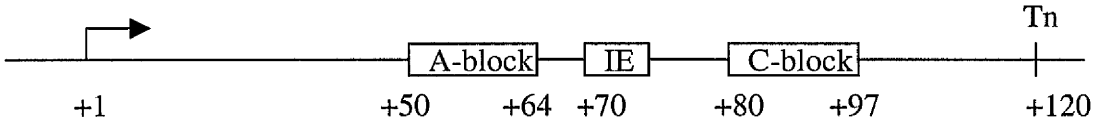
Figure 1.1

Promoter structure of class III genes

Schematic depiction of the three general types of promoter utilised by pol III. The sites of transcription initiation and termination are indicated by +1 and Tn, respectively. Also represented are the relative positions of the various promoter elements that characterise each promoter type. Included are the intermediate element (IE) of the type I promoter and the proximal and distal sequence elements (PSE and DSE, respectively) found in type III promoters.

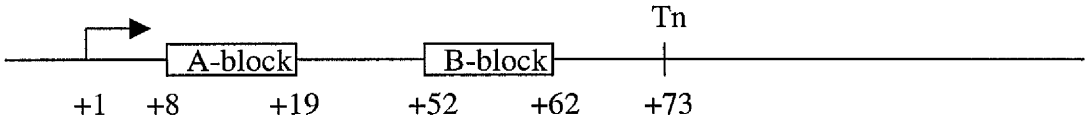
Type I promoter

e.g. *Xenopus* somatic 5S rRNA genes



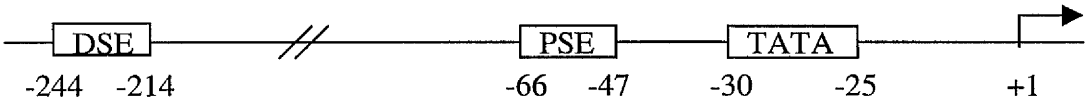
Type II promoter

e.g. *Saccharomyces* SUP4 tRNA gene



Type III promoter

e.g. Human U6 snRNA gene



Type I promoters

Type I promoters are unique to 5S rRNA genes. The *Xenopus laevis* somatic 5S rRNA gene serves as a classic example, possessing two functional domains located within the transcribed region, the 5' A-block (+50 to +64) and a second domain comprising an intermediate element (+67 to +72) and the 3' C-block (+80 to +97) (Pieler et al., 1987). The particular bases in between these sequence elements are not influential on transcription efficiency; however, they collectively act as spacers, with variation in the spacing of the essential elements being poorly tolerated (Pieler et al., 1987). Studies have demonstrated that the region between the A-block and transcriptional start site is an important determinant of 5S rRNA expression, particularly under conditions suboptimal for transcription (Fradkin et al., 1989; Keller et al., 1990). However, the three essential elements contained within the minimal ICR (+50 to +97) suffice for efficient transcription (Pieler et al., 1987). These ICRs are highly conserved between species and mutations in the A- and C-blocks abolish transcription (Keller et al., 1990). In contrast, the flanking sequences display limited conservation and, despite having strong modulatory effects, they are significantly more resilient to mutations (White, 1998a).

Type II promoters

The majority of class III genes, including the tRNA genes, the adenovirus VA genes and numerous middle repetitive gene families such as Alu, B1 and B2, utilise a type II promoter (White, 1998a). The ICRs of type II promoters consist of two essential and highly conserved sequence elements termed the A- and B-blocks. Each block is ~10bp and they are separated by 30-40bp (White, 1998a). The A-blocks of type I and II promoters are homologous and in certain cases are interchangeable, although they

differ in their location relative to the start site (Ciliberto et al., 1983b). The A-block of type II promoters is located at approximately +10 to +20, contrasting with its location in type I promoters, in which it is commonly found ~40bp further upstream (Galli et al., 1981). The location of the B-block is immensely variable, which is partly a manifestation of the presence of short introns within the coding region of some tRNA genes. Although the interblock separation for optimal transcription is ~30-60bp, distances of up to 365bp can be tolerated (Baker et al., 1987; Fabrizio et al., 1987). This flexibility is remarkable given that a single transcription factor, TFIIIC, binds simultaneously to both the A- and B-blocks (Schultz et al., 1989).

The A- and B-blocks have consensus sequences TGGCNNAGTGG and GGTTCGANN-CC, respectively, and constitute the essential elements of a type II promoter. Point mutations in the A- and B-blocks have been found to confer a substantial effect on transcription efficiency (Newman et al., 1983; Nichols et al., 1989; Traboni et al., 1984) and have lead to a general observation that mutations capable of reducing template activity also impinge on factor binding (Gaeta et al., 1990). However, additional internal or flanking sequences commonly confer modulatory effects. Indeed, although the site at which initiation can occur is dictated primarily in relation to the A-block (Baker et al., 1987; Ciliberto et al., 1983b), the precise start site within that region is determined by local sequence. Thus, pol III favours initiation at a purine preceded by a pyrimidine (Ciliberto et al., 1983a; Fruscoloni et al., 1995) and the upstream flanking region can also be influential. In most cases the 5' flanking sequences have an overall stimulatory influence upon transcription, although repressive effects can also occur (DeFranco et al., 1981; Dingermann et al., 1982; Hipskind and Clarkson, 1983). Despite their modulatory effects, flanking regions are generally poorly conserved. Indeed, the 5' flanking

regions of tRNA genes display little or no homology, even between different genes encoding the same tRNA isoacceptor (White, 1998a). However, this variation may provide a mechanism for differential regulation of tRNA genes in response to differing codon and amino acid demands in various cell types.

The tRNA genes classically define type II promoters and variations in type II promoters of different genes are minimal. The A- and B-blocks remain the essential components and, as with tRNA genes, the B-block is the major quantitative determinant of VA_1 promoter activity (Railey and Wu, 1988; Wu et al., 1987). Similarly, like tRNA genes, modulatory effects on VA_1 transcription are conferred by interblock and external sequences (Railey and Wu, 1988; Wang and Roeder, 1996). The influence of these additional effects could account, at least in part, for the VA_1 gene being more strongly transcribed than tRNA genes and, indeed, as a general feature influencing differential promoter strength of different class III genes with type II promoters.

Type III promoters

A small proportion of vertebrate pol III templates possess type III promoters. While retaining some A-block homologies, they have no equivalent B- or C-block domains. Indeed, they lack any requirement for intragenic promoter elements and type III promoter function pertains to the extragenic sequence elements located in the 5' flanking region of the gene (Gabrielsen and Sentenac, 1991; Willis, 1993). Thus, mouse U6 snRNA as well as human U6 snRNA, 7SK and MRP RNA genes, which all rely on type III promoters, retain full transcriptional activity in the absence of all sequences downstream of +1 (Das et al., 1988; Kunkel and Pederson, 1989; Murphy et al., 1987; Yuan and Reddy, 1991). Although this feature of type III promoters is an

obscurity for class III genes, it is standard promoter structure for class I and II genes, as well as bacteria.

The human U6 gene is a well characterised type III promoter. Efficient expression is dependent upon 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 (Bark et al., 1987; Carbon et al., 1987; Das et al., 1988; Kunkel and Pederson, 1988). These upstream regions show considerable homology to the promoters of the class II snRNA genes. Indeed, the PSEs of the human U2 and U6 promoters are identical at 13 out of 17 positions and are functionally interchangeable (Lobo and Hernandez, 1989). This feature is reflected by the DSEs, with DSEs from U2 and U6 genes being at least partially interchangeable in supporting expression (Bark et al., 1987; Kunkel and Pederson, 1988). However, while U6 DSEs show some positional flexibility, they do not display the extreme position- and orientation-independence which is commonly demonstrated by the enhancers of many class III genes (Das et al., 1988; Kunkel and Pederson, 1988).

The TATA box is a principal determinant of polymerase specificity for the U snRNA genes (Lobo et al., 1991; Mattaj et al., 1988). The TATA element is not a feature normally associated with class III genes. It is, however, a general component of promoters transcribed by pol II, although interestingly, the promoters of class II U snRNA genes notably lack a TATA box. Paradoxically, insertion of a TATA box into a U2 gene can convert it into a pol III template, while inactivation of the TATA element allows U6 to be transcribed by pol II (Lobo and Hernandez, 1989; Mattaj et al., 1988). Meticulous point mutational studies have demonstrated that the exact sequence requirements for a class III TATA box differ from those of a TATA box in a

class II gene (Simmen and Mattaj, 1990). Nonetheless, there is considerable overlap and class II TATA elements can substitute for the U6 TATA box in directing pol III transcription (Lobo et al., 1991). Despite this, polymerase specificity of the U1 gene cannot be converted by the U6 TATA box alone (Lescure et al., 1991) and a 4bp shift upstream of the PSE is also required, making the distance between the TATA box and PSE equivalent to that in the U6 promoter (Lescure et al., 1991). This observation reflects the rigid spacing requirement of the U6 promoter. Start site selection is influenced by both the TATA box and PSE and alteration in the separation of these elements severely compromises pol III transcription (Goomer and Kunkel, 1992).

In contrast to the upstream promoter elements of vertebrate U6 genes, the yeast U6 promoters resemble type II promoters of tRNA and VA genes (Willis, 1993), with transcription of the *S. cerevisiae* U6 gene being dependent on downstream A- and B-blocks (Brow and Guthrie, 1990). A TATA box is located upstream at –30 to –25, which influences start site selection and stimulates transcription *in vitro*, although has little effect upon *in vivo* expression (Chalker and Sandmeyer, 1993; Gerlach et al., 1995). A region around –55 displays partial homology to PSEs of vertebrate U6 promoters, but similarly confers little or no effect on expression levels (Burnol et al., 1993; Gerlach et al., 1995). The promoter arrangements of other type III genes closely resemble that of vertebrate U6 genes and the type I and II promoters are reasonably well conserved between yeast and vertebrates (White, 1998a).

Mixed promoters

Some class III genes possess promoters that do not fall into any of these promoter categories. Instead, they rely on a combination of internal and upstream sequences for efficient expression. For example, the EBER2 gene of Epstein-Barr virus has

intrgenic A- and B-blocks that are essential for transcription (Howe and Shu, 1989). While these elements are typical of type II promoters, the EBER2 promoter differs in its requirement for upstream sequences, which are thought to allow binding of Sp1 and activating transcription factor (ATF) (Howe and Shu, 1989). Additionally, a TATA box located between –28 and –23 can stimulate transcriptional activity 5-fold (Howe and Shu, 1989).

Similarly, the human 7SL gene depends on both internal and external promoter elements. It possesses A- and B-blocks, although they are fairly degenerate compared to those of type II promoters (Allison et al., 1983). Efficient expression requires upstream sequences which, like the EBER2 promoter, include an ATF binding site at –51 to –44 and a putative TATA box between –28 to –24 (Bredow et al., 1990; Howe and Shu, 1993). Thus, along with silkworm $tRNA^{Ala}$ genes (Sprague et al., 1980), the *Xenopus* $tRNA^{Sec}$ gene (Carbon and Krol, 1991) and the rat vault RNA gene (Vilalta et al., 1994), these genes utilise the A- and B-block elements, homologous to those of type II promoters, in combination with sequence elements such as ATF- and Sp1-binding sites from type III promoters, as the 5' flanking sequences of the human 7SL gene can be efficiently substituted by those of the 7SK gene (Kleinert et al., 1988).

1.4 RNA polymerase III

Although pol III is responsible for the transcription of a less extensive and diverse range of DNA templates than pol II, it is, nonetheless, the largest nuclear RNA polymerase, with a molecular weight of 600-700kD (Sentenac, 1985). Pol III has

been purified from a variety of organisms, including human (Jaehning et al., 1977; Wang and Roeder, 1997), mouse (Sklar and Roeder, 1976), frog (Engelke et al., 1983), silkworm (Sklar et al., 1976), fruitfly (Gundelfinger et al., 1980), wheat (Jendrisak, 1981) and yeast (Valenzuela et al., 1976). There is significant similarity in the polypeptide composition of pol III between the different species.

The two largest polypeptides of pol III are immunologically related to the corresponding subunits of pols I and II and, indeed, 11 of the 12 subunits of *Saccharomyces* pol II are related or identical to a subunit in pol I and/or III (Sadhale and Woychik, 1994). However, pol III also has many unique subunits and seven of these pol III-specific subunits associate with the evolutionarily conserved core of the yeast pol III enzymes, which is comprised of C160, C128, AC40 and AC19. It possesses seventeen subunits in all, ranging between 10 to 160kD in size (Chedin et al., 1998). The genes for sixteen of these have been cloned and proved to be essential for yeast cell viability (Chedin et al., 1998). In contrast, several pol II subunits are dispensable for growth (Woychik et al., 1990; Young, 1991). The C37 and C25 subunits of pol III have been found absent from some preparations that remain viable for accurate transcription (Werner et al., 1992). However, genetic disruption of C25 inhibits tRNA synthesis *in vivo* (Sadhale and Woychik, 1994), suggesting that it remains a necessary component of pol III. The catalytic region and binding sites for the DNA template and nascent RNA lie in the 'active site', which is thought to incorporate three blocks of homology that are conserved between the largest subunits in all RNA polymerases that have been analysed. Another homology domain of the largest pol III subunit in *S. cerevisiae*, C160, is thought to play an important role in maintaining structural integrity. The second largest subunit, C128, appears to be involved in the response of pol III to termination signals, as mutations introduced in

its conserved regions can affect transcription termination (Shaaban et al., 1995). AC40 and AC19, the remaining two subunits forming the enzymatic core of yeast pol III along with C160 and C128, show some sequence homology to the prokaryotic α subunit, which functions in polymerase assembly (Dequard et al., 1991). This role is reflected in pols I and III, where assembly *in vivo* is defective as a result of a temperature-sensitive mutation in AC40 (Mann et al., 1987).

Three of the yeast pol III-specific subunits, C82, C34 and C31, are believed to form a subcomplex (Valenzuela et al., 1976). C31 also interacts with C160 (Thuillier et al., 1995) and may, therefore, serve to tether the subcomplex to the polymerase core. C62, C39 and C32 are human homologues of the yeast subcomplex subunits and although human pol III depleted of this subcomplex remains active for transcriptional elongation and termination, the ability to support promoter-directed initiation is lost (Wang and Roeder, 1997). Consequently, this subcomplex appears to be involved in directing pol III to the preinitiation complex, a contention supported by the fact that yeast C34 and human C39 bind directly to the initiation factor TFIIIB (Wang and Roeder, 1997; Werner et al., 1993). Purified nuclear RNA polymerases have no sequence-specificity for DNA and interactions with transcription factors such as TFIIIB are therefore necessary to direct accurate transcription (Parker and Roeder, 1977)

1.5 Transcription factors utilised by RNA polymerase III

The process of transcription involves a complex array of *trans*-acting factors that work in conjunction with the *cis*-acting DNA elements. These factors serve to recruit pol III to the appropriate start sites of the class III gene set and direct initiation (Parker and Roeder, 1977); without them, transcription by pol III would initiate randomly (Cozzarelli et al., 1983; Weil et al., 1979). Although a variety of such factors, for example TFIIIA, confer an element of regulation on pol III transcription in a gene-specific manner, there are a host of general transcription factors which are common to pol III recruitment.

TATA-binding protein

The TATA-binding protein (TBP) epitomises the general transcription factor. It is utilised by all three nuclear RNA polymerases (Hernandez, 1993; Rigby, 1993; White and Jackson, 1992b). Moreover, it is required not only for genes possessing a TATA box, but unexpectedly for many genes devoid of a TATA box as well (White et al., 1992a; White et al., 1992b). This is a significant finding in view of the fact that the majority of pol III templates lack a TATA-like sequence.

The requirement of TBP for transcription of TATA-less class III genes was first suggested by White *et al.* They observed that transcription of the TATA-less pol III templates, 5S, tRNA, VA, Alu, B1 and B2, in addition to the TATA-containing U6 and EBER2 genes, is severely inhibited by competition with TATA box sequences from class II promoters; however, this could be restored by addition of pure recombinant TBP (White et al., 1992a; White et al., 1992b). Support for this finding

was provided by a variety of studies, including the observation that recombinant TBP restores transcription of TATA-less pol III templates where TBP has been depleted by fractionation or heat-treatment (Huet and Sentenac, 1993; White et al., 1992a; White et al., 1992b). Indeed, in the cases of all three nuclear RNA polymerases, extracts prepared from yeast expressing mutant TBP are unable to support transcription (Cormack and Struhl, 1992; Poon et al., 1993; Schultz et al., 1992). This deficiency can be relieved by addition of recombinant TBP, confirming the specific requirement for TBP for transcription by each polymerase (Poon et al., 1993; Schultz et al., 1992). Consequently, it constitutes a clear potential target for the co-ordinated regulation of transcription by pols I, II and III.

The fundamental role of TBP is highlighted by the fact that it is the most conserved eukaryotic transcription factor. All TBP genes encode a small (27-38kD) polypeptide. The N-terminal region is variable in both size and sequence and it has been suggested that it may modulate the activity of the extremely highly conserved C-terminal domain (Kuddus and Schmidt, 1993; Lescure et al., 1994; Mittal and Hernandez, 1997). The C-terminal domain of TBP possesses two 66-67 residue direct repeats, to which the TATA-box binding function has been ascribed (Heard et al., 1993; Reddy and Hahn, 1991; Strubin and Struhl, 1992). TATA binding is a slow, temperature-dependent step, which occurs through contacts in the minor groove (Lee et al., 1991; Starr and Hawley, 1991) and induces DNA bending (Horikoshi et al., 1992; Kuddus and Schmidt, 1993). X-ray crystallography of *Arabidopsis* TBP has revealed the nature of DNA binding by TBP, with an antiparallel β-sheet motif at the DNA interface and a region of α-helices forming a site for protein interaction (Nikolov et al., 1992). Tolerance of the resulting DNA distortion is an underlying

criterion for TBP binding and may constitute as important a role as base-specific recognition. This may account, in part, for the ability of TBP to bind sequences displaying poor TATA homology (Singer et al., 1990).

TBP interacts with other polypeptides, termed TBP-associated factors (TAFs), to form a number of different TBP-TAF complexes. This has provided the basis for a model which proposes that polymerase specificity is achieved through TBP interaction with sets of TAFs to give rise to distinct complexes. These, in conjunction with appropriate initiation factors, form class-specific complexes at promoters and facilitate recruitment of the correct RNA polymerase (Hernandez, 1993; Rigby, 1993; White and Jackson, 1992b). Thus, the TBP-containing complex SL1 serves to recruit pol I (Comai et al., 1992; Eberhard et al., 1993; Zomerdijk et al., 1994), while the equivalent complexes in the pol II and pol III systems are TFIID (Greenblatt, 1991; Pugh and Tjian, 1991) and TFIIIB (Huet and Sentenac, 1993; Taggart et al., 1992; White and Jackson, 1992a), respectively.

TFIIIB

Although TFIIIB itself is unable to bind directly to TATA-less class III genes (Klekamp and Weil, 1986), it is, nonetheless, capable of independent recruitment of polymerase and, thus, able to dictate the site at which transcription is initiated (Kassavetis et al., 1990). Considerable focus has been given to elucidating the composition of this central transcription factor. Using cation exchange chromatography Kassavetis *et al.* split a TFIIIB preparation into two components, termed B' and B" (Kassavetis et al., 1991), both of which proved necessary for tRNA transcription in the presence of purified TFIIIC and pol III. Work by Bartholomew *et al.* exploited the ability of TFIIIC to recruit TFIIIB into the vicinity of DNA, in order

to probe the polypeptide composition of TFIIIB by photocrosslinking. This allowed the detection of two polypeptides within the TFIIIB fraction which are recruited to the promoter by TFIIIC (Bartholomew et al., 1991), a 70kD polypeptide which was associated with the B' fraction of TFIIIB and a second polypeptide of 90kD found in the B" fraction (Kassavetis et al., 1991).

Three groups independently cloned the BRF1 (TDS4/ PCF4) gene encoding the 70kD yeast TFIIB-related factor (yBRF) protein product (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-de-Leon et al., 1992), so-named on account of the extensive sequence homology of the N-terminal half to the general pol II transcription factor TFIIB (Colbert and Hahn, 1992). Disruption of BRF1 resulted in a rapid decline in tRNA synthesis without compromising *in vivo* expression of either class I or class II genes (Buratowski and Zhou, 1992; Lopez-de-Leon et al., 1992). Addition of recombinant yBRF to BRF-deficient extracts was able to restore compromised pol III transcription; furthermore, in wild-type extracts, recombinant yBRF stimulates class III transcription, identifying it as a normally limiting factor (Colbert and Hahn, 1992; Lopez-de-Leon et al., 1992).

BRF1 encodes a protein of 596 amino acids with a predicted molecular mass of 67kD (Colbert and Hahn, 1992). The majority of the homology that exists between yBRF and TFIIB pertains to a putative zinc finger at the extreme N-terminus and two imperfect repeats of 76 amino acids within the remainder of the amino half (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-de-Leon et al., 1992). In contrast, the C-terminal half of yBRF displays no obvious homology to any other known protein and is significantly less well conserved. However, three discreet regions of strong conservation have been identified, yeast homology regions I, II and

III (HI, HII and HIII), and are likely to be essential for BRF function (Khoo et al., 1994).

Extensive mutagenesis has demonstrated that BRF contains two distinct TBP-binding domains, which interact with opposite faces of the TBP-DNA complex (Colbert et al., 1998; Kassavetis et al., 1998). The HII domain of the C-terminal half of BRF is thought to mediate one interaction with TBP (Andrau et al., 1999; Colbert et al., 1998; Khoo et al., 1994). The second TBP-binding site is ascribed to the conserved direct repeat region in the N-terminal half, which has also been shown to directly contact RNA polymerase III (Khoo et al., 1994) and interact with the largest subunit of yeast TFIIIC (Chaussivert et al., 1995). Remarkably, BRF can be divided into two separate halves that remain functional when mixed (Kassavetis et al., 1998).

Both yBRF and TBP can be detected in the B' fraction of TFIIIB by western analysis. Additionally, antibodies against either recombinant TBP or yBRF are able to specifically supershift a TFIIIB/tDNA complex in a gel retardation assay (Buratowski and Zhou, 1992; Kassavetis et al., 1992). Furthermore, Kassavetis *et al.* (Kassavetis et al., 1992) showed that recombinant TBP and yBRF are necessary and sufficient to reconstitute all known properties of the B' fraction, conferring strong evidence that TBP and yBRF interact to form part of TFIIIB.

The activity of the other fraction identified by Kassavetis *et al.* (Kassavetis et al., 1991), B", was found to co-migrate with the 90kD polypeptide in a SDS-polyacrylamide gel (Kassavetis et al., 1992). Cloning of the TFC5 gene encoding the 90kD subunit confirmed its responsibility for the B" activity (Kassavetis et al., 1995; Roberts et al., 1996). The B" gene encodes a 594 amino acid protein with a predicted mass of ~68kD but that migrates at ~90kD (Kassavetis et al., 1995; Roberts et al.,

1996). It shows no significant homology to other known proteins with the exception of a putative SANT domain (Aasland et al., 1996; Kassavetis et al., 1995; Roberts et al., 1996). B" proves to be remarkably resistant to truncation and a core domain of 176 amino acids (residues 224-400) will continue to support U6 transcription (Kumar et al., 1997). However, two distinct domains (residues 270-305 and 390-460) are both required for tRNA synthesis, either of which can function in the case of U6 (Kumar et al., 1997). Protein footprinting suggests that B" is folded such that these two domains are in close proximity when TFIIIB is assembled onto DNA (Kumar et al., 1997). B" makes numerous contacts within the transcription complex (Bartholomew et al., 1991; Joazeiro et al., 1994; Kassavetis et al., 1991; Roberts et al., 1996), possibly accounting for its resilience to deletion mutagenesis if the loss of any individual contact is compensated for by other interactions made by the protein. Protein-protein interactions of B" also appear to function co-operatively, as although recombinant B" binds weakly to TBP alone, binding affinity is substantially greater in the presence of yBRF and in the absence of TBP, no interaction is observed between B" and yBRF (Roberts et al., 1996).

Although TFIIIB, reconstituted from recombinant TBP, yBRF and B", is able to support both TFIIIC-dependent and TATA-dependent DNA binding and transcription (Roberts et al., 1996; Ruth et al., 1996), the reconstituted factor was found to be less active than native TFIIIB (Kassavetis et al., 1995; Ruth et al., 1996). This could possibly be due to the recombinant polypeptides being folded incorrectly or lacking important post-transcriptional modifications. The possibility also exists that a component(s) missing from the reconstituted TFIIIB could be non-essential but stimulatory or essential but present in residual amounts as a contaminant of the purified complementary fractions used to reconstitute transcription. Alternatively,

TFIIIE, which is present in both native B' and B" fractions (Dieci et al., 1993) has been shown to augment transcriptional ability of recombinant TFIIIB (Ruth et al., 1996). Consequently, it highlights the potential for a set of additional regulatory subunits that are distinct from TFIIIB and not requisites for basal transcription.

Characterisation of mammalian TFIIIB is less advanced than that of yeast. A variety of chromatographic procedures have been employed in its purification. Using anti-TBP antibodies, a TAF of 88-90kD could be specifically immunoprecipitated from fractions containing TFIIIB (Mital et al., 1996; Wang and Roeder, 1995). The cDNA encoding this polypeptide was isolated by peptide sequencing and established as encoding a 677 amino acid protein (Mital et al., 1996). On account of the N-terminal 280 residues being 24% identical to human TFIIB and 41% identical to yBRF from *S. cerevisiae*, this TAF was termed human TFIIB-related factor (hBRF) (Mital et al., 1996). It shares regions of extensive homology with other BRF species, including a zinc finger motif and two direct repeats (Mital et al., 1996). With the exception of yeast homology region II (HII), the C-terminal half of hBRF displays little homology to yBRFs (Mital et al., 1996). However, like its yeast homologues, hBRF possesses two TBP-binding sites, a weak site in its N-terminal half and a stronger site which may be ascribed to the conserved HII domain in its C-terminal half; transcriptional activity is abolished by deletion of either half (Wang and Roeder, 1995). Transcription of VA and tRNA genes was found to be severely inhibited following immunodepletion of hBRF and was restored by addition of recombinant TBP and hBRF, proving its essential role in human pol III transcription (Mital et al., 1996; Wang and Roeder, 1995).

A significant advance in determining the composition of hTFIIIB was made by the cloning of a human homologue of yeast B" (Schramm et al., 2000). Human B" (hB") is required for transcription of both TATA-less tRNA-type promoters and TATA-containing snRNA-type pol III promoters (Schramm et al., 2000). With a calculated molecular mass of 156kD, it is significantly larger than its yeast counterpart. It possesses three principal regions of sequence homology: a putative SANT domain (residues 415-472), which shows 43% identity to 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, which displays 17% identity to yB" (Schramm et al., 2000). In addition, a striking feature of hB" is a region of 19 repeats of 26-28 amino acids in the C-terminal domain, which is absent from yB". The functional significance of hB" was demonstrated by the severe inhibition of class III gene expression following its immunodepletion from cell extracts, which could be restored by addition of recombinant hB". This was shown to be unique to pol III transcription, as hB" immunodepletion conferred no effect on pol II transcription of either the adenovirus major late promoter or the human U1 snRNA promoter (Schramm et al., 2000).

Given that essential homologues of all three core yeast TFIIIB subunits, TBP, BRF and B", are found in human TFIIIB, TFIIIB appears to be strongly conserved between these species. However, varying forms of TFIIIB appear to function at different class III promoters in humans, setting it apart from the yeast system in which a single form of TFIIIB is necessary and sufficient for transcription of all class III genes. Thus, the TFIIIB requirement of type III promoters varies from that of types I and II, with TFIIIB fractions capable of supporting VA_1 expression being inactive for U6 or 7SK (Lobo et al., 1992; Mital et al., 1996; Teichmann and Seifart, 1995). Teichmann and

Seifart (Teichmann and Seifart, 1995) separated two forms of hTFIIIB using chromatography on EMD-DEAE-Fractogel (EDF). One form, hTFIIIB-α, was free of hBRF and active for U6 but not VA_1 expression. However, the second form, hTFIIIB-β, co-eluted with hBRF and efficiently supported VA_1 but not U6 transcription (Teichmann et al., 1997; Teichmann and Seifart, 1995). Indeed, despite being essential for transcription of type I and II genes, the requirement of hBRF for transcription of type III genes has been controversial. Wang and Roeder observed inhibition of U6 and 7SK expression following immunodepletion using anti-BRF antibodies (Wang and Roeder, 1995). Conversely, Mital *et al.* demonstrated that immunodepletion of hBRF inhibited VA_1 but not U6 transcription (Mital et al., 1996). The recent cloning of a novel gene that encodes a protein termed BRFU (Schramm et al., 2000) may account for these observations. BRFU is highly related to hBRF and hTFIIB but is required for transcription of human U6 and 7SK genes, although not VA_1 (Schramm et al., 2000). Consequently, it is plausible that anti-BRF antibodies utilised by Wang and Roeder recognised both hBRF and BRFU, while those of Mital *et al.* depleted extracts only of hBRF. Furthermore, other splice variants of hBRF have also been cloned, all of which are able to complex with TBP. They are, however, differentially required for transcription at structurally distinct promoter types. hBRF1 is the most active variant in transcription of type I and II promoters, while hBRF2 serves in U6 gene expression (McCulloch et al., 2000). Thus, it may be the case that both hBRFU and hBRF2 are components of TFIIIB required for U6 transcription.

As a principal factor in the transcription of pol III templates, TFIIIB constitutes a major determinant of biosynthetic capacity that is frequently targeted for regulation of

pol III transcription levels. Some of the influencing factors of TFIIIB are summarised in figure 1.2.

TFIIIC

Another of the general transcription factors associated with pol III transcription is TFIIIC. TFIIIC is responsible for recruiting TFIIIB, which has no sequence-specific affinity for DNA, to genes possessing type I or II promoters. As with TFIIIB, it was first purified from yeast, where it is also referred to as τ factor. Consequently, the polypeptides of approximately 138, 131, 95, 91, 60 and 55kD, that co-purify with TFIIIC transcriptional and DNA-binding activity are often referred to as τ138, τ131, τ95, τ91, τ60 and τ55, respectively (Bartholomew et al., 1990; Braun et al., 1992; Swanson et al., 1991). Direct visualisation studies using scanning transmission electron microscopy (STEM) revealed that TFIIIC/tDNA complexes have a dumbbell-shaped appearance, with individual protein domains, termed τA and τB, bound separately to the A- and B-blocks, respectively (Schultz et al., 1989). Although the τB domain constitutes the principal quantitative determinant of binding, interaction of the τA domain with the A-block is also required for transcription (Marzouki et al., 1986). The remarkable ability of TFIIIC to bind to A- and B-blocks simultaneously while separated by highly variable distances is due to the flexible linker connecting the two DNA-binding domains (Marzouki et al., 1986). Figure 1.3 displays a schematic illustration of the relative positions of the various subunits of *S. cerevisiae* TFIIIC when bound to a tRNA promoter.

In contrast to the situation for TFIIIB, human and yeast TFIIIC display significant differences. Human TFIIIC can be resolved by ion exchange chromatography on

Figure 1.2

Antagonistic influences targeting TFIIIB

The growth suppressors RB, p107, p130, p53 and Dr1 can all repress TFIIIB by direct interactions. In contrast, a variety of oncogenic influences have been shown to stimulate TFIIIB activity; in some of these cases the effect is achieved without a direct interaction. Activators of TFIIIB include the E1A protein of adenovirus, SV40 large T antigen, the Tax protein of HTLV-1, the X protein of HBV, activated Ras and the tumour-promoting phorbol ester TPA.

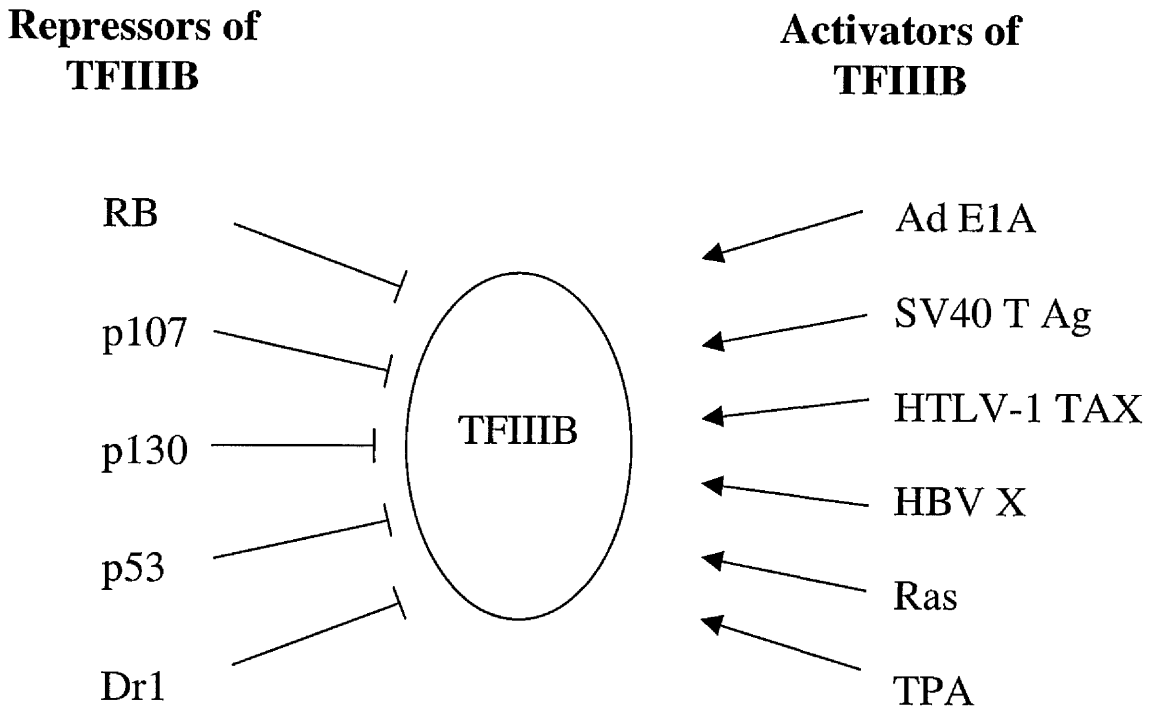
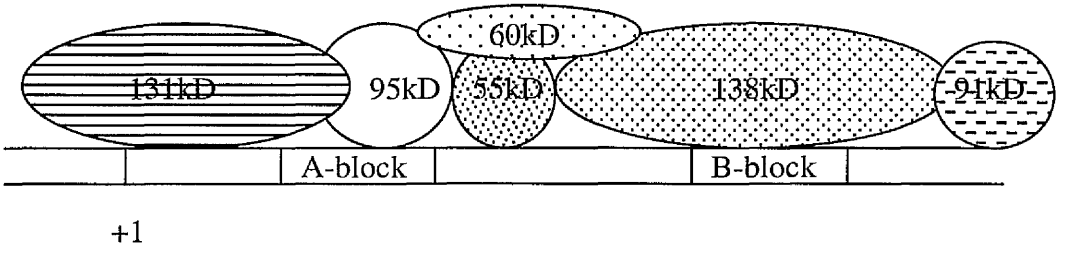

Repressors of TFIIIB
Activators of TFIIIB
RB
p107
p130
p53
Dr1
TFIIIB
Ad E1A
SV40 T Ag
HTLV-1 TAX
HBV X
Ras
TPA

Figure 1.3

TFIIIC interactions with a tRNA promoter

Diagrammatic illustration of the interactions made between the various subunits of *S. cerevisiae* TFIIIC and the promoter elements of a tRNA gene. The molecular mass of each subunit is indicated (kD), as is the site of transcription initiation (+1).


60kD
131kD
95kD
55kD
138kD
91kD
A-block
B-block
+1

Mono Q into two components termed TFIIIC1 and TFIIIC2 (Dean and Berk, 1987; Yoshinaga et al., 1987). Both components are essential for transcription of 5S, VA_1 and tRNA genes (Dean and Berk, 1987; Lagna et al., 1994; Oettel et al., 1997; Yoshinaga et al., 1987); however, TFIIIC2 is dispensable for U6 and 7SK transcription (Lagna et al., 1994; Oettel et al., 1997). Sedimentation analysis suggests a mass of up to 200kD for TFIIIC1, assuming it to be globular, although its subunit compostion has yet to be defined (Yoshinaga et al., 1987). The initial recognition of type I and II promoters is achieved by TFIIIC2, which binds specifically with high affinity to the B-block (Boulanger et al., 1987). It, in turn, serves to recruit TFIIIB and TFIIIC1, which enhances and extends the protein footprint produced by TFIIIC2 to include the A-block (Dean and Berk, 1988; Wang and Roeder, 1996; Yoshinaga et al., 1987). Human TFIIIC2 has been highly purified and consists of five polypeptides of 220, 110, 102, 90 and 63kD, giving a cumulative mass approaching 600kD (Sinn et al., 1995; Wang and Roeder, 1996; Yoshinaga et al., 1989).

Hoeffler *et al.* resolved two DNA-binding forms of HeLa cell TFIIIC2 on non-denaturing gels. The two forms, termed TFIIIC2a and TFIIIC2b, produce identical footprints on VA_1 and have comparable DNA-binding affinities (Hoeffler et al., 1988; Sinn et al., 1995). However, transcriptional activity is associated only with TFIIIC2a (Hoeffler et al., 1988).

All five subunits of TFIIIC2 have been cloned; hTFIIIC220, hTFIIIC110 and hTFIIIC90 show no significant homology to any of the subunits of yeast TFIIIC. The hTFIIIC63 and hTFIIIC102 subunits display weak homology of 22% to τ95 and 31% to τ131, respectively.

Additional general transcription factors

Although apparently not an essential factor in basal transcription, TFIIIE is another general transcription factor that has been identified in yeast (Dieci et al., 1993). TFIIIE is required for the efficient transcription of 5S and tRNA genes using partially purified factors (Dieci et al., 1993). However, as yet, a defined role of TFIIIE in transcription has not been established; proposals include roles in TFIIIB recruitment, stabilisation of the transcription complex and conformational rearrangements in TFIIIB (Ruth et al., 1996).

Ottonello *et al.* (Ottonello et al., 1987) resolved an activity, termed TFIIID, which is required for transcription of tRNA and 5S genes in silkworm. This remains the only system in which its activity has been identified, although it has been suggested that silkworm TFIIIC and TFIIID represent a subdivision of the components associated with TFIIIC in other organisms (Sprague, 1992; Young et al., 1991). Thus, it may be that silkworm TFIIIC and TFIIID bear some equivalence to human TFIIIC1 and TFIIIC2.

TFIIIA

In contrast to the general transcription factors, TFIIIA is a gene-specific factor, required only for the expression of 5S genes (Engelke et al., 1980). It has a predicted sequence of 344 amino acids and is predominantly composed of 9 tandem 27 amino acid repeats, each characterised by pairs of cysteine and histidine residues at precisely repeated positions (Ginsberg et al., 1984; Miller et al., 1985). Each repeat forms an independent domain enclosing a central zinc ion within a loop-like structure that directly contacts DNA, termed a "zinc finger" (Miller et al., 1985). These are

essential for the DNA-binding activity of TFIIIA (Hanas et al., 1983) and an α-helix contained within each finger domain makes contacts in the major groove of DNA (Pavletich and Pabo, 1991). The DNA-binding domain is divided into three distinct regions with clusters of zinc fingers binding to separate regions of the 5S gene ICR (Clemens et al., 1992; Vrana et al., 1988). Fingers 1-3 and 7-9 form two compact regions, binding the C- and A-blocks, respectively (Christensen et al., 1991; Clemens et al., 1992; Liao et al., 1992). The third region, composed of fingers 4-6, is extended with finger 5 binding to the intermediate element and fingers 4 and 6 acting as spacer elements spanning these promoter regions (Christensen et al., 1991; Clemens et al., 1992; Liao et al., 1992). Mutation of finger 3 has a strong detrimental effect on binding and disruption of finger 9 confers a severe loss of transcription (Del Rio and Setzer, 1993). Indeed, data imply that while fingers 1-6 are required purely for binding to the ICR, fingers 7-9 perform an additional structural or catalytic role.

TFIIIA, although essentially a transcription factor serving as an adaptor molecule between TFIIIC and the 5S gene promoter, also functions in both the storage and transport of 5S rRNA (Tafuri and Wolffe, 1993). Through the export of 5S RNA from the nucleus, a negative feedback loop is generated. This arises from the transport of TFIIIA to the cytoplasm in the form of a RNP complex, thus depleting the level of TFIIIA available in the nucleus to function in transcription of 5S genes (Guddat et al., 1990).

SNAPc/PTF

SNAPc/PTF is essential for transcription of 7SK and vertebrate U6 genes (Yoon et al., 1995). It binds to the PSE of type III promoters and can greatly enhance the recruitment of TFIIIB to the TATA box (Yoon et al., 1995). SNAPc/PTF comprises

five subunits of 190, 50, 45, 43 and 19kD (Henry et al., 1995; Sadowski et al., 1993), with a native mass of ~200kD (Sadowski et al., 1993). Both the largest subunit, SNAP190 (PTFα), and the second largest, SNAP50 (PTFβ), can be crosslinked to PSE DNA and are responsible for contacting DNA (Henry et al., 1996; Yoon et al., 1995). The other subunits do not bind independently to DNA (Yoon and Roeder, 1996) but are present in the complex binding to the PSE (Yoon et al., 1995). Immunodepletion of extracts of SNAP50 (PTFβ), SNAP43 (PTFγ) or SNAP45 (PTFδ) specifically repressed transcription from the 7SK and U6 promoters while conferring no effect on the VA_1 and AdML promoters (Henry et al., 1996; Sadowski et al., 1996; Yoon and Roeder, 1996). However, transcription of the pol II U1 and U2 genes was also inhibited by this immunodepletion and could be restored by addition of highly purified SNAPc/PTF, demonstrating that this PSE-binding protein is required specifically by both pol II- and pol III-transcribed U snRNA genes.

SNAPc/PTF interacts directly with TBP (Sadowski et al., 1993; Yoon and Roeder, 1996) and, like TBP, hBRF can be co-immunoprecipitated with SNAPc/PTF (Bai et al., 1996). Indeed, antisera against SNAPc/PTF immunoprecipitate TBP and hBRF in equimolar amounts, suggesting that SNAPc/PTF interacts with a form of TFIIIB, as opposed to free TBP (Bai et al., 1996).

1.6 Preinitiation complex formation on class III genes

The formation of transcription complexes involves the assembly of factors, which bind to DNA at the relevant gene promoter in an ordered stepwise manner

(summarised in figure 1.4). In the case of type I and II promoters, it has been established that TFIIIB is only able to bind to the promoter after the binding of TFIIIC (Bieker et al., 1985; Lassar et al., 1983). The 5S gene, possessing a type I promoter, requires an additional initial step, whereby TFIIIA binds to the promoter prior to TFIIIC (Bieker et al., 1985; Lassar et al., 1983). Subsequent studies provided further insight by revealing that TFIIIC2 is the first factor to bind VA_1 or tRNA genes through its B-block-binding activity. TFIIIC1 and TFIIIB then interact, in either order, to form a preinitiation complex (Dean and Berk, 1988), a situation which is also likely to be true for 5S genes. Consequently, the sequence-specific DNA-binding function of TBP is not required for transcription of the TATA-less class III genes (Martinez et al., 1995; Schultz et al., 1992). However, once recruited as a component of TFIIIB, TBP is positioned on the DNA such that it can discriminate between different upstream sequences (Joazeiro et al., 1996), possibly providing an explanation for the general preference for A/T-richness in the 5'-flanking regions of class III genes. Pol III recruitment is the final step in initiation complex formation and is mediated through protein-protein interactions with TFIIIB (Bieker et al., 1985; Setzer and Brown, 1985).

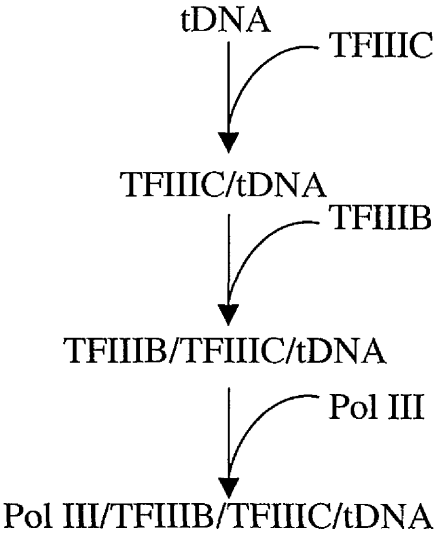
The array of interactions involved in preinitiation complex assembly is most fully characterised in yeast, where positions of the various TFIIIC subunits relative to a tRNA promoter have been established by photocrosslinking (Bartholomew et al., 1990). The τ138 subunit of TFIIIC is associated with the B-block, the τ95 and τ55 subunits with the A-block region (Bartholomew et al., 1990), while the τ91 subunit is located at the downstream end of class III genes (Braun et al., 1992). τ131 is the only subunit of TFIIIC that is located, in part, upstream of the start site (Bartholomew et

Figure 1.4

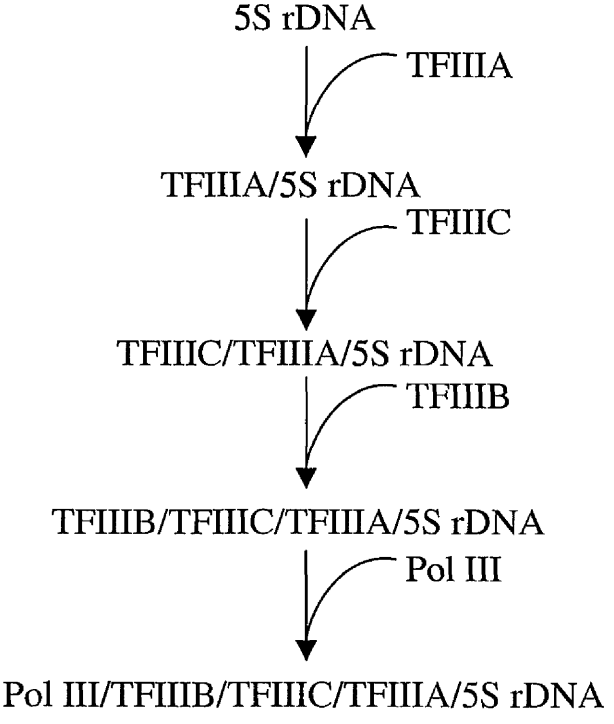
The order of promoter assembly on class III genes

Flow charts depicting the order of interaction of transcription factors and pol III with either a typical type II promoter such as that of a tRNA gene (panel A) or with the class I promoter of a 5S gene (panel B).

(A)


tDNA
TFIIIC
TFIIIC/tDNA
TFIIIB
TFIIIB/TFIIIC/tDNA
Pol III
Pol III/TFIIIB/TFIIIC/tDNA

(B)


5S rDNA
TFIIIA
TFIIIA/5S rDNA
TFIIIC
TFIIIC/TFIIIA/5S rDNA
TFIIIB
TFIIIB/TFIIIC/TFIIIA/5S rDNA
Pol III
Pol III/TFIIIB/TFIIIC/TFIIIA/5S rDNA

al., 1990; Bartholomew et al., 1991). It contains 11 tetratricopeptide repeats (TPRs), which function in protein-protein interactions, making it a likely candidate for interacting and positioning TFIIIB (Bartholomew et al., 1991). Indeed, TFIIIB extends the upstream footprint produced by τ131 (Braun et al., 1989; Kassavetis et al., 1989). The initial contact is made with the B' component of TFIIIB, with B" requiring B' to be present in order to be recruited (Kassavetis et al., 1991). yBRF has been shown to bind directly to τ131 via an interaction with its N-terminal half (Chaussivert et al., 1995; Khoo et al., 1994) and its binding to the TFIIIC/tDNA complex is stabilised by the addition of TBP before B" is recruited (Kassavetis et al., 1992).

The sequential assembly of TFIIIB components on DNA results in changes in photocrosslinking efficiency of τ131 with these subunits, suggesting a series of conformational changes (Kassavetis et al., 1992). These possibly account for the ability of TFIIIB, which alone has no affinity for DNA, to bind tightly to DNA once fully assembled on the DNA. Thus, interaction with τ131 may unmask the high-affinity TBP-binding site located in the C-terminal half of BRF (Khoo et al., 1994) and the subsequent binding of TBP may also reveal a cryptic DNA-binding site in yBRF (Huet et al., 1997). Consequently, following the recruitment of TFIIIB, TFIIIA and TFIIIC are dispensable for transcription in yeast (Kassavetis et al., 1990). In humans, a less avid interaction is made between TFIIIB and DNA despite it occupying a similar position in transcription complexes (McBryant et al., 1995; Tapping et al., 1994). TFIIIB is responsible for the subsequent recruitment of pol III. All three TFIIIB components are necessary for the stable recruitment of pol III, although pol III interacts directly, through the C34 and C17 subunits, with yBRF only

(Ferri et al., 2000; Werner et al., 1993). Similarly, the C39 subunit of human pol III, the homologue of yeast C34, binds directly to hBRF, as well as forming an additional interaction with TBP (Wang and Roeder, 1997).

The assembly of transcription complexes at type III promoters differs due to lack of ICRs, which consequently eliminates the need for TFIIIA and TFIIIC2 (Bernues et al., 1993; Lagna et al., 1994; Oettel et al., 1997). While these promoters still utilise TFIIIC1 (Oettel et al., 1997; Yoon et al., 1995), they employ a form of TFIIIB which is chromatographically separable from that used by types I and II (Lobo et al., 1992; Teichmann and Seifart, 1995). PSE occupancy is of primary importance in transcription complex assembly on a type III promoter, with SNAPc/PTF and TBP enhancing each others recruitment, an effect dependent on the N-terminal of TBP (Mittal and Hernandez, 1997).

Once assembled, class III transcription complexes are extremely stable and their components remain associated after transcription initiation (Bogenhagen et al., 1982; Lassar et al., 1985). The polymerase is recycled without being released from the template, generally committing the gene to multiple rounds of transcription which are able to proceed 5- to 10-fold more rapidly than the initial round (Bogenhagen et al., 1982; Dieci and Sentenac, 1996; Lassar et al., 1983).

1.7 Regulation of RNA pol III transcription

Pol III is subject to regulation, both positive and negative, by a variety of mechanisms which function to ensure that gene expression progresses within a carefully controlled system.

1.7.1 Activities that reduce pol III transcription

Dr1

In considering the repression of pol III transcription, the transcription factor TFIIIB serves as the principal player. One of the cellular proteins capable of targeting TFIIIB for repression is the 19kD nuclear phosphoprotein DrI, the activity of which can be stimulated by a 28kD co-repressor termed DRAP1, which on its own is inactive (Mermelstein et al., 1996). Dr1 has little affinity for DNA and functions through binding the essential TFIIIB component TBP (Inostroza et al., 1992; Kim et al., 1997).

Using recombinant proteins, it has been shown that Dr1 can prevent the binding of TBP to BRF (White et al., 1994). BRF binds to TBP through two distinct sites (Khoo et al., 1994; Wang and Roeder, 1995). It is believed that disruption of TBP-binding to the N-terminal direct repeats of BRF is achieved through conformational changes induced in TBP by Dr1. The second TBP-binding site, located in the C-terminal half of BRF, appears to make a pol III-specific high-affinity interaction with the basic repeat region of TBP (Khoo et al., 1994). Point mutations in TBP that can prevent this interaction with BRF similarly block binding of Dr1 (Khoo et al., 1994; Kim et

al., 1995), strongly suggesting that Dr1 and BRF compete directly for over-lapping binding sites in the basic repeat region of TBP. Thus, both TBP-binding sites can be blocked by Dr1, preventing essential interactions required for formation of an active preinitiation complex.

Dr1 demonstrates a similar ability to inhibit pol II transcription but pol I transcription appears to be immune to the repressive effects of Dr1 (Inostroza et al., 1992; White et al., 1994). Consequently, when rRNA levels are limiting, this protein presents a potential mechanism for shifting the balance of nuclear transcription in favour of pol I.

Retinoblastoma protein

Another protein that can target TFIIIB is RB, the 105kD nuclear phosphoprotein product of the retinoblastoma susceptibility gene, Rb. It was initially isolated because of its association with an inherited predisposition to retinoblastoma, a rare pediatric tumour of the retina (Friend et al., 1986). However, inactivating mutations in Rb have also been found in many other types of human tumour (Weinberg, 1995; Whyte, 1995) and it is now widely recognised as a potent tumour suppressor.

RB is expressed almost ubiquitously in normal human and mouse cells and plays a vital role in the control of growth and proliferation, ensuring that cell cycle progression through the restriction (R) point in late G_1 phase occurs only under favourable conditions. Under such conditions, cyclin D- and E-dependent kinases hyperphosphorylate RB, causing its inactivation and the subsequent progression into S phase (Weinberg, 1995; Whyte, 1995). The ability of cells to arrest growth and

proliferation is severely compromised when RB is inactivated (Weinberg, 1995; Whyte, 1995), potentiating the progression towards carcinogenesis.

The growth-suppressive function of RB lies in the pocket region (residues 380 to 785) (Weinberg, 1995; Whyte, 1995). The two pocket subdomains, A and B, are separated by a non-essential spacer and mutations within the pocket domain frequently arise in cancers (Weinberg, 1995; Whyte, 1995).

The ability of RB to repress pol III transcription was initially demonstrated by White *et al.* (White et al., 1996). The overexpression of RB was found to inhibit transcription of VA_1 without compromising transcription of a co-transfected CAT gene under the control of the pol II-transcribed human immunodeficiency virus promoter (White et al., 1996). This *in vivo* observation could potentially have resulted from an indirect effect arising from cell cycle changes. Evidence to support a direct inhibition of pol III transcription was provided by the finding that recombinant RB repressed the expression of a range of class III genes when transcription was reconstituted *in vitro* using partially purified factors (White et al., 1996). Furthermore, analysis of the pol III transcriptional activity in two human osteosarcoma cell lines, U2OS, which contain functional wild-type RB, and SAOS2, which only express a non-functional truncated form of RB, demonstrated that RB could inhibit pol III when present at physiological concentrations within a cell (White et al., 1996). The RB-deficient SAOS2 cells were found to express a transfected VA_1 gene ~5-fold more actively than U2OS and, similarly, SAOS2 cell extracts showed higher pol III activity *in vitro* (White et al., 1996). Moreover, synthesis of pol III products was shown to be ~5-fold higher in fibroblasts derived from $Rb^{-/-}$ mice than the equivalent cells from wild-type $Rb^{+/+}$ mice, providing conclusive evidence of a

major role for endogenous RB in suppression of pol III transcription *in vivo* (White et al., 1996). Indeed, RB has been shown to repress transcription of all pol III templates tested, demonstrating an ability to regulate transcription from all class III promoter types (Larminie et al., 1997; White et al., 1996). This strongly suggested that its mode of action pertained to modulation of a general pol III transcription factor or to pol III itself. Larminie *et al.* subsequently showed that TFIIIB is a specific target for repression by RB and pull-down assays identified that GST-RB was able to interact with TBP and hBRF to deplete extracts of TFIIIB activity (Larminie et al., 1997). Furthermore, immunoprecipitation experiments provided evidence of an interaction between endogenous RB and TFIIIB when these factors are present at physiological ratios and demonstrated that this interaction occurs specifically with the hypophosphorylated (active) form of RB (Larminie et al., 1997). This evidence is consistent with previous data demonstrating that TFIIIB activity increases as cells progress from G_1 into S phase, the interval through which RB is inactivated by hyperphosphorylation (White et al., 1995a). Subsequent studies identified more specifically the exact mechanism allowing RB to repress class III gene expression by demonstrating that when TFIIIB is repressed by RB, it is unable to form the necessary interactions with TFIIIC and pol III that are required for pol III transcription (Sutcliffe et al., 2000). The form of TFIIIB utilised by type III promoters differs from that used by types I and II. Although RB may also be able to inhibit this form recent data suggest that RB can repress transcription of class III genes with external promoters, such as the type III promoter gene U6, by targeting TBP and/or SNAPc/PTF (Hirsch et al., 2000). SNAPc/PTF, which is not required for transcription of genes possessing type I or II promoters, interacts with RB. This presents the possibility that more than

one mechanism of RB-mediated repression may regulate expression of genes with type III promoters.

RB has indeed been shown to repress transcription from all three nuclear RNA polymerases (Larminie et al., 1998; White, 1997). However, although a general repressor of pol III transcription, the class II genes inhibited by RB are very limited (Larminie et al., 1997). Significantly though, a key target of RB is the factor E2F (Adams and Kaelin, 1995; Dyson, 1998), which is responsible for activation of a variety of pol II-transcribed genes that are important for cell cycle progression (Adams and Kaelin, 1995; Dyson, 1998). Taken together with the repressive effects of RB on pols I and III, which are responsible for the production of important components required for protein synthesis, RB-repression presents a vital mechanism for controlling both cell growth, through inhibition of pols I and III, and cell proliferation, through its inactivation of E2F (Adams and Kaelin, 1995; Dyson, 1998). This regulation, along with a schematic illustration of RB and the interactions that allow it to repress transcription of pols I, II and III, is displayed in figure 1.5.

p53

Another important tumour suppressor, which is unrelated to RB, is p53. The gene encoding p53 is highly conserved among vertebrate species and with more than half of all human malignancies displaying a loss or mutation of the p53 gene, it represents the most frequently mutated gene in human cancers (Hollstein et al., 1991). Wild-type p53, like RB, can arrest cell growth and proliferation (Crook et al., 1994; Mercer et al., 1990). However, in contrast to RB, p53 is not an intrinsic cell cycle regulator and instead functions as an important checkpoint control mechanism protecting against aberrant growth and neoplastic transformation (Donehower et al., 1992). Its

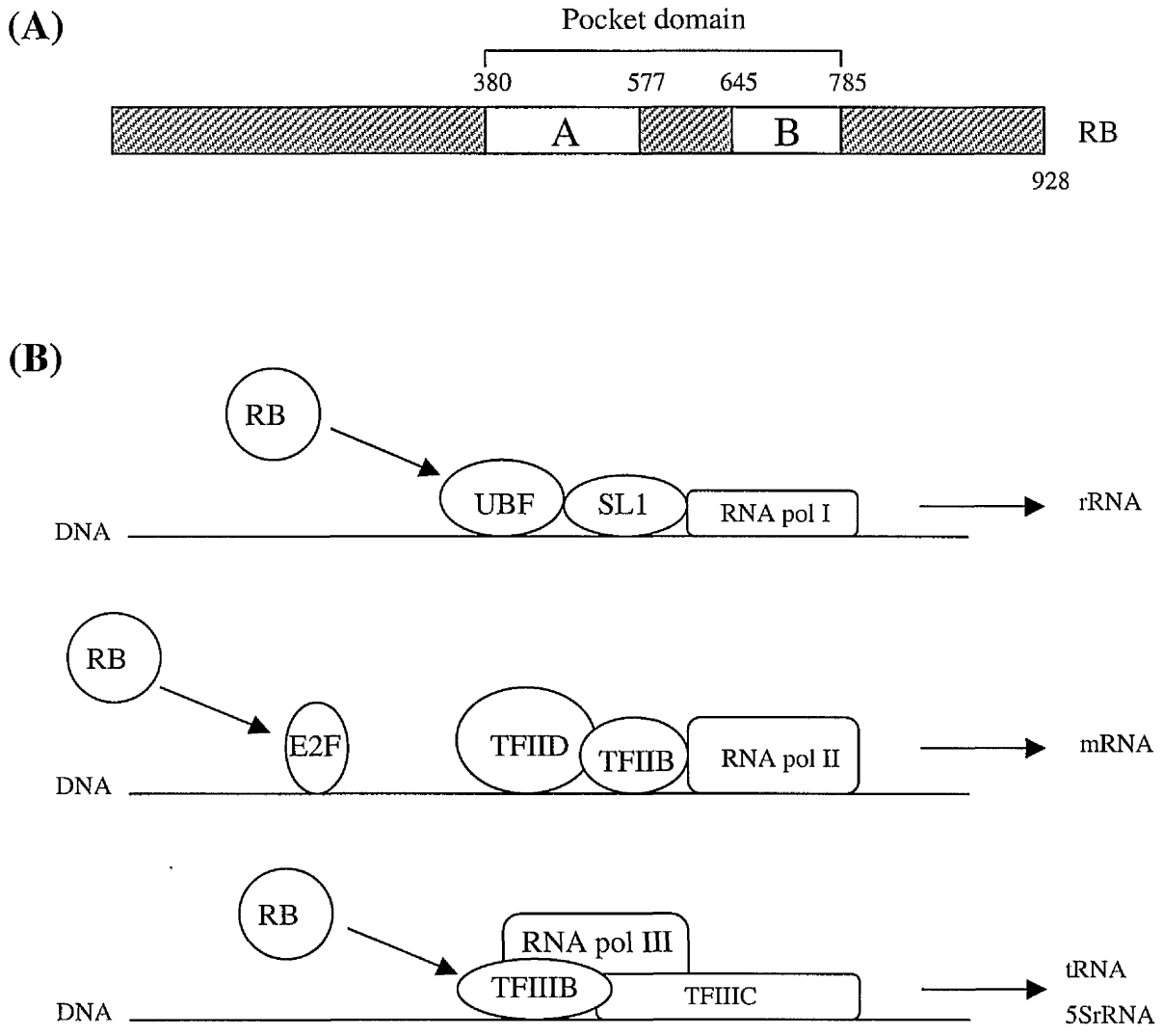
Figure 1.5

The retinoblastoma protein and its repressive effects

Panel A shows a schematic diagram of the retinoblastoma protein (RB), indicating the A and B subdomains within the pocket domain.

RB is able to regulate transcription by RNA polymerases I, II and III (panel B). Key transcription factors are indicated. RB represses pol I transcription through the general factor UBF. RB is able to repress a subset of pol II templates through gene-specific regulatory factors such as E2F and RB-mediated repression of pol III transcription is achieved through an interaction with the general factor TFIIIB.

The pol II transcription factor E2F promotes cell cycle progression, apparently through activating the expression of genes that encode products required for DNA replication, such as thymidine kinase (TK), dihydrofolate reductase (DHFR) and DNA polymerase α, in addition to proteins that drive the cell cycle, including cdc2 and cyclins. RB may limit the synthesis of these products through repressing E2F, providing a brake on proliferation. The transcription factors UBF and TFIIIB can also be repressed by RB, reducing the synthesis of rRNA and tRNA, which are important determinants of the biosynthetic capacity of the cell. Consequently, this may present a mechanism by which RB is able to limit the rate of translation and cellular growth.


(A)
Pocket domain
380
577
645
785
A
B
RB
928
(B)
RB
UBF
SL1
RNA pol I
DNA
rRNA
RB
E2F
TFIID
TFIIB
RNA pol II
DNA
mRNA
RB
RNA pol III
TFIIIB
TFIIIC
DNA
tRNA
5SrRNA
(C)
RB
E2F
UBF,
TFIIIB
+
+
DNA pol α,
DHFR, TK,
cdc2, cyclins
PROLIFERATION
(Cell Number)
+
+
rRNA,
tRNA
GROWTH
(Cell Mass)

ability to do this is highlighted by the fact that Li-Fraumeni individuals, who possess a germ-line mutation in p53, are highly prone to cancer (Ko and Prives, 1996).

The p53 protein displays a variety of biochemical activities, amongst which is the ability to regulate transcription (Haffner and Oren, 1995). Its central core domain (residues 100 to 300) allows it to bind to DNA in a sequence-specific manner and stimulate expression of proximal class II genes with p53-binding sites (Kern et al., 1991; Zambetti et al., 1992). Transcriptional activation is mediated through an acidic domain at the N-terminus that directly binds TBP (Liu et al., 1993; Truant et al., 1993) and several TAFs in TFIID (Farmer et al., 1996). Such activation can influence pol II-transcribed genes encoding proteins that are instrumental in inhibition of cell cycle progression. These include the cyclin-dependent kinase inhibitor p21/WAF1, which is able to prevent both G_1/S and G_2/M transitions in the cell cycle, and pro-apoptotic genes such as Bax (Ko and Prives, 1996; Levine, 1997). In addition, p53 is also able to specifically repress promoters lacking a p53-response element, including those that encode c-fos (Kley et al., 1992), PCNA (Jackson et al., 1994) and cyclin A (Yamamoto et al., 1994), which are implicated in promoting progression of the cell cycle. Consequently, the ability of p53 to both activate and repress transcription of specific genes is likely to contribute to its role as a tumour suppressor (Cox and Lane, 1995; Ko and Prives, 1996).

Transcriptional regulation by p53 does, however, extend beyond the confines of pol II-transcribed genes and pol III-transcribed genes are also subject to p53-repression (Chesnokov et al., 1996). p53 appears to function as a general repressor of class III gene expression; however, these genes display differential sensitivity to the repressive

effects of p53, with genes such as Alu and U6, possessing weak promoters, appearing most susceptible (Cairns and White, 1998; Chesnokov et al., 1996).

As a key general transcription factor, TFIIIB again serves as a direct target for repression by p53. Co-fractionation and co-immunoprecipitation experiments demonstrated that p53 associates with endogenous TFIIIB in a relatively stable complex at physiological ratios (Cairns and White, 1998). In wild-type fibroblasts TFIIIB is limiting, but disruption of the p53 gene conferred a specific increase in TFIIIB activity and pol III transcription. Furthermore, the inhibition by p53 of *in vitro* pol III transcription can be specifically relieved by the addition of excess TFIIIB (Cairns and White, 1998). It has also been shown that the N-terminal region of p53, which possesses a TBP-binding site, is sufficient to bind TFIIIB (Chesnokov et al., 1996). Point mutations that abolish the binding of free TBP similarly abolish TFIIIB binding and, moreover, also abrogate the ability of p53 to repress pol III transcription (Chesnokov et al., 1996). These data suggest that p53-repression of pol III transcription involves a direct interaction with TBP within the TFIIIB complex. Once TFIIIB has been assembled into a transcription complex, however, it becomes significantly less susceptible to p53-repression (Cairns and White, 1998).

A number of studies have implicated p53 in protein synthesis regulation (Ewen et al., 1995; Fontoura et al., 1997; Marechal et al., 1994). Its ability to control pol III transcription, specifically targeting TFIIIB, a major determinant of biosynthetic capacity of cells, supports this contention. Furthermore, that TFIIIB is targeted by two independent major tumour suppressors, RB and p53, both of which perform crucial physiological roles in regulating growth under specific environmental

conditions, confers considerable weight to the role of pol III regulation in the control of growth.

1.7.2 Activities that stimulate pol III transcription

TAP1

The TAP1 gene has been cloned from yeast and shown to be essential for cell viability (Di Segni et al., 1993). Immunoblotting with anti-TAP1 antibodies has demonstrated that TAP1 does not co-purify with any known class III factors but is thought to act on DNA in chromatin to facilitate both transcription and transcript release, on account of its homology to a yeast DNA strand transfer protein with riboexonuclease activity (Aldrich et al., 1993; Di Segni et al., 1993).

Staf and Oct-1

Staf is a 65kD polypeptide of 600 amino acid residues (Schuster et al., 1995). It possesses seven tandemly repeated zinc fingers which are responsible for specifically binding to activator elements of DNA (Schuster et al., 1995). Staf was found to bind to the DSE elements of vertebrate U snRNA and 7SK genes and substitution of the Staf-binding motif resulted in reduced expression *in vivo* (Schaub et al., 1997). The majority of DSE elements contain an octamer motif within 28bp of a Staf site. It is believed that the ubiquitous Oct-1 factor, which has been shown to stimulate both pol II and pol III transcription (Murphy et al., 1989) may function co-operatively with Staf, since their respective binding motifs can activate synergistically, if appropriately spaced (Schaub et al., 1997). Staf alone, however, is similarly able to stimulate both pol II and pol III transcription (Schaub et al., 1997).

Oct-1 was originally identified as a pol II factor and several other such factors have been implicated as potential regulators of pol III transcription, on the basis of sequence motifs linked with class III genes. Members of the ATF and CREB family that bind ATF sites may interact with a component of the pol III transcriptional machinery. ATF sites present in promoters of EBER2 and 7SL genes contribute to their expression (Bredow et al., 1990; Howe and Shu, 1989). Extracts prepared from HeLa cells treated with forskolin in order to raise cAMP levels and consequently activate a subset of these factors, displayed elevated levels of 7SL transcription, but not 7SK, which lacks an ATF motif in its promoter (Bredow et al., 1990). This suggests that these factors may be able to regulate pol III transcription.

Casein kinase II

Casein kinase II (CKII), which has been implicated in growth and cell cycle control, is a highly conserved serine/threonine protein kinase that is also able to stimulate pol III transcription in yeast (Hockman and Schultz, 1996). Extracts from the yeast strain $cka2^{ts}$, which carries a mutation in the catalytic α' subunit of CKII, are compromised for transcription of tRNA and 5S rRNA while pol I and basal pol II transcription remain unaffected (Hockman and Schultz, 1996). Furthermore, compromised transcription could be relieved by the addition of purified CKII and also by a synergistic action of CKII and recombinant TBP (Ghavidel et al., 1999; Hockman and Schultz, 1996). Thus CKII is thought to stimulate pol III transcription through phosphorylation of TFIIIB, most likely through specifically phosphorylating the TBP component of TFIIIB. However, other components of TFIIIB or of the remaining pol III machinery that also possess CKII target motifs may be subject to stimulation by CKII (Ghavidel et al., 1999; Hockman and Schultz, 1996).

La can stimulate recycling of the human pol III transcription complex and transcription from isolated complexes preassembled on the VA_1 promoter (Fan et al., 1997; Maraia, 1996). CKII phosphorylates La; however, in contrast to the situation in yeast, CKII appears to confer an inhibitory effect on La (Fan et al., 1997), although the effect of this inhibition on the rate of pol III transcription under physiological conditions remains to be established.

Protein phosphatase 2A

A yeast strain $tpd3^{ts}$, which possesses a temperature-sensitive mutation in the TPD3 gene encoding the regulatory A subunit of protein phosphatase 2A (PP2A), was found to cease synthesis of tRNA at the non-permissive temperature and, similarly, extracts were unable to support transcription of tRNA genes (Van Zyl et al., 1992). Mixing experiments revealed that the defect in $tpd3^{ts}$ cells is attributed to an inhibitory activity, as opposed to the loss of a transcription component (Van Zyl et al., 1992). It is believed that PP2A confers a positive regulation on pol III transcription in yeast through stimulating TFIIIB, and to a lesser degree, pol III (Van Zyl et al., 1992). A kinase that inactivates TFIIIB, and possibly pol III, through phosphorylation could account for the inhibitory effect observed in the tpd3 mutants, since this inhibition may normally be balanced by PP2A.

1.7.3 Repression by chromatin

The assembly of DNA into chromatin, the natural organisation of DNA *in vivo*, is achieved principally through association with highly conserved proteins called histones. They are small basic polypeptides of 11-16kD and the core histones, H2A, H2B, H3 and H4, interact with each other to form a nucleosome, the fundamental

repeating unit of chromatin (Kornberg, 1977). A nucleosome is comprised of a central $(H3/H4)_2$ tetramer around which 120bp of DNA are wrapped. A histone H2A/H2B dimer subsequently flanks each end through protein-protein interactions to result in an octamer organising a total 160bp of DNA in two left-handed superhelical turns (Thomas, 1984; White, 2001; Wolffe, 1998). Nucleosomes are generally separated by 20-30bp that is referred to as linker DNA, which is commonly associated with a linker histone such as H1, the presence of which stabilises the interaction of the nucleosome with the DNA wrapped around it (Thomas, 1984; White, 2001; Wolffe, 1998). Linker histones promote the organisation of nucleosomal arrays to coil and fold into chromatin fibres (Thomas, 1984). These fibres are believed to have a solenoidal structure, with each turn containing six or more nucleosomes and over 1000bp of DNA (Thomas, 1984). Chromatin fibres are assembled into large domains with non-histone proteins performing both structural and regulatory roles. Further folding of these domains within the chromosome compacts the length of DNA by an additional 100-fold.

Eukaryotic rRNA genes lack nucleosomes on the transcribed repeats (Lucchini and Sogo, 1998); however, nucleosomes are associated with the coding regions of many genes transcribed by pol II and pol III (Chipev and Wolffe, 1992; Englander and Howard, 1995; Gottesfeld and Bloomer, 1980; Louis et al., 1980). Such packing of DNA presents a major obstacle to transcription, severely restricting the accessibility of genes to transcription factors (Wolffe, 1995). A variety of studies have established that pol III transcription can be inhibited by the presence of histones (Almouzni et al., 1990; Gottesfeld and Bloomer, 1982; Morse, 1989). However, the susceptibility of class III genes to nucleosomal repression is highly template-dependent. The majority of tRNA genes are remarkably resistant to chromatin-mediated repression, while

middle repetitive genes such as B2 and Alu are extremely susceptible (Bouvet et al., 1994; Russanova et al., 1995). Acetylation can facilitate the access of transcription factors to chromatinised promoter sequences (Lee et al., 1993). Thus, evidence that human TFIIIC2 possesses histone acetyltransferase (HAT) activity (Hsieh et al., 1999; Kundu et al., 1999) suggests that TFIIIC performs a role, in addition to its function as an assembly factor, to weaken the interaction of nucleosomes with the transcribed region of at least some class III genes.

1.8 Physiological regulation of pol III transcription

In higher eukaryotes pol III transcription is strongly regulated in response to a variety of important physiological stimuli (White, 1998a; White, 1998b).

Differentiation

In situ hybridisation has revealed that pol III transcript levels are substantially reduced when parietal endoderm forms during early mouse embryogenesis (Vasseur et al., 1985). This can be similarly reproduced in culture using F9 embryonal carcinoma (EC) cells, which differentiate into parietal endoderm upon treatment with retinoic acid and cAMP (Murphy et al., 1983; White et al., 1989). Differentiation causes approximately a 9-fold reduction in pol III transcription rate, mediated through a specific down-regulation of TFIIIB (White et al., 1989). More specifically, it has been shown that the abundance of two of the essential components of TFIIIB, TBP and BRF, decreases during F9 cell differentiation. The assembly of TBP specifically into TFIIIB is reduced, which is not the case for all TBP-containing complexes. BRF

levels, in particular, show a dramatic decrease during F9 cell differentiation, which alone is sufficient to account for the overall decline in class III transcriptional activity that accompanies parietal endoderm formation (Alzuherri and White, 1998).

The cell cycle

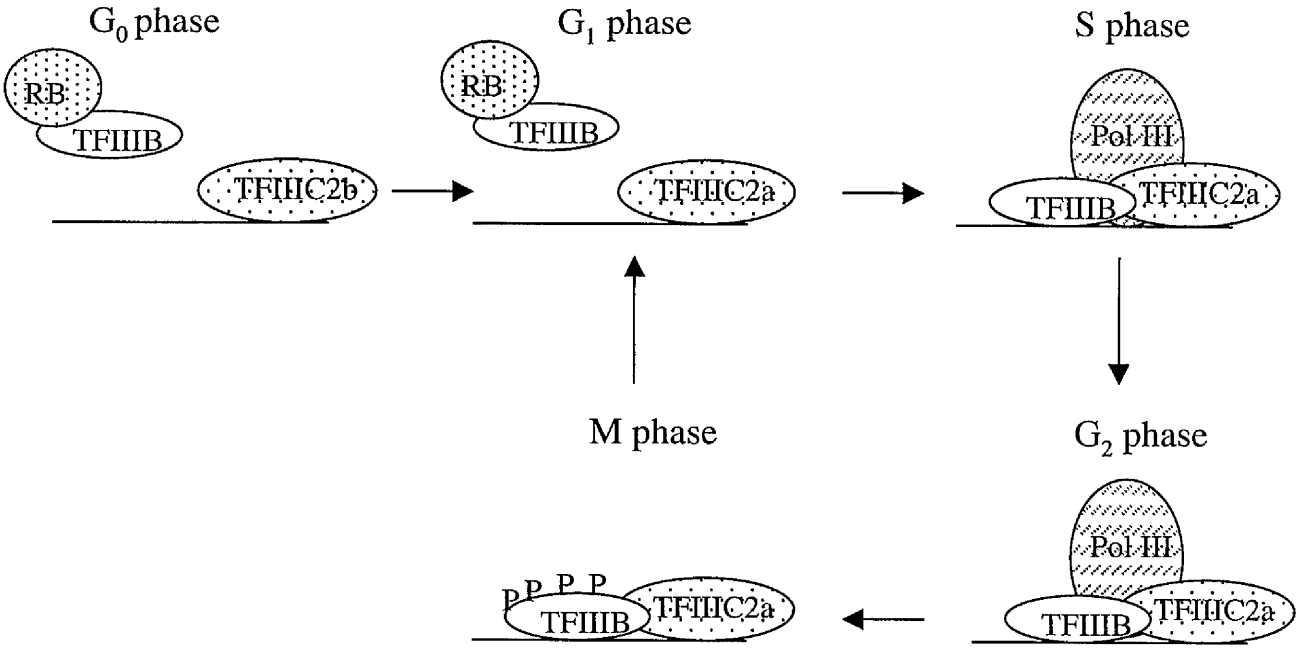
In mammalian systems, the cell cycle is subject to careful regulatory control and various mechanisms are employed to modulate pol III transcription (Figure 1.6). Indeed, all nuclear transcription is repressed at mitosis (Gottesfeld and Forbes, 1997), with inhibition of pol III transcription being achieved through the phosphorylation and inactivation of TFIIIB (Gottesfeld et al., 1994; Leresche et al., 1996; White et al., 1995b). Gottesfeld *et al.* demonstrated that TFIIIB from metaphase-arrested *Xenopus* eggs was incapable of supporting transcription without prior treatment with phosphatase (Gottesfeld et al., 1994). Nonetheless, cyclin B/cdc2-depleted mitotic extracts still display repressed pol III transcription. This can be relieved by the general kinase inhibitor DMAP, suggesting that additional kinases may be present that can similarly inhibit pol III activity in metaphase-arrested *Xenopus* eggs (Hartl et al., 1993).

TFIIIB is also subject to specific inhibition in mitotic HeLa cells (White et al., 1995b). In both *Xenopus* and HeLa cells, the TBP subunit of TFIIIB becomes hyperphosphorylated during mitosis, the functional significance of which, however, remains unclear (Leresche et al., 1996; White et al., 1995b). Recombinant TBP is unable to restore transcriptional activity in mitotic extracts and repression appears to be attributed to a specific loss of TAF activity (Leresche et al., 1996; White et al., 1995b). Indeed, given that the BRF subunit of TFIIIB is also found to be hyperphosphorylated in mitotic HeLa cells, it may be that specific inactivation of this

Figure 1.6

Cell cycle control of pol III transcription

Model illustrating the regulatory mechanisms that contribute to the cell cycle control of pol III transcription in mammalian systems. During quiescence (G_0), when expression of pol III transcripts is low, TFIIIC may be present in its transcriptionally inactive TFIIIC2b form and TFIIIB activity is subject to repression by RB. In cycling cells TFIIIC predominates in its active TFIIIC2a form, however, TFIIIB remains associated with RB, sustaining low transcription levels. Following the G_1/S transition, transcription levels are significantly elevated until the end of G_2 phase. Transcription is repressed again during mitosis through the phosphorylation-mediated inactivation of TFIIIB.


G0 phase
RB
TFIIIB
TFIIIC2b
G1 phase
RB
TFIIIB
TFIIIC2a
S phase
Pol III
TFIIIB
TFIIIC2a
G2 phase
Pol III
TFIIIB
TFIIIC2a
M phase
P P P P
TFIIIB
TFIIIC2a

TAF could account for the repression of transcription. However, the highly condensed state of chromosomes, that is characteristic of mitosis, is likely to be a contributing factor (White et al., 1995b).

As cells exit mitosis, TFIIIB hyperphosphorylation is rapidly reversed, although TFIIIB activity remains low and is limiting in early G_1, a situation that reflects the low TAF activity during this phase (White et al., 1995a). The rate of pol III transcription only increases gradually as cells progress through G_1 phase (Scott, 2001; White et al., 1995a). Transcription levels are maximal in S and G_2 phases when TAF activity is no longer limiting and active TFIIIB is in relative excess over TFIIIC (White et al., 1995a).

Detailed time courses have established that the significant rise in TFIIIB activity and pol III transcription in late G_1 phase closely correlates to the time when RB is inactivated through hyperphosphorylation by the cyclin D- and E-dependent kinases around the restriction (R) point (Johnson et al., 1974; Mauck and Green, 1974). Indeed, co-immunoprecipitation experiments revealed that RB dissociates from TFIIIB shortly before S phase entry, reflecting its ability to bind and inactivate TFIIIB only when in its active underphosphorylated form (Scott, 2001). RB remains in its inactive hyperphosphorylated state throughout the S and G_2 phases, until being dephosphorylated at the start of the next G_1 phase. It is then again able to bind and inactivate TFIIIB, likely accounting for the low transcriptional activity during this phase of the cell cycle (Grana et al., 1998; Herwig and Strauss, 1997; Mittnacht, 1998). Consequently, RB plays a major role in cell cycle regulation of pol III transcription.

In addition to its active period during early G_1, RB is similarly underphosphorylated and active in quiescent cells (White et al., 1995a). During quiescence, when cells exit the cell cycle and growth arrest, the synthesis of tRNA and 5S rRNA is dramatically reduced (Johnson et al., 1974; Mauck and Green, 1974). This reflects the reduced biosynthetic demands of cells at rest and the likely important role of RB in suppressing pol III transcription during G_0 phase. In support of the crucial function of RB in this context, a decrease in TFIIIB activity has been observed in serum-starved mouse fibroblasts (Tower and Sollner-Webb, 1988). However, in $Rb^{-/-}$ fibroblasts the ability to down-regulate pol III transcription is compromised (Scott, 2001), strongly implicating RB-mediated repression of TFIIIB in the reduced pol III transcription levels characteristic of G_0 phase. Despite this, some decline in transcription, albeit less substantial, is still observed in quiescent $RB^{-/-}$ cells, indicating the involvement of additional mechanisms in the control of pol III activity in quiescent cells (Scott, 2001). The RB-related pocket proteins p107 and p130 possess the ability to repress pol III transcription both *in vitro* and *in vivo* (Sutcliffe et al., 1999). Furthermore, p107 p130 double knockout mouse fibroblasts are compromised in their ability to decrease pol III transcription in response to serum withdrawal, suggesting that one or both of these proteins contribute to the control of pol III transcription during quiescence (Sutcliffe et al., 1999). It would appear that p130 is likely to be the more prominent player in this regard, as while p107 is poorly expressed in serum-starved mouse fibroblasts, p130 is relatively abundant and in an active form (Grana et al., 1998).

1.9 Deregulation of pol III transcription by transformation

An accumulating mass of evidence shows that the abundance of pol III transcripts is abnormally elevated in transformed and tumour cell lines (Brickell et al., 1983; Kramerov et al., 1990; Majello et al., 1985; Scott et al., 1983). Numerous transforming agents can stimulate pol III transcription, including a range of chemical carcinogens when applied to cells (Garber et al., 1991; Garber et al., 1994; Scott et al., 1983). Moreover, recent studies have established that elevation of pol III transcripts is a feature that can also be observed in tumours *in vivo* (Chen et al., 1997a; Chen et al., 1997b; Winter et al., 2000), providing substantial support for the contention of a critical role of pol III transcriptional activation in tumourigenesis. However, in many cases the mechanistic basis of pol III deregulation remains elusive. Given the prominent roles of RB and p53 in pol III repression and the propensity for their inactivation during neoplastic transformation, inactivation of these tumour suppressors could possibly account for such deregulation of pol III in transformed and tumour cell types. Indeed, although p53 inactivation has yet to be established as contributing to the elevated pol III activity found in tumours, evidence that this is the case for RB has already been documented.

Most of the naturally occurring RB mutations in tumours encompass the pocket domain (Hu et al., 1990), which is essential for RB regulation of pol III activity (White et al., 1996). Moreover, direct evidence linking naturally occurring RB mutations with the elevation of pol III activity consistently observed in transformed and tumour cells has been documented. Studies have shown that subtle mutations within the pocket domain arising in small cell lung carcinomas, which inactivated RB

function, also conferred a loss of the ability to repress pol III transcription (White et al., 1996). Additionally, TFIIIB is unable to bind to a mutant form of RB that is found in the osteosarcoma cell line SAOS2 (Larminie et al., 1999). It seems highly likely that the release of TFIIIB from RB-mediated repression will arise whenever RB function is compromised and it has been suggested that this may be the case in all human malignancies (Weinberg, 1995).

Regulation of RB activity in normal cells can be achieved through the action of the cyclin D- and E-dependent kinases (Grana et al., 1998). In many human cancers, which retain wild-type RB, cyclin D-dependent kinases are hyperactive, providing an alternative mechanism for RB inactivation. In addition to the overexpression of cyclin D1 in 30-40% of primary breast tumours (Bates and Peters, 1995), the gene for p16, a specific repressor of the cyclin D-dependent kinases, is deleted in many other cancers such as oesophageal, bladder, lung and pancreatic carcinomas (Hirama and Koeffler, 1995; Hunter and Pines, 1994). Thus, these mechanisms provide two distinct means of elevating cyclin D-dependent kinase activity in cancers, ensuring inactivation of RB by phosphorylation.

While inactivation of RB can release TFIIIB from repression, increase in TFIIIB activity can similarly result from constitutively active Ras (Wang et al., 1997). Given that activating mutations in Ras are also very frequently found in human tumours, this presents yet another potential mechanism for deregulating pol III transcription in transformed and tumour cells (Lowry and Willumsen, 1993).

1.10 Regulation of pol III transcription by viruses

Following infection, viruses are able to subvert the host cells translational and replication machinery towards mass synthesis of viral proteins and the viral genome. A variety of viruses have been shown to stimulate pol III transcription, frequently as a means to meet an increase in biosynthetic demands. Despite the evolution of various deregulating mechanisms, there are a number of common features centralising around the key aspects of pol III transcriptional control.

Adenovirus

Several viral genomes contain class III genes which are necessary for viral replication (White, 1998a). Adenovirus is one such example and encodes two pol III products, VA_I and VA_{II} (Soderlund et al., 1976; Weinmann et al., 1976). These short RNAs are expressed at very high levels late in infection (Soderlund et al., 1976) and contribute to manipulation of the host cell's translational apparatus, ensuring the synthesis of viral proteins (Thimmappaya et al., 1982).

The deregulation of pol III transcription pertains largely to the adenovirus oncoprotein E1A. Indeed, mutant virus strains lacking E1A show little or no activation of VA (Berger and Folk, 1985; Hoeffler and Roeder, 1985; Sollerbrant et al., 1993) while transfection of the E1A gene is alone sufficient to transactivate VA (Aufiero and Schneider, 1990). Furthermore, purified recombinant E1A can stimulate VA_I transcription by up to 50-fold in HeLa extracts (Datta et al., 1991). However, E1A does not bind to the VA_I gene to exert a direct effect (Datta et al., 1991) but influences transcription through the general pol III factors. HeLa cells infected with

wild-type adenovirus display a significant elevation in TFIIIC2 activity (Hoeffler et al., 1988; Hoeffler and Roeder, 1985). This is a manifestation of a selective increase in the level of the TFIIIC110 subunit, seemingly through an induction of TFIIIC110 mRNA by E1A (Sinn et al., 1995) that raises the proportion of the transcriptionally active TFIIIC2a form (Hoeffler et al., 1988). However, E1A has also been shown to stimulate pol III transcription *in vitro* (Datta et al., 1991), implying that alternative mechanisms, that do not require *de novo* protein synthesis, are also utilised by adenovirus in order to stimulate pol III transcription. Indeed, E1A can bind and inactivate the RB family of pocket proteins and thereby release TFIIIB from repression (White et al., 1996), as well as disrupting the interaction between Dr1 and TBP (Kraus et al., 1994). That E1A can overcome the important inhibitory functions of RB and Dr1 that regulate TFIIIB activity provides additional routes by which adenovirus may deregulate pol III transcription (White et al., 1994; White et al., 1996).

In addition to the viral VA_I and VA_{II} transcripts, adenovirus-infected cells have been shown to overexpress endogenous Alu genes (Panning and Smiley, 1993; Russanova et al., 1995). This may be attributed, at least in part, to the increased proportion of Alu genes that are accessible to transcription factors through changes in the chromatin structure (Russanova et al., 1995). Full induction of Alu genes, however, requires another adenovirus oncoprotein, E1B (Panning and Smiley, 1993). E1B is able to bind and inactivate p53, suggesting that adenovirus infection may also overcome the regulatory effects of p53 on pol III transcription (Ko and Prives, 1996). Taken together, these observations demonstrate that a range of mechanisms can be employed by adenovirus in order to achieve elevated pol III transcription levels.

Hepatitis B virus

Hepatitis B virus (HBV) is strongly associated with the development of hepatocellular carcinoma and its X gene induces liver cancer in transgenic mice (Kim et al., 1991). Pol III transcription can be significantly increased by transfection into a variety of cell lines, including rat 1A, *Drosophila* S-2 and Chang liver (CL) cells (Aufiero and Schneider, 1990; Kwee et al., 1992; Wang et al., 1995). As with the adenovirus E1A protein, the X gene products are unable to bind DNA directly and use protein-protein interactions with general transcription factors to influence pol III transcription. The X protein is able to interact directly with the conserved core of the TFIIIB subunit TBP and the human RPB5 subunit of pols I, II and III (Cheong et al., 1995; Qadri et al., 1995). Furthermore, a specific increase in TFIIIB activity that is observed could be accounted for by an X-induced rise in cellular TBP, since TBP is the limiting factor for pol III transcription in the cell lines utilised in these studies (Trivedi et al., 1996; Wang et al., 1997; Wang et al., 1995).

Additionally, the X gene products have also been found to stimulate kinase pathways, with both the protein kinase C (PKC) and Ras-Raf-MEK-MAP kinase signalling cascades being activated (Benn and Schneider, 1990; Kekule et al., 1993). Inhibitors of either PKC or Ras are able to block the X-mediated increase in TBP abundance and pol III transcription (Wang et al., 1997; Wang et al., 1995). A dominant negative mutant form of Ras achieves the same effect but can be relieved by coexpression of constitutively active Raf-1 (Wang et al., 1997). These results provide evidence that X activates pol III transcription via cellular signalling pathways. Notably, in the absence of the X protein, the PKC pathway has a negative effect on pol III activity (James and Carter, 1992; Wang et al., 1995).

Human papillomavirus

Human papillomaviruses (HPVs) have an aetiological role in most cervical malignancies (Vousden, 1995). HPV expresses a viral oncoprotein called E7 which has been shown to bind to the pocket domain of RB causing its inactivation (Dyson et al., 1989). Significantly, the affinity with which E7 binds RB is greater in the highly malignant HPV strains such as HPV-16 and HPV-18 (Heck et al., 1992). Pol III transcription can be dramatically elevated *in vivo* by E7, which cannot be attributed to an indirect response to cell transformation since a non-transforming mutant E7 protein, that retains its RB-binding activity, is similarly able to stimulate pol III transcription (Larminie et al., 1999; Sutcliffe et al., 1999). This supports the fact that the ability of E7 to activate pol III transcription is dependent on the integrity of the LXCXE RB-binding motif of E7, since deletions or substitutions within this domain abolish its capacity to stimulate expression of a class III gene (Sutcliffe et al., 1999). Consequently, it seems highly probable that the ability of the HPV E7 oncoprotein to deregulate pol III transcription is achieved through release of TFIIIB from repression by RB and its p107 and p130 relatives.

Human T-cell leukaemia virus type I

Human T-cell leukaemia virus type I (HTLV-I) is responsible for the aggressive malignancy called adult T-cell leukaemia and a neurodegenerative disease known as tropical spastic paraparesis/HTLV-I-associated myelopathy (Jacobson et al., 1988; Poiesz et al., 1980). The pathogenic effects of HTLV-I are ascribed to the Tax protein it expresses. This protein has been shown to transactivate VA_I when co-expressed in transient transfection assays and recombinant Tax stimulates expression of various pol III genes when added to crude extracts (Gottesfeld et al., 1996; Piras et al., 1996). In

HTLV-I-infected T cells TFIIIB activity and the synthesis of tRNA and 5S rRNA are elevated (Gottesfeld et al., 1996). Tax is believed to stimulate pol III transcription through raising the effective concentration of active TFIIIB, although the precise mechanisms involved have yet to be elucidated.

Simian virus 40

The small DNA tumour virus Simian virus 40 (SV40) is able to transform a variety of rodent cell types, causing a stimulation of pol III transcription (Carey et al., 1986; Scott et al., 1983). Several comparisons can be drawn with adenovirus as regards the mechanisms employed to achieve this. Like adenovirus, both TFIIIB and TFIIIC2 activities are substantially elevated in SV40-transformed cell extracts when compared to extracts of the untransformed parental cell line (Larminie et al., 1999). While only the TFIIIC110 subunit of TFIIIC2 is overexpressed in adenovirus-infected HeLa cells, both the TFIIIC220 and TFIIIC110 subunits are significantly more abundant following SV40 transformation, suggesting that overexpression of TFIIIC2 may account for its increased activity (Larminie et al., 1999). Furthermore, the increased abundance of these subunits reflects an overproduction of the corresponding mRNAs (Larminie et al., 1999). SV40 appears to mimic adenovirus more closely in its ability to increase the proportion of TFIIIC2 in its active TFIIIC2a form (White et al., 1990).

SV40 stimulation of TFIIIB activity, however, stems from an alternative mechanism, with expression levels of the TBP and BRF subunits of TFIIIB remaining unaffected following SV40 transformation (Larminie et al., 1999). As with adenovirus, TFIIIB activity is raised through release from RB-mediated repression. SV40 encodes a large tumour (T) antigen that resembles E1A in being able to bind and inactivate RB (DeCaprio et al., 1988; Ewen et al., 1989; Ludlow et al., 1989). Consequently, this

abolishes the repressive influence of RB on TFIIIB and the proportion of TFIIIB bound by RB in SV40-transformed fibroblasts is severely diminished (Larminie et al., 1999). In contrast to adenovirus, where a second oncoprotein, E1B, binds and inhibits p53, in SV40, this function also pertains to the large T antigen, despite another t antigen being expressed (Ko and Prives, 1996). However, the effect of this large T antigen interaction on the function of p53 in pol III transcriptional repression remains to be determined.

1.11 Objectives

Polyomavirus is another small DNA tumour virus and is closely related to SV40. It was shown to stimulate pol III transcription by Majello *et al.* in 1985; however, the mode of action involved remained to be determined. Consequently, the focus of this study has been to elucidate the mechanisms by which Polyomavirus exerts its effects on pol III transcription.

Several features of viral transformation can be observed among different viruses. Nonetheless, while common mechanisms of deregulation exist, viruses capable of stimulating pol III transcription display variable oncogenic capacities pertaining to the transforming proteins they express.

It was therefore of interest to establish which aspects of the pol III transcriptional machinery are targeted by Polyomavirus, identifying mechanisms common to SV40 or other transforming viruses and those unique to Polyomavirus.

Furthermore, establishing which of the three oncoproteins expressed by Polyomavirus is responsible for each deregulating activity was also a principal objective.

Chapter 2

Materials and Methods

2.1 Cell culture

All cell lines, Balb/c 3T3 Clone A31, Py3T3 (transformed by wild-type Polyomavirus) (Scott et al., 1983), Pytsa3T3 (transformed by the tsa mutant which has a mutation in the gene encoding the large T-antigen) (Scott et al., 1983), SV3T3 Cl38 and SV3T3 Cl49 (transformed by Simian Virus 40)(Rigby et al., 1980) were routinely cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal calf serum (FCS, Sigma), 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. Cell culture was performed in a class II hood, using aseptic technique and sterile equipment and reagents.

Cells were passaged when subconfluent; approximately every 2 to 3 days. After media was aspirated from the flask, 2ml of buffered trypsin-EDTA (0.05% trypsin, 0.02% EDTA) was added to the cells, then aspirated immediately. A further 2ml was added and left for approximately 2 minutes at 37°C. Following trypsinisation, fresh media was immediately added to the dissociated cells in order to neutralise the trypsin. Cells were centrifuged at 1200g for 2 minutes at 4°C and the media removed. The pelleted cells were then resuspended in fresh 10% FCS DMEM solution at a ratio of 1:10 or as appropriate.

Cyro-freezing was used for storage of all cell lines. Cells were trypsinised as described and, following pelleting by centrifugation, cells were resuspended in a solution of 82% DMEM (10% FCS), 10% neat FCS and 8% dimethylsulphoxide (DMSO). Cells were aliquotted into cyro-tubes and frozen in stages by initially being placed at –80°C overnight and subsequently being transferred to liquid nitrogen storage. Thawing of cells was performed rapidly by placing cyro-tubes in a waterbath at 37°C until just thawed. Cells were then mixed with fresh media, centrifuged and the supernatant aspirated off to ensure removal of DMSO prior to resuspension in 10% FCS DMEM.

2.2 [^{3}H] Thymidine incorporation

Cells were plated out onto 24-well plates at a concentration of 5 x 10^3 cells/well in 1ml of media. Cells for serum withdrawal or serum stimulation treatment, were incubated in 400µl of serum-free media for 24 hours before addition of 100µl of serum-free media or neat serum, respectively. Following a 3 hour incubation period, cells were treated with [^{3}H] thymidine (0.1µCi/ml) and incubated for a further 3 hours prior to harvesting. Cells were washed 3 times with 500µl of ice-cold phosphate-buffered saline (PBS), 3 times with 500µl of 5% trichloroacetic acid (TCA) to precipitate the DNA and twice with 500µl of ethanol. Samples were then solubilized in 500µl of 0.3M NaOH, transferred to scintillation vials and 3ml of Optiflow (Fisons chemicals) added to allow the incorporation of [^{3}H] thymidine into DNA to be

measured by liquid scintillation counting. Results are expressed as disintegrations per minute.

2.3 Preparation of whole cell extracts

All whole cell extracts were prepared from cells grown in 10cm tissue culture dishes to facilitate scraping and were harvested at approximately 80% confluency. Preparation was performed on ice as rapidly as possible and all solutions and tubes were kept ice-cold to maintain cell activity. Cells were washed twice with 5ml of PBS before being scraped with a plastic spatula into 5ml of ice-cold PBS. Cells were collected in 50ml Falcon tubes and pelleted by slow centrifugation at 1200g for 8 minutes at 4°C. A small volume of fresh ice-cold PBS was used to resuspend the cell pellets and allow the cells to be transferred to eppendorf tubes. These were then microcentrifuged briefly at 4°C to re-pellet the cells and the PBS removed. The volume of cell pellets were then measured by comparison with pre-measured volumes of water. Mircroextraction requires pellets to be between 50 - 150µl, giving approximately 0.5 – 3 x 10^7 cells; larger pellets were subdivided. An equal volume of freshly made pre-cooled microextraction buffer (450mM NaCl, 50mM NaF, 20mM Hepes pH 7.8, 25% glycerol, 1mM DTT, 0.5mM PMSF, 0.2mM EDTA, 40µg/ml bestatin, 1µg/ml trypsin inhibitor, 0.7µg/ml pepstatin, 0.5µg/ml aprotinin, 0.5µg/ml leupeptin) was added to the cells and, following resuspension, the cells were immediately snap-frozen on dry ice. Cells were then placed in a 30°C waterbath until just thawed before being immediately returned to dry-ice. This freeze-thaw procedure

was performed a total of 3 times to ensure optimal cell lysis, with cells then being microcentrifuged at 7000g for 7 minutes at 4°C after the third thaw. The supernatant was carefully decanted into a fresh tube, leaving behind the cell debris, and then promptly aliquotted and snap frozen. These whole cell extracts were then stored at –70°C.

2.4 Preparation of total cellular RNA

Total cellular RNA was isolated from cells when approximately 80% confluent using TRI reagent (Sigma), a solution of guanidine thiocyanate and phenol, in accordance with the manufacturer's instructions. Media was aspirated off cells grown in 10cm tissue culture dishes and residual media removed with two washes using 5ml ice-cold PBS. Cells from each dish were harvested by scraping in 1ml of TRI reagent per dish and transferred to a sterile eppendorf tube. Cells were left to stand for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes. 0.2ml of chloroform was then added to each tube and the samples vortexed for 15 seconds. The samples were then allowed to stand for a further 15 minutes at room temperature prior to being centrifuged at 13 000g for 15 minutes at 4°C. This resulted in separation of the samples into three phases: a lower red organic phase containing protein, a middle white interphase containing precipitated DNA and an upper colourless aqueous phase which contains the RNA. These upper phases were carefully removed, ensuring no contamination from the remaining phases and transferred to fresh eppendorf tubes. Isopropanol (500µl) was added to each of these

tubes containing the aqueous RNA and thoroughly mixed by repeated inverting. Following 5 – 10 minutes incubation at room temperature to allow maximal precipitation of RNA, samples were centrifuged at 13 000g for 10 minutes at 4°C. The supernatant was then removed and the remaining RNA pellet was washed with 1ml of 75% ethanol made up with diethypyrocarbonate (DEPC)-treated dH_2O (0.1% DEPC), thoroughly mixed into solution, left overnight at room temperature and then autoclaved to inactivate the remaining DEPC. The samples were vortexed briefly, subsequently microcentrifuged at 7500g for 5 minutes at 4°C and the supernatant aspirated off. Residual supernatant was removed with a P20 pipette following pulse microcentrifugation. Appropriate volumes of DEPC-dH_2O, in the range of 10 - 30µl, were added to the RNA pellets and the samples were heated in a 65°C waterbath for 10 – 15 minutes to facilitate resuspension of the RNA. The samples were stored at – 70°C.

RNA concentration was determined by UV spectrophotometry using the calculation: RNA concentration (µg/ml) = absorbance at 260nm x 40 x dilution factor. A ratio of absorbance at 260nm to 280nm in the range of 1.8 – 2, indicated the RNA samples were relatively free from contamination with DNA or protein.

2.5 Northern blot analysis of total cellular RNA

Typically RNA samples of 10 - 30µg were used in analysis, made up to a total volume of 10µl with DEPC-dH_20. 10µl of 2 x RNA sample buffer (1 x MOPS comprised of solutions made up with DEPC-dH_20 (20mM MOPS pH 7.0, 8mM sodium acetate,

1mM EDTA pH 8.0), 4.4M formaldehyde, 54% formamide) was added to each sample prior to heating at 65°C for 15 minutes to denature the RNA secondary structure. The samples were immediately transferred to ice to prevent any renaturation and 2µl of 1mg/ml ethidium bromide and 2µl of 10 x RNA loading buffer (50% glycerol, 1mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF) were added to each sample. Following a 20 minute pre-run at 40V of a denaturing gel (1% agarose, 2.2M formaldehyde, 1 x MOPS) in 1 x MOPS, samples were loaded and run for approximately 5 hours at 40V in order to electrophoretically separate the different species of RNA according to size. The gel was visualised under a UV transilluminator in order to confirm separation and photographed. It was then washed for 20 minutes in 20 x SSC buffer (3M NaCl, 0.3M sodium citrate pH 7.0) prior to capillary transfer as described by Maniatis et al. (Maniatis et al., 1982).

The transfer procedure required the prepared gel to be placed, inverted, on a bridge of Whatmann 3MM chromatography paper supported on a glass plate and suspended over a reservoir of 20 x SSC buffer. An appropriate size of Hybond N nylon membrane optimised for nucleic acid transfer (Amersham) was pre-soaked in 20 x SSC and positioned over the gel, followed by a further two layers of pre-soaked Whatmann paper; at each stage of layering, care was taken to ensure removal of air bubbles. This arrangement was surmounted with a stack of paper towels and an appropriate weight in order to allow efficient capillary action. During transfer, the migration of the RNA from the gel to the nylon membrane is facilitated by the passive movement of the transfer solution through the gel. Plastic wrap was used to prevent a direct contact between the paper towels and the Whatmann bridge; this ensured movement of the buffer was only through the gel. In order to achieve high-transfer efficiency, the capillary action was allowed to proceed overnight. Following transfer,

the RNA was fixed to the membrane by UV-crosslinking at 1200μJ and was then washed for 5 minutes in DEPC-dH_20.

Radiolabelled DNA probes complementary in sequence to a particular RNA of interest were used to locate it on the membrane. The pol III B2 gene probe was a 240bp *Eco*RI-*Pst*I fragment from pTB14 (White et al., 1989) and the pol II acidic ribosomal phosphoprotein P0 (ARPP P0) probe, a 1kb *Eco*RI-*Hind*III fragment from the mouse cDNA (Hurford et al., 1997). The probes were labelled using a Megaprime DNA labelling system (Amersham) according to the random oligonucleotide priming method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1984). This method involved the addition of 5μl of random hexamer oligonucleotide sequences and the appropriate volume of DEPC-dH_20 for a final volume of 50μl in the total reaction to 25ng of purified DNA template, which was subsequently denatured by heating at 95°C for 5 minutes. DNA synthesis is primed by the hexamer oligonucleotides which are able to anneal to the DNA during slow cooling to room temperature. Labelling was carried out at 37°C for 1hour in 1 x reaction buffer (containing Tris-HCl pH 7.5, 2-mercaptoethanol, $MgCl_2$)(Amersham) following the addition of 4μl each of dATP, dGTP, dTTP (in Tris-HCl pH 8.0, 0.5mM EDTA), 50μCi of [α^{32}P] dCTP (10mCi/ml, 3000Ci/mmol) and 2U DNA polymerase I Klenow fragment (in 100mM potassium phosphate pH 6.5, 10mM 2-mercaptoethanol, 50% glycerol). The reaction was stopped by heating at 80°C for 5 minutes and kept at 4°C until the nylon membrane with bound RNA had been pre-hybridised. This involved rotation in a hybridisation oven at 45°C for 45 minutes in 20ml of hybridisation buffer (0.2M sodium phosphate buffer pH 7.2, 1mM EDTA, 1% (w/v) BSA, 7% (w/v) sodium dodecyl sulphate (SDS), 45% (w/v) formamide in DEPC-dH_20). Following this, the

radiolabelled probe was added to 20ml of fresh hybridisation buffer, in which the membrane was incubated with rotation at 45°C overnight. The nylon membrane was then washed with rotation in wash buffer (40mM sodium phosphate buffer pH 7.2, 1mM EDTA, 1% (w/v) SDS in DEPC-dH_2O) at room temperature for 2 minutes and then twice for 15 minutes at 65°C in order to remove non-specific radioactivity before being exposed to autoradiography film overnight at –70°C. Membranes were stripped by incubating in boiling water for 5 minutes and pre-hybridised again prior to being reprobed. Quantification of the RNA was achieved using a phosphoimager (Fujix Bas 1000).

2.6 Preparation of cDNAs

cDNAs were prepared from 3μg of RNA. Primer annealing was carried out in a final volume of 24μl with 0.67 x hexanucleotide mix (Roche) (diluted in DEPC-dH_20) and allowed to proceed for 10 minutes before transferral to ice. 8μl of 5 x First Strand Buffer (Life Technologies), 4μl of 0.1M DTT, 2μl of 10mM dNTP mix (made up in DEPC-dH_20) and 1μl (200U) of Superscript II Reverse Transcriptase (Life Technologies) was added to initiate reverse transcription, which was performed for 1hour at 47°C before the reaction was stopped by heating at 70°C for 15 minutes.

2.7 Reverse transcriptase – Polymerase chain reaction (RT-PCR)

PCRs were carried out using a PTC-100 thermal controller (MJ Research, Cambridge, MA). 2μl of cDNA was amplified with 20pmol of either ARPP P0 primers (5'-GCACTGGAAGTCCAACTACTTC-3' and 5'-TGAGGTCCTCCTTGGTGAACAC-3') to give a 265bp product; $tRNA^{Leu}$ primers (5'-GTCAGGATGGCCGAGTGGTC-TAAG-3' and 5'-CCACGCCTCCATACGGAGACCAGAAGACCC-3') to give an 88bp product; U6 RNA primers (5'-GCTCGCTTCGGCAGCACATATAC -3' and 5'-TATCGAACGCTTCACGAATTTGCG-3') to give a 96bp product; B'' primers (5'-GCTGATAGAGATACTCCTC-3' and 5'-CCAGAGACAAGAATCTTCTC-3') to give a 293bp product; TFIIIC220 primers (5'-TCCAGCGAGACCTTCACACC-3' and 5'-GGATTGAGTGTTGCTGGGCT-3') to give a 144bp product; TFIIIC110 primers (5'-CCAGAAGGGGTCTCAAAAGTCC-3' and 5'-CTTTCTTCAGAGAT-GTCAAAGG-3') to give a 303bp product; TFIIIC102 primers (5'-CCTACTAATGT-CCGTTATCTGTGG-3' and 5'-GCAGAAGTAACATCATTGGC-3') to give a 184bp product; TFIIIC90 primers (5'-AAACAGAAGTTGCTGAGTGA-3' and 5'-ATGGTCAGGCGATTGTCC-3') to give a 210bp product; TFIIIC63 primers (5'-CGGCAGATGTTCTACCAGTTATGCG-3' and 5'-ATGGCTTGAAGTCCTCCTC-C-3') to give a 300bp product. Amplification reactions contained 0.5U of *Taq* DNA polymerase (Promega) in 20μl of 1 x *Taq* DNA polymerase buffer (Promega) containing 1.5mM $MgCl_2$, 0.2mM of each dNTP and 1.8μCi of [α^{32}P] dCTP (10mCi/ml, 3000Ci/mmol).

PCR was performed under the following cycling parameters:

ARPP P0: 95°C for 2 minutes, 18 cycles of [95°C for 1 minute, 58°C for 30 seconds, 72°C for 1 minute], 72°C for 3 minutes.

$tRNA^{Leu}$: 95°C for 2 minutes, 30 seconds, 25 cycles of [95°C for 30 seconds, 68°C for 30 seconds, 72°C for 20 seconds], 72°C for 5 minutes.

U6 RNA: 95°C for 3 minutes, 16 cycles of [95°C for 1 minute, 60°C for 30 seconds, 72°C for 1 minute], 72°C for 5 minutes.

B'': 95°C for 2 minutes, 35 cycles of [95°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute], 72°C for 5 minutes.

TFIIIC220: 95°C for 3 minutes, 20 cycles of [94°C for 20 seconds, 62°C for 30 seconds, 72°C for 30 seconds], 72°C for 10 minutes.

TFIIIC110: 94°C for 3 minutes, 5 cycles of [95°C for 1 minute, 66°C for 40 seconds, 72°C for 40 seconds], 22 cycles of [95°C for 1 minute, 62°C for 40 seconds, 72°C for 40 seconds], 72°C for 5 minutes.

TFIIIC102: 95°C for 3 minutes, 30 cycles of [95°C for 30 seconds, 61°C for 30 seconds, 58°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute], 72°C for 5 minutes.

TFIIIC90: 95°C for 3 minutes, 23 cycles of [95°C for 1 minute, 55°C for 30 seconds, 72°C for 1 minute], 72°C for 5 minutes.

TFIIIC63: 95°C for 3 minutes, 22 cycles of [95°C for 1 minute, 69°C for 1 minute, 62°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute], 72°C for 5 minutes. In this case, 2mM $MgCl_2$ was used in the PCR mix.

Reaction products were resolved on 7% polyacrylamide sequencing gels containing 7M urea and 0.5 x TBE (45mM Tris, 45mM boric acid, 0.625mM EDTA pH 8.0). Gels were pre-run for 30 minutes at 40W in 0.5 x TBE and 2μl of each sample was loaded after being boiled at 95°C for 2 minutes and quenched on ice. Electrophoresis was carried out for a further 1hour at 40W and the gel subsequently vacuum-dried at 80°C for 1hour before being exposed to autoradiography film in order to detect the radiolabelled products. Quantification of results was achieved by phosphoimaging (Fujix Bas 1000).

2.8 Measuring protein concentration

The protein concentration of samples was determined using Bradford's reagent (Biorad). Quantification of the colour reaction produced upon mixing 1ml of diluted reagent (1:4 in distilled water) with a volume of sample containing protein in the range of ~1 –12μg gave an accurate indication of protein concentration. This was achieved by measuring the absorbance of these samples at 595nm in a UV spectrophotometer, as absorbance in response to increasing amounts of protein under these conditions is approximately linear. Absorbance readings obtained were then compared to a set of standards of known protein concentration using bovine serum albumin (BSA) measured at 595nm. A range of sample dilutions were measured and

compared in this manner in order to obtain an average, which would provide a more accurate measurement of protein concentration. Where sample absorbance readings fell outside the linear range of the standard set, appropriate dilutions were made and the samples re-measured.

2.9 Random polymerase assay

Assays of RNA polymerisation activity were based on a method described by Roeder (Roeder, 1974). Reactions were performed in a total volume of 50µl with a final concentration of 6mM HEPES pH7.9, 30mM KCl, 3.6mM $MgCl_2$, 6mM Tris pH 7.9, 200µM EDTA, 7.5mM ammonium sulphate, 800µM manganese chloride, 600µM rATP, rCTP and rGTP, 50µM UTP, 0.6mM DTT and 5% glycerol. This was supplemented with 5µg of poly(dA.dT) (2µl) to provide a non-specific template, 20µg BSA, 10µCi [α-^{32}P] UTP (400Ci/mmol) and typically 10 -15µg of cell extract (up to 15µl). For inhibition of pol II activity, α-amanitin (diluted in DEPC-dH_2O) was added to give a final concentration of 1µg/ml or of 100µg/ml for inhibition of both pol II and pol III activities. Reactions were performed at 30°C for 20 minutes and stopped by transferral to 2cm^2 Whatmann DE51 paper discs, which were then subjected to serial 5 minute washes: 6 washes in 0.5M Na_2HPO_4, twice in distilled water, twice in 96% ethanol and finally once in ether. Discs were then dried at room temperature for a few minutes and transferred to scintillation vials containing 5ml of Optiflow scintillation fluid (Fisons chemicals). Levels of incorporated activity were then measured in a scintillation counter counting ^{32}P for 5 minutes.

2.10 Transformation of competent cells

E.coli XL-1 Blue supercompetent cells (Stratagene) were transformed for plasmid storage and propagation. Cells, which were stored at –80°C and highly temperature sensitive, were thawed on ice to prevent loss of transformation ability. 0.4µl of β-mercaptoethanol, which enhances transformation efficiency, was added to the 50µl of cells that were required per transformation reaction to give a final concentration of 25mM. Typically 10 – 20ng of plasmid DNA was then gently mixed into the chilled cells. The contents were gently tapped occasionally during a 30 minute incubation on ice, before being heat shocked at 42°C for exactly 45 seconds and then transferred to ice for a further 2 minutes. Cells were incubated at 37°C for 1hour on an orbital shaker (225 - 250rpm) following the addition of 450µl of preheated (42°C) SOC medium (LB broth, 0.04% glucose, 10mM $MgSO_4$, 10mM $MgCl_2$). Typically 150µl of the transformation mixture was then plated on LB agar (2% LB, 2% agar) plates containing 50µg/ml ampicillin (Amp) and the plates were incubated at 37°C overnight to allow growth and colony-formation of the transformed cells.

2.11 Preparation of plasmid DNA

For large scale plasmid DNA preparation, a single isolated bacterial colony was selected from a freshly-streaked plate and used to inoculate 4ml of LB medium containing the selective antibiotic (50µg/ml ampicillin). This was allowed to incubate

with vigorous shaking at 37°C for ~6 hours to form a mini-culture and was subsequently used to inoculate 250ml of LB medium containing 50μg/ml ampicillin. Following an overnight incubation at 37°C on an orbital shaker (~300rpm), cells were harvested by centrifugation at 6000g for 15 minutes at 4°C and plasmid DNA retrieved using the QIAGEN Plasmid Maxi Kit.

The bacterial pellet was resuspended in 10ml of Buffer P1 (50mM Tris pH 8.0, 10mM EDTA, 100μg/ml RNase A) and then gently but thoroughly mixed with 10ml of Buffer P2 (200mM NaOH, 1% SDS) to initiate an alkaline lysis reaction. This reaction was allowed to proceed for 5 minutes at room temperature before neutralising the lysate by the addition of 10ml of chilled Buffer P3 (3M potassium acetate, pH 5.5) which subsequently resulted in formation of a precipitate of potassium dodecyl sulphate. The SDS-denatured proteins and chromosomal DNA were co-precipitated with the detergent whilst the plasmid DNA remained in solution due to a lack of close protein associations. Precipitation was enhanced by a 20 minute incubation on ice and the precipitate pelleted by centrifugation at 20 000g for 30 minutes at 4°C. The supernatant containing plasmid DNA was promptly removed and applied to a QIAGEN-tip 500 pre-equilibrated with 10ml of Buffer QBT (750mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100). Gravity flow allowed the supernatant to pass through the anion-exchange resin to which plasmid DNA is able to tightly bind. The resin was then washed twice with 30ml of Buffer QC (1M NaCl, 50mM MOPS pH 7.0, 15% isopropanol) and the purified plasmid DNA was subsequently eluted with 15ml of Buffer QF (1.25M NaCl, 50mM Tris pH 8.5, 15% isopropanol) and precipitated with 10.5ml (0.7 volume) of room-temperature isopropanol. This was immediately followed with a 15 000g centrifugation at 4°C for

30 minutes. The plasmid DNA pellet was then washed with 70% ethanol, dried at room-temperature for 5 – 10 minutes and resuspended in an appropriate volume of sterile water or TE buffer, pH 8.0 (10mM Tris pH 8.0, 1mM EDTA).

2.12 RNA pol III *in vitro* transcription assay

In vitro transcription of class III genes was reconstituted using 20μg of cell extracts to provide the basal pol III transcription components. This was supplemented with the addition of 250ng of plasmid DNA to supply a specific pol III template and reactions were carried out in a 25μl volume with a final concentration of 12mM HEPES pH 7.9, 60mM KCl, 7.2mM $MgCl_2$, 0.28mM EDTA, 1.2mM DTT, 10% (v/v) glycerol, 1mM creatine phosphate, 0.5mM each of rATP, rCTP and rGTP and 10μCi [α-^{32}P] UTP (400mCi/mmol) (Amersham). Transcription components were assembled on ice and the reaction was performed at 30°C for 1hour. In the case of assays incorporating additional reagents, a 15 minute pre-incubation at 30°C was carried out prior to adding the nucleotides required to initiate transcription. Transcription was terminated by the addition of 250μl of 1M ammonium acetate/0.1% SDS containing 20μg of yeast tRNA which acts as a stabiliser for the synthesised RNA. Phenol-choloroform extraction of the samples was performed to remove protein and DNA by adding 250μl of a 25:24:1 ratio solution of PhOH/$CHCl_3$/IAA. The samples were vortexed, microcentrifuged at 13 000g for 5 minutes and 200μl of the upper aqueous layer was then transferred to a fresh eppendorf tube containing 750μl of 96% ethanol in order to precipitate the RNA. The samples were thoroughly mixed by repeated inversion, left

at –20°C overnight before being microcentrifuged at 13 000g for 20 minutes to pellet the precipitated RNA. The supernatant was carefully removed and 750µl of 70% ethanol was added to each sample to wash the pellet. This was also carefully removed to avoid dislodging the pellet and the samples were heated at 47°C for 5 – 10 minutes to dry. 4µl of formamide loading buffer (98% formamide, 10mM EDTA pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol FF) was added to each sample, which was then vortexed for 20 minutes to ensure the RNA was fully redissolved. 2µl of each sample was loaded on a pre-run 7% polyacrylamide sequencing gel containing 7M urea and 0.5 x TBE (45mM Tris, 45mM boric acid, 0.625mM EDTA pH 8.0) after being boiled at 95°C for 2 minutes and quenched on ice. Electrophoresis was performed at 40W for 1hour in 0.5 x TBE before being dried and exposed to autoradiography film in order to detect the radiolabelled transcripts. Quantification of results was achieved by phosphoimaging (Fujix Bas 1000).

The plasmid templates used for *in vitro* transcription assays were as follows: pVA_1 is a 221bp *Sal*I-*Bal*I fragment of adenovirus 2 DNA containing the VA_1 gene subcloned into pUC18 (Dean and Berk, 1988). pTB14 is a 0.2kb *Bgl*II fragment containing a mouse B2 gene subcloned into pUC18 (White et al., 1989). pE2-160 contains the EBER2 gene (Howe and Shu, 1989). pMcet1 contains a *C. elegans* $tRNA^{Pro}$ gene (Ciliberto et al., 1982). pGlu6 is a 490bp *Bam*H1-*Eco*R1 fragment of genomic DNA containing the human $tRNA^{Glu6}$ gene subcloned into pAT153 (White et al., 1995b).

2.13 TFIIIB activity assay

TFIIIB activity assays were performed using 8μg of cell extracts made up to a volume of 10μl with microextraction buffer (450mM NaCl, 50mM NaF, 20mM Hepes pH 7.8, 25% glycerol, 1mM DTT, 0.5mM PMSF, 0.2mM EDTA, 40μg/ml bestatin, 1μg/ml trypsin inhibitor, 0.7μg/ml pepstatin, 0.5μg/ml aprotinin, 0.5μg/ml leupeptin) and LDB_0 (20mM Hepes pH 7.9, 12mM $MgCl_2$, 0.1mM EDTA, 17% glycerol, 2mM DTT) to achieve a final optimal salt concentration of 60mM. These were heat-treated to specifically inactivate TFIIIC and TBP by a 15 minute incubation at exactly 47°C. 5μl of a 1:4 mix of TBP to PC-C (a TFIIIC-containing phosphocellulose-column fraction), required for reconstitution of pol III transcription, was added to each sample. These heat-treated extracts were then analysed by *in vitro* transcription assay.

2.14 Immunoprecipition and immunodepletion

Antibodies for immunoprecipitation experiments were coupled to protein A-Sepharose beads. 20μl of packed beads was used per sample and beads were washed twice with 1 x TBS (2.5mM Tris-HCl pH 7.6, 15mM NaCl) prior to incubation with the appropriate antibody on a shaker for 1hour at 4°C. The beads were then washed twice with 1 x TBS to ensure removal of unbound antibody.

For immunodepletion experiments, these beads, carrying equivalent amounts of prebound immunoglobulin (IgG) were incubated on ice with approximately 150μg of cell extract (made up to a volume of 30μl with 1 x TBS) for 2 hours, gently mixed by tapping every 5 minutes. Beads were pelleted by gentle pulse microcentrifugation and 4μl of the supernatant (immunodepleted extract) was analysed by *in vitro* transcription assay.

For co-immunoprecipitation reactions, typically 200 - 500μg of cell extract (made up to a total volume of ~300μl with 1 x TBS) was incubated with the prepared protein A-Sepharose beads at 4°C for 3 hours on an orbital shaker. The beads were gently pelleted by pulse microcentrifugation and the supernatants carefully removed. The beads were then subjected to extensive washing (once in 300μl of 1 x TBS, 0.25mM NaCl, 0.5% Triton X-100 and a further 3 – 4 washes in 300μl of 1 x TBS) before the bound material was released by the addition of an equal volume of 2 x protein sample buffer (125mM Tris pH 6.8, 1% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.25% bromophenol blue) and analysed by SDS-PAGE and subsequent western blotting for the protein of interest.

2.15 Separation of proteins by polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved on denaturing polyacrylamide gels according to molecular weight by electrophoresis. Typically, 7.8% polyacrylamide resolving minigels

(375mM Tris pH 8.8, 0.1% SDS) were used with a stacking layer comprised of 4% polyacrylamide gel (125mM Tris pH 6.8, 0.1% SDS) based on the discontinuous buffer system described by Laemmli (Laemmli, 1970). Samples were boiled for 2 minutes in 1 x protein sample buffer (62.5mM Tris pH 6.8, 0.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.125% bromophenol blue) prior to loading. Electrophoresis was performed in 1 x SDS running buffer (0.1% SDS, 76.8mM glycine, 10mM Tris, pH 8.3) at an initial voltage of 70V while the bromophenol dye front moved through the stacking gel and a subsequent voltage of 140V after reaching the resolving gel. Electrophoresis was allowed to proceed until the dye front had reached the bottom of the gel, approximately 1 – 1.5 hours.

2.16 Western blotting

Electrophoretic transfer of proteins resolved by SDS-PAGE to PVDF membrane was achieved using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system. Transfer was carried out in 1 x transfer buffer (76.8mM glycine, 10mM Tris, pH 8.3, 16.5% methanol) at 50V for 1 hour. Following transfer, the membrane was blocked in milk buffer (32.5mM Tris, 150mM NaCl, 0.2% Tween-20, 4% skimmed milk powder (Marvel)) for 1hour at room temperature. Membranes were incubated with primary antibodies (typically a 1:1000 dilution in milk buffer) overnight at 4°C. Excess primary antibody was removed by washing the blot 3 times for 3 minutes in fresh milk buffer before incubating for 1hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000 dilution in

milk buffer) (DAKO). To ensure removal of excess secondary antibody, the blot was sequentially washed in batches of fresh milk buffer, 3 times for 3 minutes, followed by 2 washes for 15 minutes. After one further 5 minute wash using 1 x TBS (2.5mM Tris-HCl pH 7.6, 15mM NaCl), the blot was developed using the enhanced chemiluminescence method (ECL, Amersham) as directed by the manufacturers.

2.17 Antibodies

Ab2E	anti-TFIIIC220 (Shen et al., 1996)
AC19	anti-AC19 (Generous gift from J. Zomerdijk)
C11	anti-actin (Santa Cruz)
C15	anti-RB (Santa Cruz)
C18	anti-p70S6 kinase (Santa Cruz)
C18	anti-TFIIB (Santa Cruz)
F4	anti-Py T Ag (Oncogene science)
K-23	anti-ERK2 (Santa Cruz)
M19	anti-TAF_I48 (Santa Cruz)
MTBP-6	anti-TBP (Pruzan et al., 1992)
p44/42 MAPK	anti-total ERK (New England Biolabs)

phosphoERK	anti-active ERK (New England Biolabs)
R-113	anti-Phas1 (Santa Cruz)
SL-1	anti-TBP (Lobo et al., 1992)
SL30	anti-TBP (Ruppert et al., 1996)
TVG710Y	anti-HPV16-E7 (Santa Cruz)
T4-7220	anti-TFIIIC110 (Transduction laboratories)
113	anti-BN51 (Ittmann et al., 1993)
128-4	anti-BRF (Cairns and White, 1998)
330-4	anti-BRF (Alzuherri and White, 1998)
2663-4	anti-B" (Schramm et al., 2000)

2.18 Transient transfection

Expression vectors coding for Polyomavirus large T antigen (pSV-LT), middle T antigen (pSV-MT) and a transforming mutant (pSV-NG59) were kindly provided by Kurt Ballmer-Hoffer. An "empty" pSV expression vector was produced by a double restriction digest of the pSV-MT insert with *Eco*R1 and *Hind*III and the linearised vector was then separated from the insert by electrophoresis through a 1% low melting point agarose gel. Gel-purification of the vector was achieved using a QIAquick gel extraction kit (Qiagen) and *Eco*R1 and *Hind*III 5' protruding ends were

blunted using Klenow DNA polymerase. The vector was then re-ligated overnight at 15°C using T4 DNA ligase and the ligation product used to transform E.coli XL-1 Blue supercompetent cells (Stratagene). 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 digests performed with *Eco*R1 to verify that cells contained the correct vector and lacked the insert prior to large scale plasmid preparation using a QIAGEN Plasmid Maxi kit. Again, plasmid preparations were verified and DNA concentrations determined by UV spectrophotometry.

Balb/c 3T3 Clone A31 cells for transient transfection were plated out at 3 x 10^5 cells/well on 6-well plates 24 hours prior to transfection, resulting in a confluency of ~70 –80% at the time of transfection. Two wells were transfected per treatment with a total of 4μg of plasmid DNA per well of cells; this was comprised of 0.5μg of VA_1, 0.5μg of SV40 CAT (Promega) and 3μg of "empty" pSV expression vector, pSV-LT, pSV-MT or pSV-NG59. Mastermixes for each set of wells were made up comprising the appropriate plasmid DNA and neat DMEM media to give a volume of 100μl per well. These were incubated at room temperature for 5 minutes and 100μl vortexed with 8μl of Superfect (Qiagen) for each well to be treated. Following a 10 minute incubation period at room temperature, 600μl of DMEM (10% fetal calf serum (Sigma), 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin) was used to dilute each Superfect mix. Media was removed from the cells prior to addition of the mix and plates swirled to ensure the wells were evenly covered. Treatment was carried out for 3 hours at 37°C before removing the mix, washing the cells once with warm PBS and applying fresh media. Cells were incubated for a further 48 hours to allow expression of transfected DNA; with media being renewed

again after 24 hours. Cells were harvested and total RNA extracted for analysis by primer extension.

2.19 Primer extension

Expression levels of the transfected pol III template VA_1 and the CAT gene, which was co-transfected as an internal control for transfection efficiency, were analysed by primer extension. VA_1 (5'-CACGCGGGCGGTAACCGCATG-3') or CAT (5'-CGA-TGCCATTGGGATATATCA-3') oligonucleotides were γ-^{32}P end-labelled using T4 polynucleotide kinase (PNK). For each primer extension reaction, 10μg of total RNA (made up to 10μl with DEPC-dH_20) and incubated at 80°C for 10 minutes with 9μl of 5 x First Strand Buffer (Life Technologies) and 1μl (2.5ng) of the relevant probe to act as a primer. Samples were immediately transferred to a 50°C hotblock for a further 2 hours incubation. 30μl of an elongation mix (23μl DEPC-dH_20, 0.5μl 1M DTT, 5μl 5mM dNTP mix (5mM in DEPC-dH_2O), 0.5μl 4mg/ml actinomycin D, 0.5μl RNasin, 0.5μl (100U) of Superscript II Reverse Transcriptase (Life Technologies)) was then added to the samples to initiate reverse transcription and the reaction was allowed to proceed for 1hour at 42°C. Reaction products were precipitated overnight at –20°C by the addition of 5μl of 3M sodium acetate and 125μl of ethanol and subsequently pelleted by microcentrifugation at 13 000g for 15 minutes. Pellets were washed with 300μl of 75% ethanol and dried at 47°C for 5 minutes before being resuspended by vortexing for 10 minutes in 4μl of formamide loading buffer (98% formamide, 10mM EDTA pH 8.0, 0.025% bromophenol blue,

0.025% xylene cyanol FF). Electrophoresis through a 7M urea 7% polyacrylamide gel was used to resolve the samples, the reaction products detected by overnight exposure to autoradiography film at –80°C and quantitated by phosphoimaging (Fujix Bas 1000).

2.20 Electrophoretic mobility shift assay (EMSA)

TFIIIC2 DNA-binding activity was determined by EMSAs which were carried out using a γ-^{32}P labelled oligonucleotide containing a B-block consensus (5'-AGAGGT-CCTGAGTTCAAATCCCAG-3' (RJW1) annealed to the complementary 3' to 5' strand (RJW2)). An oligonucleotide that contained a consensus Sp1 binding site (5'-ACTTGATTAACTGGGCGGAGTTATGATTGA-3' (M1) annealed to the complementary 3' to 5' strand (M2)) was employed to assay for Sp1 DNA-binding activity. For use in EMSAs, oligonucleotides were 5' end-labelled using T4 polynucleotide kinase (PNK). 40ng of RJW1 or M1 was assembled on ice with 10U of PNK in 1 x PNK buffer (Promega) and following the addition of 20μCi of [γ-^{32}P] dATP (10mCi/ml, 3000Ci/mmol) to give a total volume of 10μl, the reaction was performed at 37°C for 1hour. This was stopped by heating at 65°C for 10 minutes and was succeeded by phenol-chloroform extraction of the PNK enzyme; achieved by addition of 50μl of PhOH/$CHCl_3$/IAA (25:24:1) followed by vortexing and microcentrifugation at 13 000g for 5 minutes. The aqueous layer was transferred to a fresh eppendorf tube and 5μl of 3M sodium acetate and 125μl of 100% ethanol added. After a 30 minute incubation on dry ice, the precipitated oligonucleotide was pelleted

by microcentrifugation at 13 000g for 10 minutes. The supernatant was removed and the pellet washed by sequential addition and removal of 100µl of 70% ethanol to ensure removal of unincorporated label. The pellet was then dried by heating at 47°C for 10 minutes before being redissolved by incubation at 30°C for 30 minutes in 20µl of TE buffer (10mM Tris pH 8.0, 1mM EDTA). This was followed by heating in a hotblock at 90°C for 2 minutes in the presence of unlabelled complementary oligonucleotide (RJW2') which was added in 2.5 fold excess to ensure that all labelled oligonucleotide was annealed. The hotblock was then turned off and the sample allowed to cool slowly overnight, after which, it was stored at 4°C until ready for use.

Each binding reaction was performed in a total volume of 10µl, with an optimal salt concentration of 60mM KCl and contained 1µg of poly(dI.dC) (2µl), 100ng of non-specific or specific competitor oligonucleotide (2µl), typically 2 - 4µl of cell extract and 0.25 – 0.5ng of labelled probe (2µl). A pre-incubation of 15 minutes at 30°C was carried out prior to addition of the probe, followed by a further 15 minutes at 30°C. Analysis of the formation of protein-DNA complexes was achieved by electrophoresis of samples on a pre-run 4% nondenaturing polyacrylamide gel in 1 x TAE buffer (40mM Tris acetate, 1mM EDTA pH 8.0) for 1.5 -2 hours at 4°C. The gel was dried for 1.5 hours at 80°C and exposed to autoradiography film overnight at –70°C. Phosphoimaging (Fujix Bas 1000) was used for quantification.

Chapter 3

Deregulation of pol III transcription in Polyomavirus-transformed cells

3.1 INTRODUCTION

The fundamental objective of the cell cycle is the faithful replication of DNA during S phase and the accurate segregation of the sister chromatids during mitosis. In eukaryotic cells, these events are highly regulated processes and the discrete transitions through stages in the cell cycle are subject to checkpoints. In order for cells to complete a full cycle, favourable conditions during the first two thirds of the G_1 phase are required. Upon passing through the so-called R (restriction) point before the end of G_1, they commit to the remaining steps of the mitotic cycle and enter into a state of serum-independence (Weinberg, 1995). Checkpoints ensure that in conditions of low serum, cell damage or the absence of the correct signals, the cell cycle is arrested and cells enter into a state of quiescence (Elledge, 1996).

Given that pol III is responsible for the transcription of a variety of essential cellular products, including tRNA, 5S rRNA, the 7SL component of the signal recognition particle and the U6 small nuclear RNA that is required for mRNA splicing (Willis, 1993), it could be presumed that the genes encoding these housekeeping transcripts would be constitutively active. On the contrary, however, it has been clearly established that pol III transcription is strongly regulated in response to a variety of external stimuli and environmental conditions (Brown et al., 2000; White, 1998b).

Indeed, transcription levels are intrinsically linked to growth conditions, falling when serum factors or nutrients are limiting and increasing in response to mitogens. Upon mitogenic stimulation, there is an elevation of tRNA and rRNA synthesis, ribosomal protein assembly is accelerated and translation factors activated, overall resulting in a net increase in protein accumulation before cells reach S phase (Johnson et al., 1974; Rosenwald, 1996). Conversely, in quiescent cells tRNA and rRNA levels are down-regulated, polysomes disassemble and a decrease in the overall rate of protein synthesis is observed (Abelson et al., 1974; Clarke et al., 1996; Mauck and Green, 1974). This transcriptional response to quiescence is consistent with the regulation demonstrated by pol III with respect to the cell cycle. When mammalian cells begin to proliferate, pol III activity increases just prior to the G_1/S transition and maximal transcription levels are sustained throughout S and G_2 phases (White et al., 1995b). Previous studies implicate the retinoblastoma protein, RB, and its relatives p107 and p130, collectively termed the pocket proteins, in this pattern of expression (Scott, 2001; Sutcliffe et al., 1999). These regulatory proteins have been shown to associate with TFIIIB and repress pol III transcription, both *in vitro* and *in vivo* (Chu et al., 1997; Larminie et al., 1997; Sutcliffe et al., 1999). During G_0 and early G_1 phase, RB and p130 bind and repress TFIIIB, dissociating again on the approach to S phase to permit an increase in transcription (Scott, 2001).

When cells enter mitosis at the end of interphase, there is a general cessation of nuclear gene expression (Chiang et al., 1993; Prescott and Bender, 1962). With regard to pol III transcription, this is achieved through the phosphorylation of TFIIIB (Gottesfeld et al., 1994; White et al., 1995b). The components of this multisubunit complex have now been elucidated and, as in yeast, it comprises the TATA-binding protein TBP, the TFIIB-related factor BRF and a third subunit B" (Schramm et al.,

2000). Although TBP is hyperphosphorylated in extracts of mitotic HeLa cells, restoration of transcription cannot be achieved by the addition of unphosphorylated recombinant TBP, requiring instead TBP-associated components of TFIIIB. Thus, the mitotic repression of pol III activity, mediated through TFIIIB, is by a specific inactivation of one or more of the TBP-associated components of this complex (White et al., 1995b).

Reversal of TFIIIB hyperphosphorylation ensues the exit of cycling human cells from mitosis, although TFIIIB activity is not restored during early G_1 phase (White et al., 1995a). This continued repression correlates strongly with the activity of the pocket proteins, which become dephosphorylated at the end of mitosis (Mittnacht, 1998) and are, consequently, active through early G_1 phase and able to bind and inhibit TFIIIB until the restriction point is reached in mid to late G_1 phase (Scott, 2001).

In addition to the regulation conferred by growth conditions and cell cycle control mechanisms, pol III transcription is subject to another, although unrelated, tumour suppressor protein, p53. It has been demonstrated that within its array of functions, is the ability of p53 to repress pol III transcription (Cairns and White, 1998; Chesnokov et al., 1996). Similar to RB, regulation is conferred by binding and inactivating TFIIIB, although the susceptibility of TFIIIB to p53-repression is significantly blocked following assembly into a pre-initiation complex (Cairns and White, 1998). During neoplastic transformation, RB and p53 are frequently lost or mutated (Hollstein et al., 1991; Weinberg, 1995) and the consequent release of TFIIIB from important restraints may constitute a pivotal process in transformation (Brown et al., 2000).

Furthermore, TFIIIB is repeatedly targeted for deregulation during viral transformation, demonstrating another mode of deregulation for pol III transcription (White, 1998b). Most transformed and tumour cells display elevated levels of pol III products, although this is a manifestation of a variety of mechanisms employed by transforming viruses (DeCaprio et al., 1988; Piras et al., 1996; Wang et al., 1997; Yoshinaga et al., 1986). Invariably, TFIIIB and TFIIIC are the focus for deregulation, with TFIIIB often being targeted for release from repression by RB (Larminie et al., 1999; White et al., 1996) and the active or limiting subunits of TFIIIB and TFIIIC commonly being selectively increased (Larminie et al., 1999; Sinn et al., 1995; Wang et al., 1995). Other modes of action, however, include counteracting the effect of another cellular repressor, Dr1, as observed in transformation by the E1A oncoprotein of adenovirus (Kraus et al., 1994) and, in the case of hepatitis B virus (HBV), the viral X gene has been established as an activator of kinase signalling pathways which are implicated in the stimulation of pol III transcription (Wang et al., 1995). Whilst clearly a range of activation mechanisms are utilised by viruses, the polymerase itself appears to remain exempt from direct deregulation in all studies to date, with stimulation of pol III transcription, instead, being mediated through the activities of pol III-specific transcription factors. Nonetheless, as transforming mechanisms are being elucidated, an increasing abundance of evidence highlights the key role of pol III transcription in the progression towards tumourigenesis.

This chapter serves to establish the capacity of another transforming virus, Polyomavirus, to deregulate cell growth and cell cycle control. Polyomavirus, which is able to induce neoplasms in a wide variety of cell types (Eddy et al., 1958), is a nuclear icosahedral virus containing a circular genome of double-stranded DNA (Oliveira et al., 1999). Like the closely related Simian Virus 40 (SV40),

Polyomavirus is a small DNA tumour virus belonging to the papovavirus family (McCormick et al., 1982). It produces 3 oncogenic T antigens, large, middle and small, which are all encoded by a common precursor mRNA that is differentially spliced to yield multiple monocistronic mature mRNAs (Griffin et al., 1980).

It has been established that middle T antigen is necessary and sufficient to induce morphological transformation and alterations in the growth properties of established cell lines (Raptis et al., 1985), while large T antigen is essential for immortalisation of primary cells in culture (Freund et al., 1992). Although the expression of small t is not required for cell transformation, it can complement middle T for tumour induction (Asselin et al., 1983) and transformation (Asselin et al., 1986) and independent expression of small t antigen in fibroblasts enables them to grow to high cell density (Noda et al., 1986).

Elevated levels of pol III transcripts following transformation was first discovered with murine fibroblast lines that have been transformed by SV40 (Scott et al., 1983; Singh et al., 1985) and Majello *et al* subsequently established an increase in pol III transcription by Polyomavirus transformation. (Majello et al., 1985). However, while studies have provided much clarification on the mechanisms of SV40-mediated deregulation of pol III transcription (Larminie et al., 1999), the mechanisms surrounding deregulation by Polyomavirus have remained elusive. This chapter presents data documenting the effect of Polyomavirus transformation on pol III transcription.

3.2 RESULTS

3.2.1 Characterisation of 3T3 and Py3T3 cells

3.2.1a Py3T3 cells display accelerated growth

Accelerated growth and uncontrolled proliferation are the hallmark of cellular transformation. A simple experiment, establishing rates of proliferation for a Polyomavirus-transformed fibroblast cell line, Py3T3, relative to the untransformed parental 3T3 cells, was conducted to highlight this fundamental deregulation and indicate appropriate passaging conditions for cell culture. Figure 3.1A graphs cell counts over a period of 6 consecutive days following plating out at 5 x 10^5 cells/ml. 3T3 cells reached at maximum of 1.5 x 10^6 cells/ml on day 5 and subsequently showed a decline in number as cells demonstrated contact inhibition. In contrast, Py3T3 cells, which exhibited a significantly higher rate of proliferation from the outset, were still increasing in number at day 6, having reached 2.6 x 10^6 cells/ml. This behaviour is consistent with previous studies documenting the rapid proliferation of cells following transformation and their inability to contact inhibit (Aaronson and Todaro, 1968)

3.2.1b Serum-independence of Py3T3 cells

To determine the response of 3T3 and Py3T3 cells to serum-withdrawal and serum-stimulation, levels of [^{3}H] thymidine incorporation into newly synthesised DNA were measured for serum-starved, serum-stimulated or asynchronous cells. 3T3 cells have previously been shown to enter a quiescent state with serum-withdrawal and to re-enter the cell cycle upon serum-stimulation (Abelson et al., 1974; Scott, 2001). As previously shown, actively growing 3T3 cells become quiescent after serum withdrawal (Figure 3.1B). These growth arrested cells were made to re-enter the cell cycle by the addition of media containing 20% serum and [^{3}H] thymidine incorporation data demonstrates the entry of these cells into S phase on serum stimulation (Figure 3.1B). Asynchronous 3T3 cells also show an increase in [^{3}H] thymidine uptake over quiescent levels, although this value is approximately 50% lower than synchronised cells. In contrast, Py3T3 cells fail to respond to serum-withdrawal and [^{3}H] thymidine incorporation levels remain similar to levels displayed by the asynchronous population. Furthermore, it is significant that the basal level of Py3T3 cells passing through S phase in the absence of serum is similar to that of the 3T3 cells following serum-stimulation. These results imply that while 3T3 cells withdraw from the cell cycle in serum-free conditions, Py3T3 cells are able to continue to pass through S phase in a serum-independent manner.

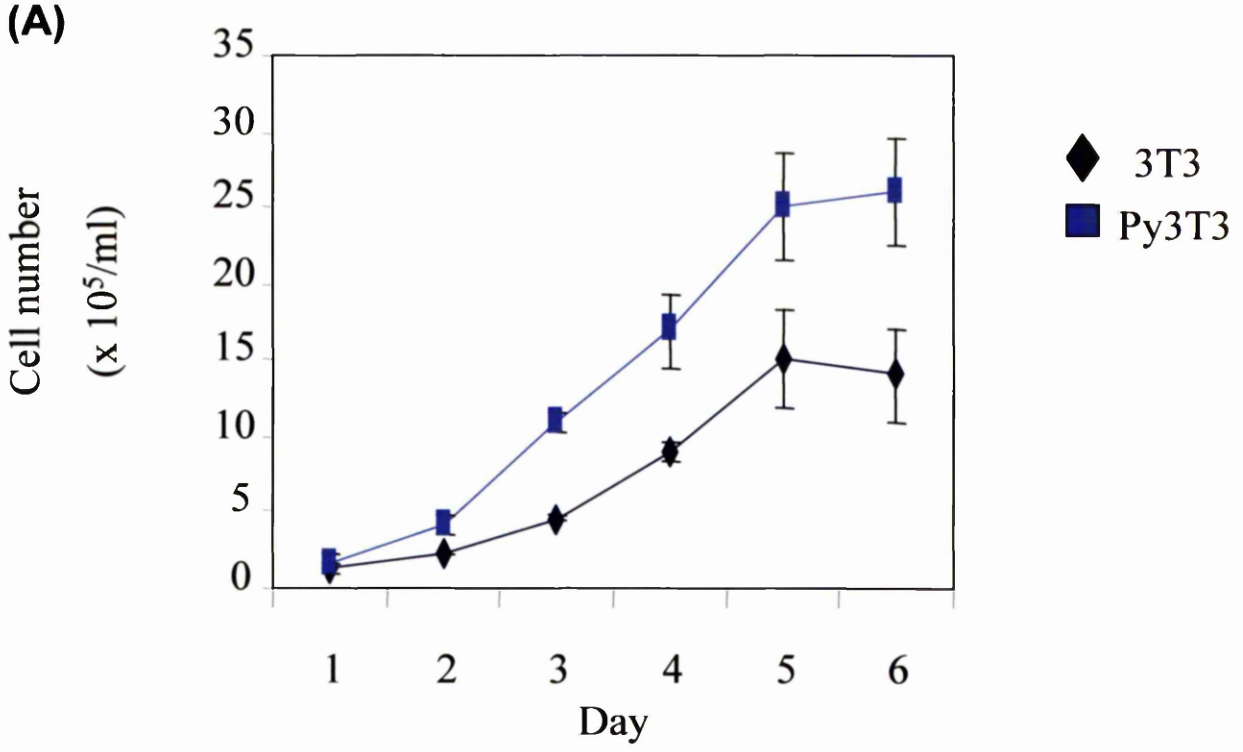
Figure 3.1

Py3T3 cells have diminished serum dependence

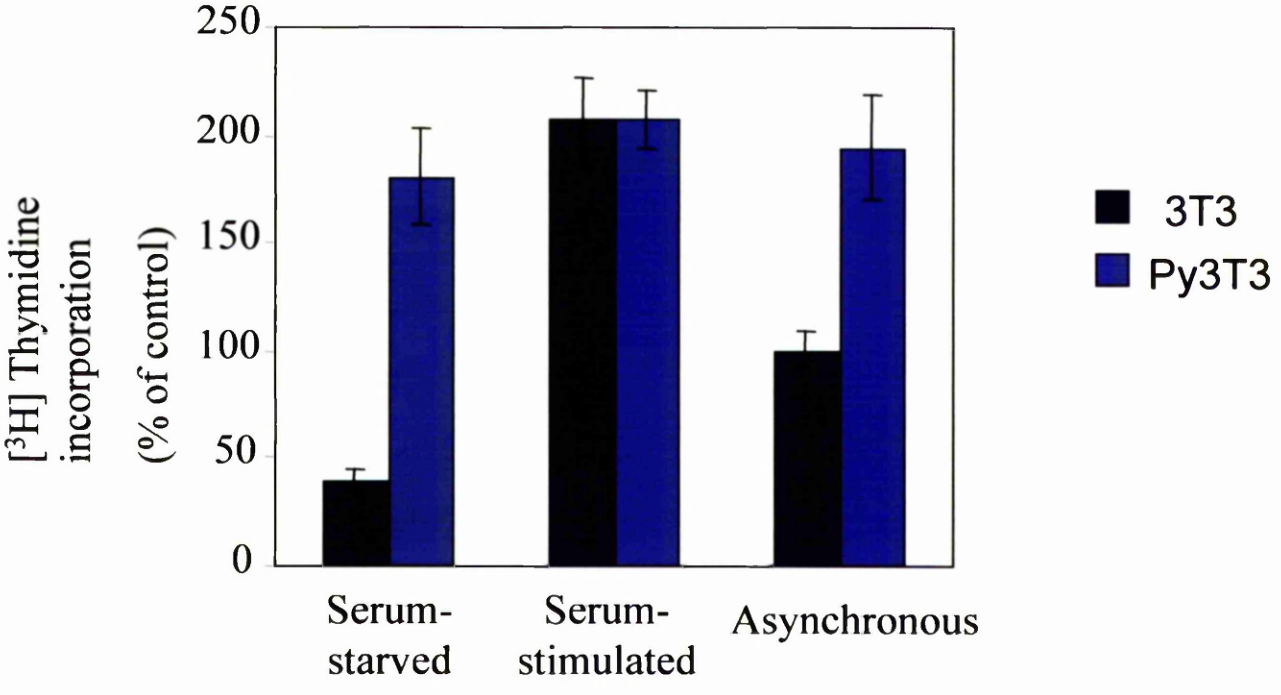
Panel A shows a growth curve of the relative rates of proliferation for 3T3 and Py3T3 cells. Cells were plated out at 5 x 10^5 cells/ml and cultured in DMEM medium supplemented with 10% fetal calf serum which was renewed daily. Counts were taken for each cell line over 6 consecutive days after plating. The values delineate the mean of two experiments ± standard deviation.

Relative levels of [^{3}H] thymidine incorporation into newly synthesised DNA of 3T3 and Py3T3 cells cultured for 24 hours in the absence of serum and subsequently stimulated with 20% serum for 3 hours prior to addition of [^{3}H] thymidine and a further 3 hour incubation before harvesting are illustrated in panel B. Asynchronous cells were cultured in 10% serum prior to [^{3}H] thymidine treatment. Values shown represent the mean of 20 counts taken over 2 experiments and are given relative to the value obtained for the asynchronous 3T3 population (designated 100%) ± standard deviation.

(A)


Cell number
(x 10^5/ml)
35
30
25
20
15
10
5
0
1
2
3
4
5
6
Day
3T3
Py3T3

(B)


[^{3}H] Thymidine incorporation
(% of control)
250
200
150
100
50
0
Serum-starved
Serum-stimulated
Asynchronous
3T3
Py3T3

3.2.2 Overexpression of pol III transcripts in Py3T3 cells

Previous studies, such as the activation of pol III transcription in SV40-transformed cells (Larminie et al., 1999; White et al., 1990), have established a precedent for a stimulation of pol III transcription by oncogenic viruses (reviewed in Brown et al., 2000). To investigate the effect of transformation by Polyomavirus on pol III transcription, RNA was extracted from 3T3 and Py3T3 cells and analysed by northern blotting. Figure 3.2A displays the steady state level of pol III transcripts derived from the B2 middle repetitive gene family. Py3T3 cells demonstrate a substantial overexpression relative to the 3T3 cells (compare lanes 1 and 2). It is evident from the levels of the pol II transcript encoding ARPP P0 that this overexpression does not extend to pol II transcripts and is instead, specific to pol III (Figure 3.2B). After values for B2 levels were normalised against those for ARPP P0 (Figure 3.2C), it was apparent that B2 transcript overexpression in the Py3T3 cells was in the region of 11-fold.

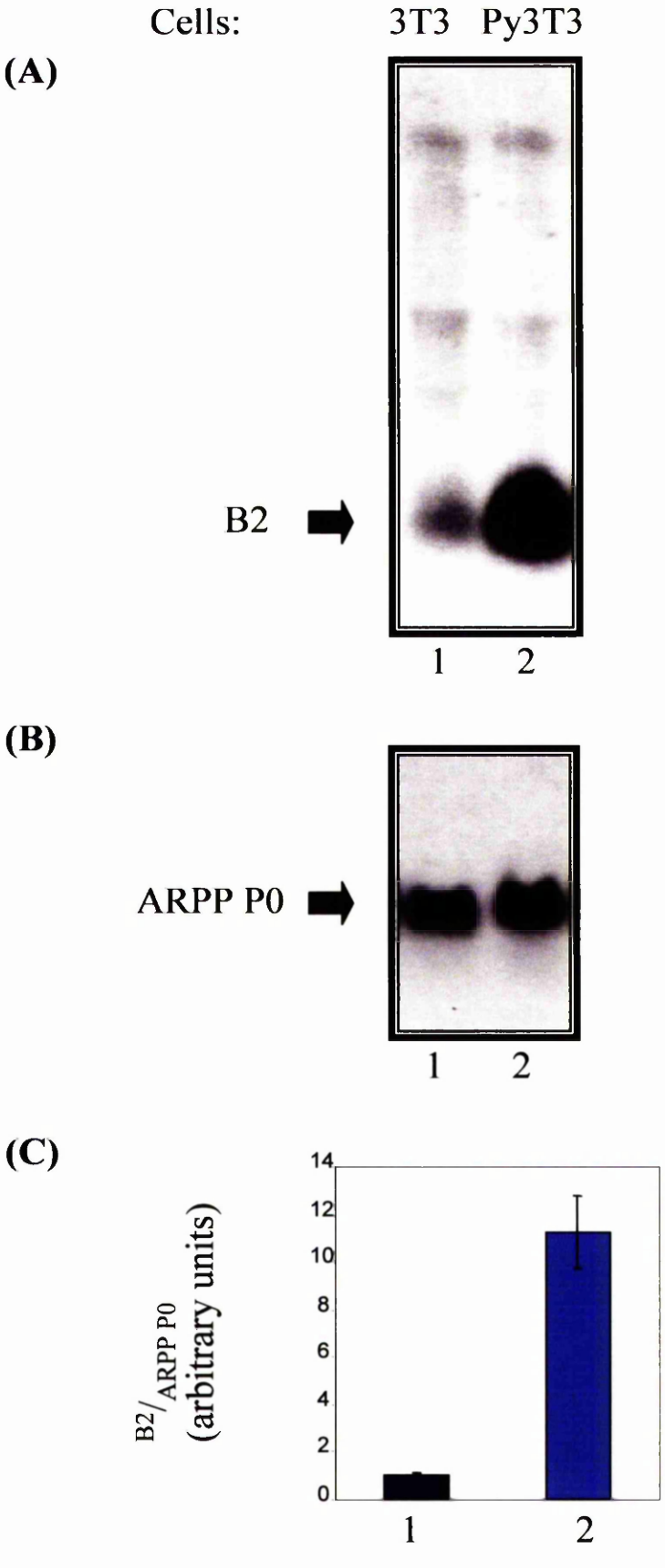
3.2.3 B2 overexpression is not dependent on cell confluency

The overexpression of the pol III B2 transcripts seen in figure 3.2 was clearly substantial. However, to ensure that this observation was not an artefact of proliferation rate and to address the implications of confluency of cells at the time of harvesting, RNA was extracted from 3T3 and Py3T3 cells over 6 consecutive days

Figure 3.2

Pol III transcripts are overexpressed in Py3T3 cells

Total RNA (30μg) was extracted from 3T3 (lane 1) and Py3T3 (lane 2) cells and used for northern blot analysis. Panel A shows the blot probed with a B2 gene. The same blot was stripped and subsequently reprobed with the ARPP P0 gene (Panel B). The levels of B2 and ARPP P0 RNA from the northern analysis were quantitated by phosphoimaging (Fujix Bas 1000); B2 levels were normalised against levels for ARPP P0 and expressed as arbitrary units. Values shown represent the mean of two experiments ± standard deviation, with the value obtained for the 3T3 cells being designated 1, as illustrated in panel C.


Cells:
3T3
Py3T3
(A)
B2
1
2
(B)
ARPP P0
1
2
(C)
B2/ARPP P0 (arbitrary units)
14
12
10
8
6
4
2
0
1
2

after plating and used for northern analysis. Figure 3.3A displays the steady state level of pol III-transcribed B2 RNA with Py3T3 cells again demonstrating a substantial overexpression relative to the 3T3 cells (compare lanes 1-6 with lanes 7-12). For both cell lines, levels of B2 transcripts start to decline at higher confluencies when growth is no longer in the exponential phase; however, the increase in Py3T3 cells of B2 transcripts holds, irrespective of confluency, and the B2 transcripts are consistently higher over the course of the 6 days. Although the levels of the pol II transcript encoding ARPP P0 exhibited some variation (Figure 3.3B), when values for B2 levels were normalised against those for ARPP P0 (Figure 3.3C), the overexpression of the B2 transcripts in the Py3T3 cells remained evident.

3.2.4 Py3T3 cells display deregulated pol III transcriptional activity

Support for the deregulation of pol III transcription is provided in figure 3.4. Specific pol III transcription reconstituted *in vitro* using the VA_1 template with 3T3 and Py3T3 cell extracts, prepared from cells harvested over a course of 4 consecutive days, shows an elevated level of transcriptional activity for the Py3T3 extracts (compare lanes 1-4 with lanes 5-8). Consistent with the increase in B2 levels seen for Py3T3 cells by northern analysis, the deregulation of reconstituted pol III transcription following transformation by Polyomavirus was apparent regardless of cell confluency at the time of harvesting.

Figure 3.3

Overexpression of pol III transcripts is not dependent on cell confluency

Northern blot analysis of total RNA (30μg) extracted from 3T3 (lanes 1-6) and Py3T3 (lanes 7-12) cells cultured in 10cm tissue culture dishes and harvested on consecutive days over a 6 day time course. Panel A shows the blot probed with a B2 gene. The same blot was stripped and subsequently reprobed with the ARPP P0 gene (Panel B). The levels of B2 and ARPP P0 RNA from the northern analysis were quantitated by phosphoimaging (Fujix Bas 1000); B2 levels were normalised against levels for ARPP P0 and expressed as arbitrary units, with the lowest value obtained for the 3T3 cells being designated 1, as depicted in panel C.

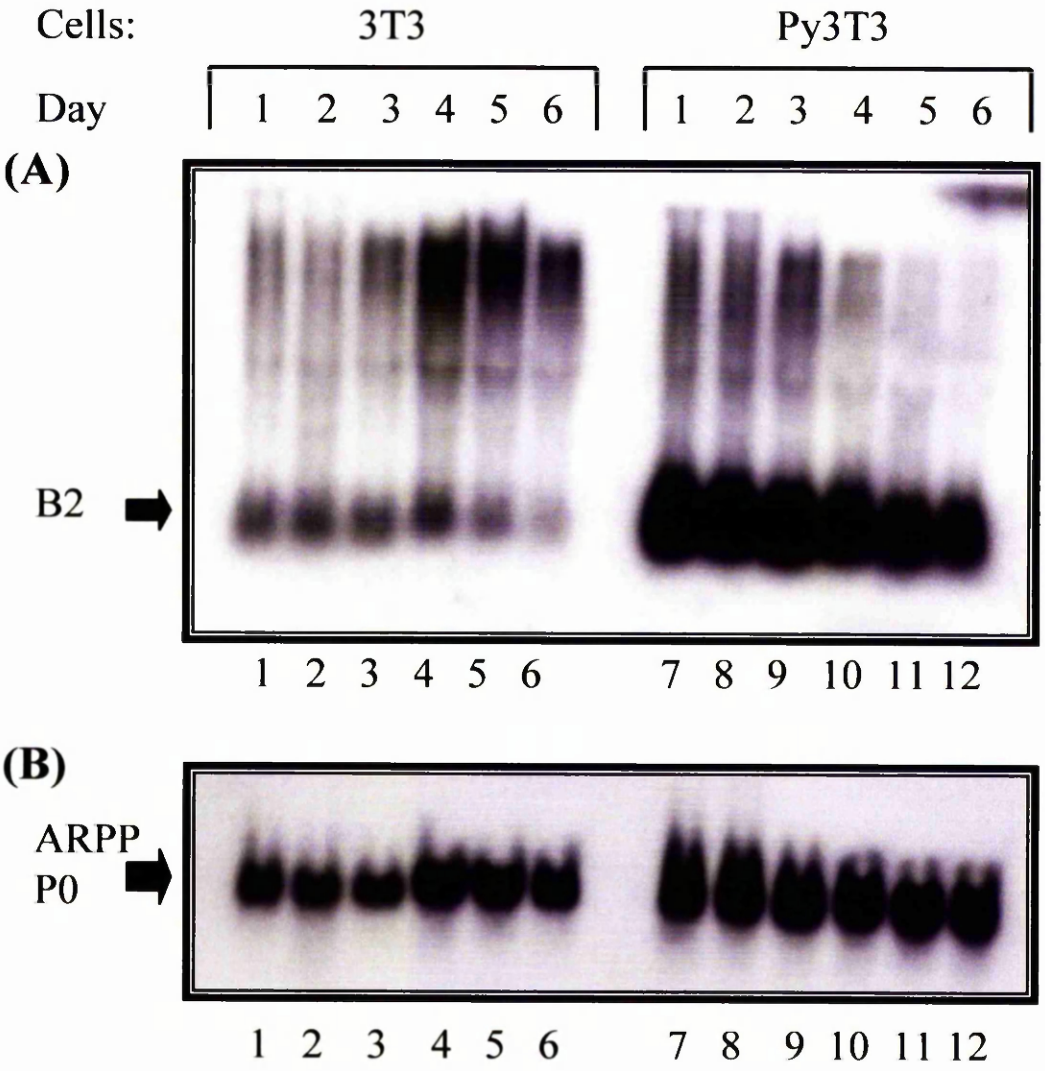
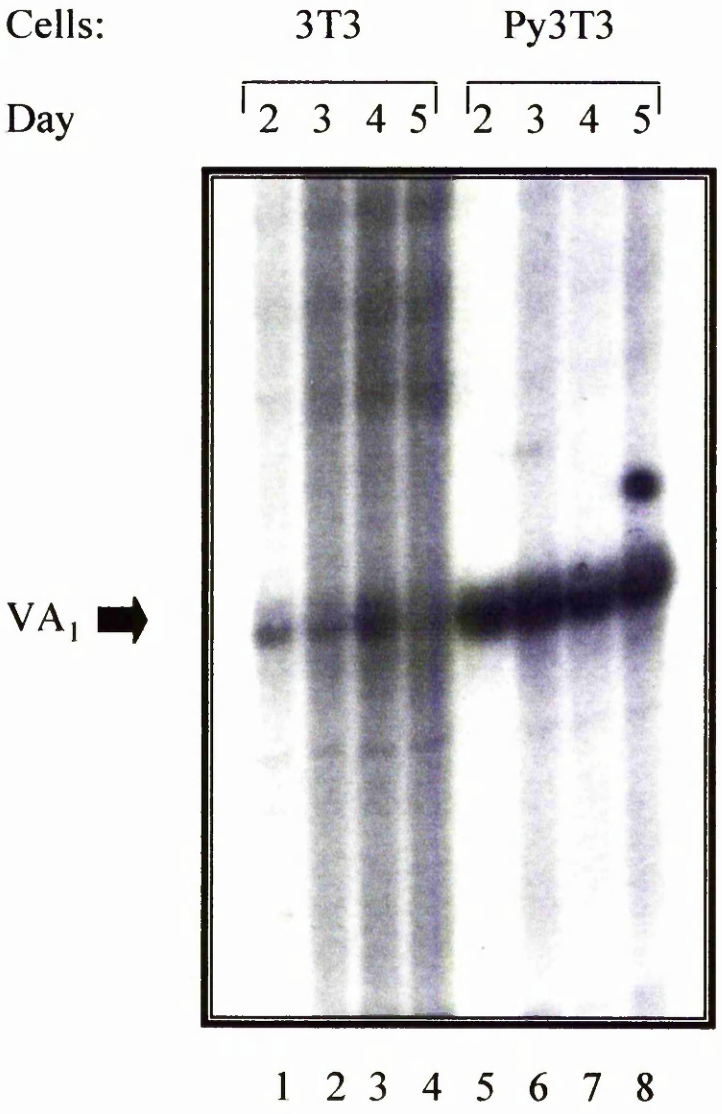

Cells:
3T3
Py3T3
Day
1 2 3 4 5 6
1 2 3 4 5 6
(A)
B2
1 2 3 4 5 6 7 8 9 10 11 12
(B)
ARPP
P0
1 2 3 4 5 6 7 8 9 10 11 12
(C)
B2/ARPP P0
(arbitrary units)
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.4

Deregulation of pol III transcription is displayed by Py3T3 cell extracts

In vitro transcription assay illustrating relative levels of pol III transcription for 3T3 (lanes 1-4) and Py3T3 (lanes 5-8) cell extracts harvested over a course of 4 days. Transcription reactions contained 250ng of pVA_1 template and 20μg of cell extract.


Cells:
3T3
Py3T3
Day
2 3 4 5
2 3 4 5
VA1
1 2 3 4 5 6 7 8

3.2.5 Transcriptional deregulation of Py3T3 cells is not gene specific

The *in vitro* transcription assay illustrated in figure 3.4 utilised the VA_1 template of adenovirus, as the VA_1 promoter is extremely well characterised and, therefore, routinely used in studies of mammalian pol III transcription. Figure 3.5 (compare lane 1 with lane 2 for each panel) demonstrates that the transcriptional deregulation observed for the Py3T3 cells is not a gene-specific phenomenon, since when *in vitro* transcription was reconstituted using a (A) B2, (B) EBER2, (C) $tRNA^{Pro}$ or (D) $tRNA^{Glu6}$ template, a significant up-regulation in transcriptional activity was seen in each case.

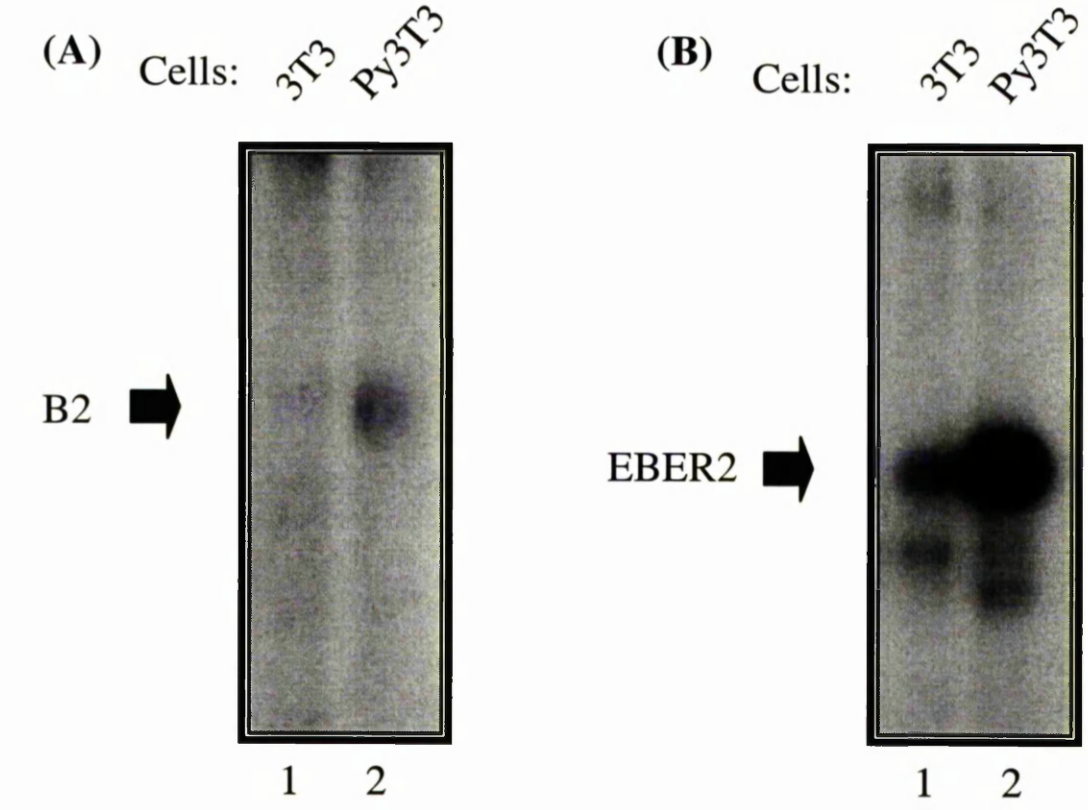
3.2.6 Elevated levels of pol III products in Py3T3 cells

Introns are processed from primary transcripts and subsequently degraded very rapidly. Consequently, their levels in a cell provide an accurate reflection of the rate of ongoing transcription (Cormack and Struhl, 1992). Primers that hybridise specifically to the intron sequence of short-lived precursors of $tRNA^{Leu}$ and $tRNA^{Tyr}$ were used in RT-PCR reactions to assay the levels of the primary transcripts in RNA extracted from 3T3 and Py3T3 cells. Py3T3 cells exhibited significantly higher levels of the primary $tRNA^{Leu}$ and primary $tRNA^{Tyr}$ transcripts when compared with the untransformed 3T3 cells (Figure 3.6A and 3.6B, respectively). This effect was specific, as no change was detected in the levels of mRNA encoding ARPP P0, which is synthesised by pol II (Figure 3.6D).

Figure 3.5

Py3T3 transcriptional deregulation is observed for a range of pol III transcripts

Comparison of the capacity of 3T3 and Py3T3 cell extracts to transcribe class III genes. Transcription reactions comprised 20μg of 3T3 (lane 1 for each panel) or Py3T3 (lane 2 for each panel) cell extract and 250ng of template. Templates utilised were pTB14 containing a mouse B2 gene (panel A), pE2-160 containing the EBER2 gene (panel B), pMcet1 containing a $tRNA^{Pro}$ gene (panel C) and pGlu6 containing a human $tRNA^{Glu6}$ gene (panel D).

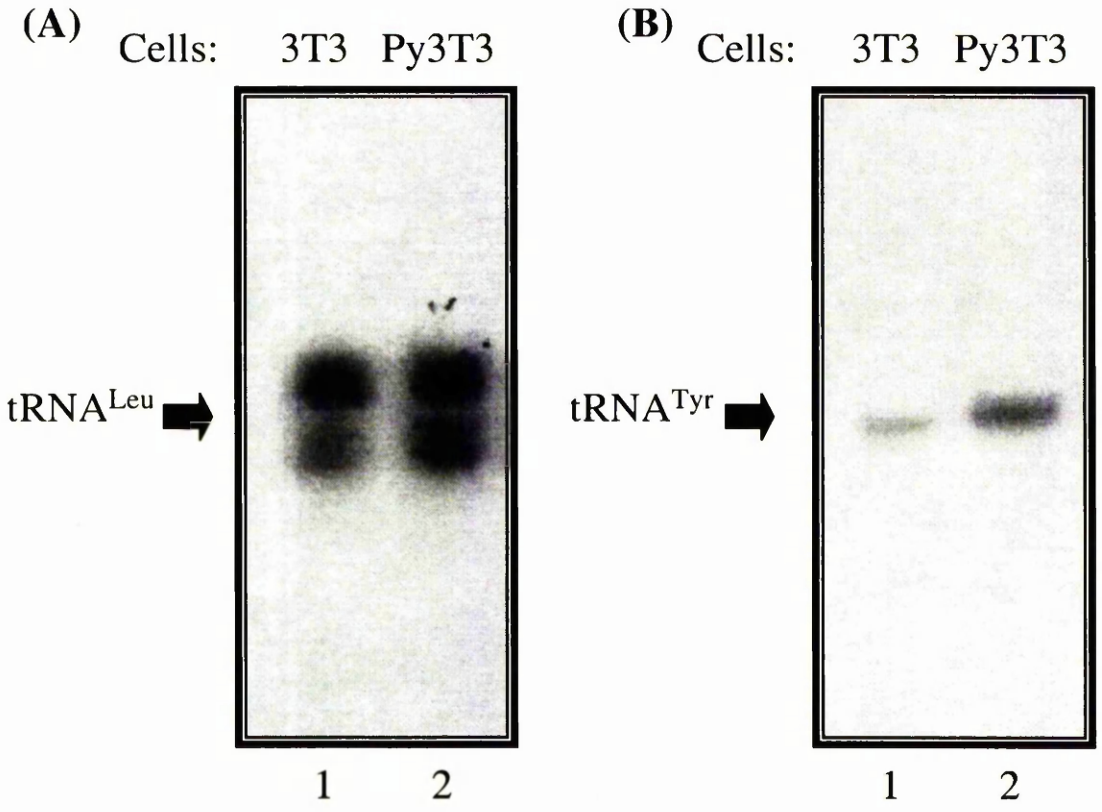

(A)
Cells:
3T3
Py3T3
B2
1
2
(B)
Cells:
3T3
Py3T3
EBER2
1
2
(C)
Cells:
3T3
Py3T3
tRNA^Pro
1
2
(D)
Cells:
3T3
Py3T3
tRNA^Glu6
1
2

To confirm that the increase observed with $tRNA^{Leu}$ and $tRNA^{Tyr}$ extended to other pol III products, U6 snRNA was tested and similarly displayed a substantial elevation in the Py3T3 cells (Figure 3.6C). Average relative increases after normalisation against the ARPP P0 control, for each product, are shown in the corresponding graphs: (E) $tRNA^{Leu}$, (F) $tRNA^{Tyr}$ and (G) U6.

Figure 3.6

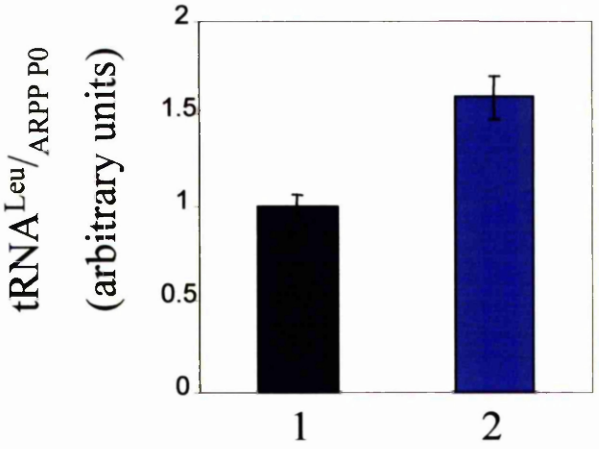
Py3T3 cells overexpress pol III transcripts

cDNAs were generated by reverse transcription of RNA from 3T3 (lane 1 in each panel) and Py3T3 (lane 2 in each panel) cells and were PCR amplified using primers for $tRNA^{Leu}$ (panel A), $tRNA^{Tyr}$ (panel B), U6 snRNA (panel C) and ARPP P0 (panel D). Levels of each transcript for both cell lines were quantitated by phosphoimaging (Fujix Bas 1000); values for pol III transcripts were normalised against ARPP P0 and delineated in graphs E, F and G which correspond to panels A, B and C above. Values shown represent the mean of three experiments ± standard deviation, with the value obtained for the 3T3 cells being designated 1.

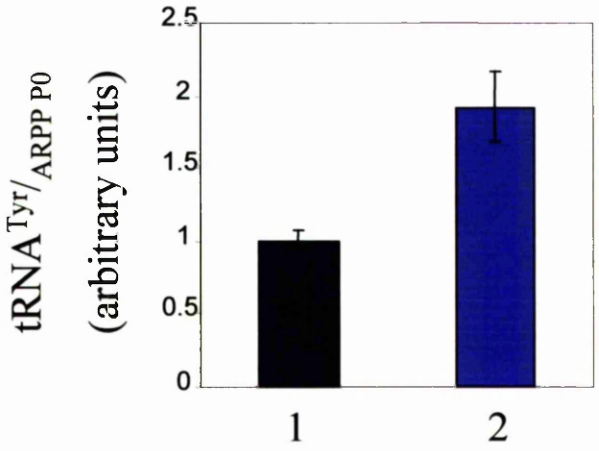

(A)
Cells: 3T3 Py3T3
tRNA^Leu
1 2
(B)
Cells: 3T3 Py3T3
tRNA^Tyr
1 2
(C)
Cells: 3T3 Py3T3
U6
1 2
(D)
Cells: 3T3 Py3T3
ARPP P0
1 2

Cells: 3T3 Py3T3

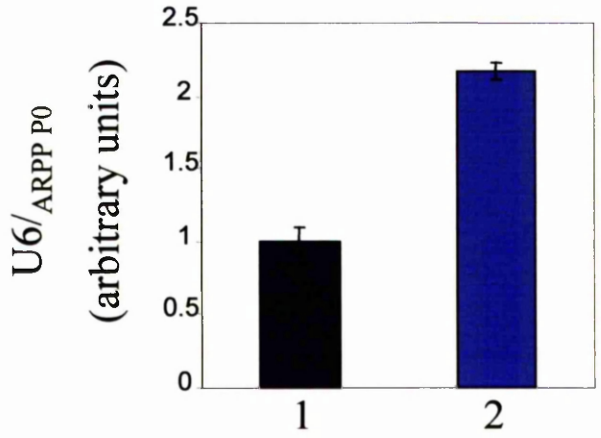
(E)



(F)



(G)



3.3 DISCUSSION

3.3.1 Py3T3 cells display accelerated proliferation and loss of cell cycle control

Proliferating cells are characterised by their ability to complete a full cell cycle and undergo cell division; events that are subject to meticulous regulation. It is well documented, however, that tumour and transformed cells display uncontrolled cell proliferation and typically employ mechanisms to deregulate the cell cycle (Sherr, 1996). Consequently, it follows that the Py3T3 cells demonstrate an elevated rate of proliferation over the untransformed 3T3 cells. This averaged as a 2.1-fold increase during the exponential phase of the two cell growth curves. The decline of 3T3 cell number upon reaching confluency was also consistent with previous studies establishing the ability of cells to contact inhibit (Aaronson and Todaro, 1968). The Py3T3 cells showed a slightly reduced rate of proliferation at post-confluency, but failed to exhibit the decline in number observed for the 3T3 cells. Although a simplistic study, these growth curves were congruous with the established characteristics of untransformed and transformed cell lines (Aaronson and Todaro, 1968) and served to demonstrate a deregulated level of cell proliferation following transformation. Additionally, appropriate passage requirements for each cell line were established on the basis of proliferation rates observed.

The tight regulation controlling progression through the cell cycle permits cells to withdraw into a quiescent state during unfavourable conditions (Larminie et al.,

1998). 3T3 cells have previously been shown to demonstrate withdrawal from the cell cycle and subsequent re-entry upon serum-stimulation (Abelson et al., 1974; Scott, 2001). This cell cycle control was demonstrated here by the levels of [^{3}H] thymidine incorporation into newly synthesised DNA, indicating cells passing through S phase. Serum-starved 3T3 cells show a marked reduction in S phase cells over the asynchronous population, indicating the withdrawal of cells into a quiescent state. As the asynchronous cells had not been subject to cell cycle co-ordination, the 50% decrease in S phase cells shown following serum-withdrawal implied that many of the asynchronous 3T3 population were already out of cycle. Conversely, 3T3 cells treated with 20% serum to induce re-entry into the cell cycle, exhibit a level approximately 2-fold greater than the asynchronous cells and in the region of 4-fold over the quiesced cells.

Previous work has illustrated a directly proportional relationship between growth rate and protein accumulation; the principal determinant of which is the rate of translation (Baxter and Stanners, 1978). A 50% reduction in the rate of protein accumulation is sufficient to cause proliferating cells to withdraw from the cell cycle and quiesce (Brooks, 1977) In considering the 2-fold elevation of proliferation rate of Py3T3 cells exhibited relative to the 3T3 cells, it follows that to sustain this increased rate, growth levels and, therefore, protein synthesis demands would have to be met. Consequently, the increase over the 3T3 cells in the asynchronous population of Py3T3 cells passing through S phase, as delineated by the 2-fold increase in [^{3}H] thymidine incorporation, suggests that growth and protein synthesis are abnormally active in Py3T3 cells.

Additionally, given that the genes commanding cell cycle control mechanisms are frequently targeted for mutation, deletion or amplification in tumours, resulting in a

loss of cell cycle regulation (Sherr, 1996), the observation that the levels of [^{3}H] thymidine incorporation were unremitting for serum-starved Py3T3 cells is indicative of the deregulated cell cycle control conferred by transformation.

Furthermore, [^{3}H] thymidine incorporation levels for serum-stimulated 3T3 cells were equalled by those for the asynchronous Py3T3 population; a notable observation bestowing further evidence of the accelerated growth and proliferation that arises as a manifestation of Polyomavirus transformation.

3.3.2 Overexpression of pol III transcripts in Py3T3 cells

Not only is the rate of protein accumulation a critical determinant of cellular growth, but it has been appreciated for a number of years that organisms adjust their translational capacity to meet, but not exceed, the requirement for protein synthesis. Central to this aspect of regulation is the control of stable RNA (tRNA and rRNA) production. *In vivo* analyses of *Saccharomyces cerevisiae* under a variety of conditions clearly established a direct link between translational load, stable RNA synthesis and ultimately ribosome biogenesis (Clarke et al., 1996).

Activation of transcription by pol III of the B2 family of middle-repetitive elements is a general feature of Simian Virus 40 (SV40) transformation (Brickell et al., 1983; Scott et al., 1983) and accompanies an elevation in growth rate. Furthermore, previous studies have shown a stimulation by Polyomavirus in the production of pol

III transcripts *in vivo* (Majello et al., 1985) which supports the contention linking growth and protein synthesis.

Northern analysis of relative B2 levels in the 3T3 and Py3T3 cell lines was used to confirm the activation of pol III transcripts in the Py3T3 cells and establish the degree of stimulation following transformation. The results clearly indicate that the Py3T3 cells overexpress B2 transcripts. To expand on this observation and address the implications of cell confluency at the time of extract preparation on stimulatory effect of transformation, cells harvested over a course of 6 consecutive days were again analysed by northern. This revealed that despite B2 expression decreasing in both cell lines at higher states of confluency, there was a sustained overexpression in the Py3T3 cells. Moreover, elevated transcription in the Py3T3 cells, demonstrated by *in vitro* transcription assays, supported the northern results with respect to both the increase in transcription and the congruity throughout a range of confluencies. *In vitro* transcription results also provided evidence of a general pol III transcription deregulatory effect by demonstrating deregulation with a variety of pol III templates.

Additional support was then obtained from RT-PCR analyses, with the transformed Py3T3 cells again demonstrating an elevation of pol III transcripts averaging just over 2-fold greater than levels expressed by the 3T3 cells, a specific effect which was not observed for the pol II transcript ARPP P0.

Together these results demonstrate, through a range of techniques, that Polyomavirus is able to deregulate proliferation, cell cycle control and pol III transcription. These features are consistent with characteristics displayed by transformed cell lines in previous studies.

Chapter 4

Up-regulation of components of the pol III transcriptional machinery

4.1 INTRODUCTION

The transcription factor TFIIIB has been strongly established as a key component of the pol III transcriptional machinery and, as such, is inextricably linked to transcription control (White, 1998b). There is a strong propensity for it to be targeted for regulation under a variety of conditions, being specifically down-regulated during differentiation (White et al., 1989) and mitosis (Gottesfeld et al., 1994; White et al., 1995b) and up-regulated as cells move into S phase (White et al., 1995a) or by oncogenic viruses (Brown et al., 2000).

Among the viruses capable of activating TFIIIB is the small DNA tumour virus Simian Virus 40 (SV40) and previous studies have documented higher specific activity of TFIIIB in the SV40-transformed cell lines SV3T3 Cl38 and SV3T3 Cl49 (Larminie et al., 1999). In untransformed murine fibroblasts, TFIIIB is subject to negative regulation by a physical interaction with the tumour suppressor protein RB (Larminie et al., 1997; White et al., 1996), but co-immunoprecipitation analyses demonstrated that in SV3T3 cells this interaction is compromised and the amount of TFIIIB associated with RB is significantly reduced (Larminie et al., 1999). Furthermore, this mode of TFIIIB activation can be ascribed to the large T antigen of SV40. It possesses an LXCXE motif, allowing it to bind RB and result in its

inactivation (DeCaprio et al., 1988; Ewen et al., 1989; Livingston, 1992). Moreover, it has been established that mutations in large T that interfere with RB binding abrogate its transforming activity (DeCaprio et al., 1988; Ewen et al., 1989). Consequently, this release of TFIIIB from RB-mediated repression contributes to the deregulation by SV40 that potentiates increased pol III transcription.

A similar ability to inactivate RB pertains in Py3T3 cells, where the large T antigen of Polyomavirus is also able to bind to RB through its LXCXE motif (Dyson et al., 1990), strongly implicating TFIIIB as a target for deregulation by Polyomavirus. Furthermore, the presence of an LXCXE motif permitting binding of RB is a feature exploited by a number of viral oncoproteins, with E1A of adenovirus and E7 of Human Papillomavirus (HPV) both demonstrating the same ability (Dyson et al., 1992; Munger et al., 1989; Whyte et al., 1989).

Interestingly though, a recent study has suggested through mutagenesis of the LXCXE-binding site of human RB, that the cell cycle arrest functions of RB are separable from binding to viral oncoproteins (Dick et al., 2000). Evidence was presented to show that the LXCXE-binding cleft of RB is not required to actively repress transcription of E2F-responsive promoters and that the repressor molecules HDAC1 and CtIP, which also contain an LXCXE consensus sequence, must possess additional RB-binding sequences that are independent of this motif. Mutation of the LXCXE-binding site did not interfere with the regulation of RB by phosphorylation and the cell cycle arrest induced by these RB mutants was insensitive to inactivation by E7 (Dick et al., 2000). These observations appear to contradict the established role of RB inactivation by transforming oncoproteins through this binding site. It is, however, possible that this paradox could be explained by a number of theories. It

may be that the interactions between RB and cellular LXCXE-containing proteins contribute to cell cycle arrest by RB without being essential or that these interactions vary between cell types or types of arrest. Alternatively, the RB LXCXE-binding cleft could be important for cell cycle arrest only under certain conditions. Furthermore, redundancy may exist between the different transcriptional repressors that associate with RB, allowing repression of E2F-dependent transcription despite some E2F target genes being regulated by LXCXE-binding proteins.

The possibility also exists that since viral oncoproteins have evolved to bind RB in order to inactivate it, they likely favour high-affinity interactions while, conversely, cellular proteins interact with RB in a regulated and reversible fashion. Consequently, interaction of viral oncoproteins with RB may depend on the LXCXE-binding site to a far greater extent than cellular proteins involved in cell cycle arrest or transcriptional repression.

The pocket domain is required for a range of RB functions, including cell differentiation (Chen et al., 1996a) and activation of transcription (Chen et al., 1996b; Nead et al., 1998). Certain RB mutants possessing uncompromised LXCXE-binding sites have been shown to retain the ability to induce differentiation and transcription while failing to regulate cell proliferation (Sellers et al., 1998). This is significant because viral oncoproteins such as E7 have demonstrated the ability to block cellular differentiation (McCaffrey et al., 1999).

Although oncoproteins like E7 overcome cell cycle regulation by RB through their LXCXE motif, evidence has yet to be presented that implicates cellular proteins possessing this motif primarily in the cell cycle function of RB.

These observations showing that mutation of this binding site allows RB to function as a cell cycle regulator while being resistant to inactivation by viral oncoproteins seem somewhat enigmatic. This is, however, a limited study and given the conservation of the LXCXE motif and its employment by a range of viral oncoproteins, it nevertheless clearly confers an important aspect of deregulation by transforming viruses.

The fact that transforming viruses exploit some of the same deregulatory mechanisms, such as the binding and inactivation of RB, is significant. However, it is equally interesting that even closely related oncogenic viruses display striking differences. The large T antigen of SV40 shares 60% amino acid sequence identity and a wide range of properties with the large T antigen of Polyomavirus (Turler, 1980). SV40 large T is able to bind to the tumour suppressor protein, p53, which is unrelated to RB, and cause its inactivation (Lin and Simmons, 1991). p53 inactivation by point mutation, deletion or MDM2 overexpression occurs frequently in Polyomavirus-induced sarcomas; however, in contrast to SV40, the large T antigen of Polyomavirus, as well as the middle and small T antigens, fail to bind p53 (Wang et al., 1989). Here another paradox manifests itself, as the large T antigen of Polyomavirus is still able to interfere with the growth suppressive activity of p53 (Doherty and Freund, 1997). Interestingly, mutant large T antigens containing a defective RB-binding domain failed to overcome growth arrest, implicating the large T interaction with RB in this function. The ability of p53 to mediate growth arrest requires overexpression of p21 (Harper et al., 1995), which binds cyclin/cdk complexes and can inhibit kinase activity of these complexes at high concentrations (Gu et al., 1993). Given, however, that RB and its family members are substrates for cyclin/cdk complexes (Hinds et al., 1992), it is suggested that p53-dependent growth arrest is a result of inhibition of

phosphorylation of RB family proteins by p21. Furthermore, a population of RB is phosphorylated in cells expressing large T, p53 and p21, implying that large T expression overrides the p21 inhibition of kinase activity or activates another RB kinase (Doherty and Freund, 1997). Large T may achieve this through a direct interaction with p21 or by inducing another protein(s) that inactivates p21.

The complexity of the Polyomavirus large T antigen functions and its interplay with cellular proteins is clearly evident. However, this chapter focuses on identifying some of the mechanisms concerned with specifically deregulating pol III transcription in Polyomavirus-transformed cells. It documents both large T -dependent and – independent effects and establishes aspects of the pol III transcriptional machinery that are targeted for deregulation. While much correlation is observed with SV40 and indeed, some features of pol III deregulation by other transforming viruses, Polyomavirus also presents novel mechanisms of deregulation.

4.2 RESULTS

4.2.1 Pol III activity is up-regulated by Polyomavirus transformation

There is a wide range of mechanisms by which pol III transcription can be modulated (reviewed in White, 1998b) and numerous transformed and tumour cell types have displayed abnormally elevated levels of pol III transcripts. However, previous studies have revealed no evidence for the polymerase itself to be controlled directly, with regulation being mediated instead through changes in the activities of pol III-specific transcription factors (Brown et al., 2000). To address the effect of Polyomavirus transformation on pol III itself, random polymerisation assays were performed using poly(dA.dT) as a non-specific template. As demonstrated in figure 4.1, the level of pol III activity was significantly higher in extracts from Py3T3 cells relative to the levels displayed in untransformed 3T3 cell extracts. With a 1.8-fold increase being produced, this would appear to be the first evidence of the pol III enzyme as a direct target for deregulation following viral transformation.

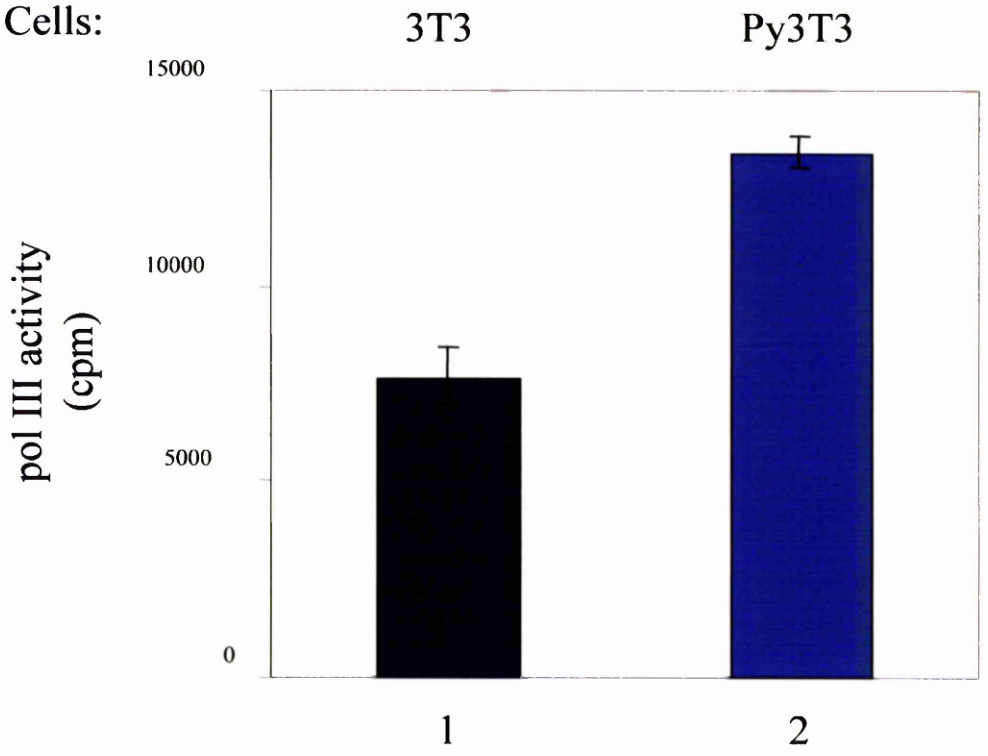
4.2.2 Elevated abundance of pol III subunits in Py3T3 cell extracts

Western blot analysis conferred support for the direct targeting of the polymerase itself by Polyomavirus. A clear elevation in the abundance of the AC19 pol III

Figure 4.1

Py3T3 cells up-regulate pol III activity

Random polymerase assays, demonstrating specific levels of activity for pol I or pols I and III combined, were performed using 3T3 and Py3T3 cell extracts and the non-specific template poly(dA.dT). Reactions were carried out in the presence of 1μg/ml α-amanitin to inhibit pol II only or in the presence of 100μg/ml α-amanitin for inhibition of both pol II and pol III activities. Values illustrated represent the level of pol III activity alone as a mean of two experiments ± standard deviation.


Cells:
3T3
Py3T3
15000
10000
5000
0
pol III activity
(cpm)
1
2

subunit is seen in extracts from Py3T3 cells relative to levels exhibited by 3T3 cell extracts (Figure 4.2A, compare lanes 1 and 2). A pol III fraction (lane 3) was run alongside to provide a positive control for the pol III subunit. Furthermore, similar elevation was seen for a second subunit of pol III, BN51 (Figure 4.2B, compare lanes 1 and 2). Blotting for actin demonstrated that the increase observed for AC19 and BN51 was specific, as levels for actin were constant for extracts from both cell lines (Figure 4.2C).

4.2.3 Elevated TFIIIB activity in Py3T3 cell extracts

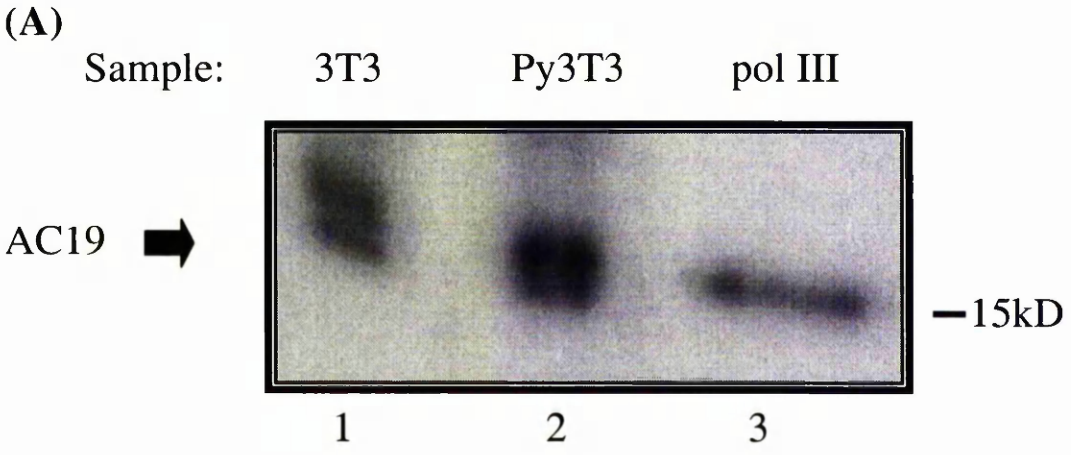
Although Polyomavirus directly targets the polymerase for deregulation, the possibility of additional mechanisms of deregulation remained likely as oncogenic viruses often target more than one of the key transcription factors (Brown et al., 2000). SV40 has not demonstrated the ability to target the polymerase itself; however, two SV40-transformed cell lines, SV3T3 Cl38 and SV3T3 Cl49, have previously been shown to display an increase in TFIIIB activity relative to the untransformed parental 3T3 cells (Larminie et al., 1999). Consequently, TFIIIB assays were conducted to establish the relative levels of TFIIIB activity in 3T3 and Py3T3 cell extracts. These assays exploit the differential sensitivity of pol III transcription factors to inactivation by mild heat treatment. Cell extracts were heat-treated for 15 minutes at 47°C, specifically inactivating TFIIIC and TBP. These were replenished by addition of PC-C, a TFIIIC- and Pol III-containing phosphocellulose-column fraction, along with recombinant TBP. The remaining components of the pol

Figure 4.2

Overexpression of pol III subunits in Py3T3 cells

Whole cell extracts prepared from 3T3 (lane 1 in each panel) and Py3T3 (lane 2 in each panel) cells and a pol III fraction (lane 3) were resolved on a SDS-7.8% polyacrylamide gel and analysed by western immunoblotting using either the AC19 antibody raised against the AC19 subunit of pol III (panel A), an antibody, 113, against the BN51 subunit of pol III (panel B) or an anti-actin antibody, C-11 (panel C).

Panels A and B show the same blot, cut and probed with the respective antibodies. The blot was stripped and reprobed for actin (panel C).


(A)
Sample:
3T3
Py3T3
pol III
AC19
15kD
1
2
3
(B)
Sample:
3T3
Py3T3
pol III
BN51
50kD
1
2
3
(C)
Sample:
3T3
Py3T3
pol III
Actin
50kD
1
2
3

III machinery are uncompromised by the heat treatment and the heat-treated extracts were then assayed for TFIIIB activity using the VA_1 pol III template. Py3T3 cell extracts displayed substantially higher TFIIIB activity than the extracts from 3T3 cells (Figure 4.3, compare lanes 2 and 3). The control lane, replacing the addition of a cell extract with buffer alone (lane 1) showed no transcription, demonstrating the requirement of TFIIIB in the extracts for pol III transcription.

This increase in TFIIIB activity in Py3T3 cell extracts provides evidence of another deregulatory mechanism employed by Polyomavirus and is consistent with the observation of elevated TFIIIB activity in cell lines transformed by SV40.

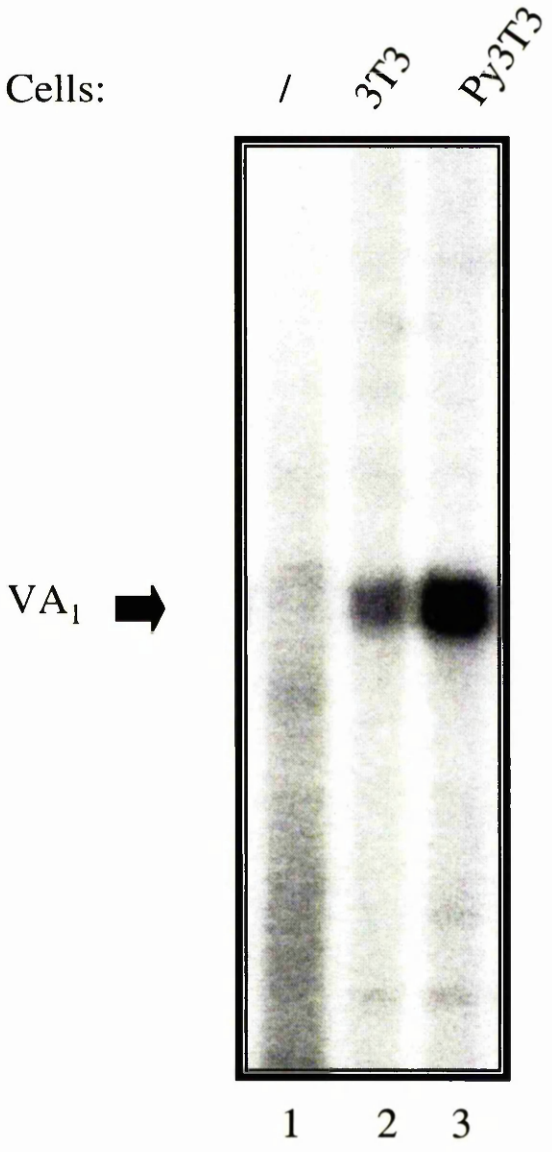
4.2.4 TFIIIB subunits TBP and BRF are not overexpressed in Py3T3 cells

The increase in TFIIIB activity accompanying SV40 transformation cannot be ascribed to an increase in the TBP and BRF subunits of TFIIIB, as levels of expression remain the same as levels observed in the parental 3T3 cells (Larminie et al., 1999). To investigate this angle in Polyomavirus-transformed cells, western analysis to determine protein levels of TBP and BRF was performed using cell extracts from 3T3 and Py3T3 cells. Synonymous with the observations in SV40-transformed cells, Py3T3 cell extracts displayed equivalent levels of both TBP and BRF to those seen in extracts of 3T3 cells (Figure 4.4, panels A and B, compare lanes 1 and 2). This demonstrated that the elevation in TFIIIB activity in Py3T3 cells was not manifested through overexpression of these subunits. Levels for TFIIB in the two

Figure 4.3

TFIIIB activity is elevated in Py3T3 cells

Relative levels of TFIIIB activity for 3T3 (lane 2) and Py3T3 (lane 3) cells, as established by TFIIIB assay performed using 20μg of cell extracts and 250ng of pVA_1 template. Extracts were heat-treated to specifically inactivate TFIIIC and TBP, by a 15 minute incubation at exactly 47°C, and analysed by *in vitro* transcription assay after the addition of TBP and PC-C (a TFIIIC-containing phosphocellulose-column fraction) required for reconstitution of pol III transcription. Lane 1 serves as a negative control where reactions were carried out in the absence of cell extract.


Cells:
/
3T3
Py3T3
VA1
1
2
3

cell lines were also assessed to confirm equal loading of extracts and, as expected, remained constant (Figure 4.4C).

4.2.5 Py3T3, Cl38 and Cl49 cells overexpress the B" subunit of TFIIIB

While yeast TFIIIB has been well defined for a number of years and known to comprise TBP (Kassavetis et al., 1992), BRF (Buratowski and Zhou, 1992) and B" (Kassavetis et al., 1995), a mammalian homologue of yeast B" had not been identified until recently. However, following the characterisation of a human homologue of yeast B" (B") (Schramm et al., 2000), it has been possible to include B" in studies for mammalian pol III transcription. Exploiting this advance, B" levels for 3T3 and Py3T3 cell extracts were determined by western analysis. Additionally, as the levels of the B" subunit had not previously been analysed in SV40-transformed cells, SV3T3 Cl38 and SV3T3 Cl49 cell extracts were similarly tested.

In blatant contrast to the observations for TBP and BRF, the level of B" in the Py3T3 cell extract substantially exceeds the level displayed by the parental cell line (Figure 4.5A, compare lanes 1 and 2). The overexpression is clearly specific, as 3T3 and Py3T3 cell extracts revealed equal protein levels of TFIIB (Figure 4.5B, lanes 1 and 2).

Similarly, protein levels of B" expressed in Cl38 and Cl49 cell extracts were clearly higher than in the extracts from 3T3 cells (Figure 4.5C, compare lanes 2 and 3 with lane 1). Blotting for TFIIB provided a control for protein loading (Figure4.5D).

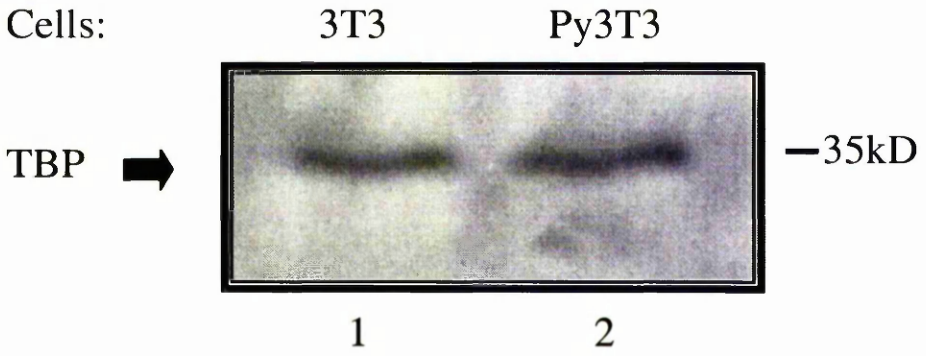
Figure 4.4

TFIIIB subunits TBP and BRF are not overexpressed in Py3T3 cell extracts

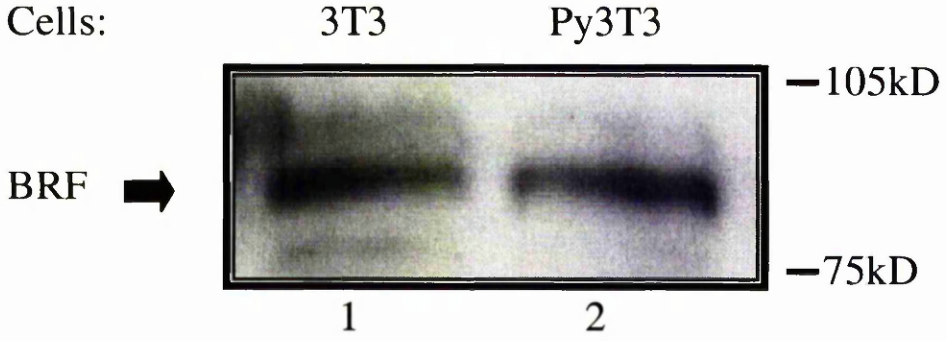
Whole cell extracts prepared from 3T3 (lane 1 in each panel) and Py3T3 (lane 2 in each panel) cells were resolved on a SDS-7.8% polyacrylamide gel and analysed by western immunoblotting using either the anti-TBP antibody SL30 (panel A), the anti-BRF antibody 330 (panel B) or the C18 antibody against TFIIB (panel C).

Panels A, B and C show the same blot, cut and probed with the respective antibodies.

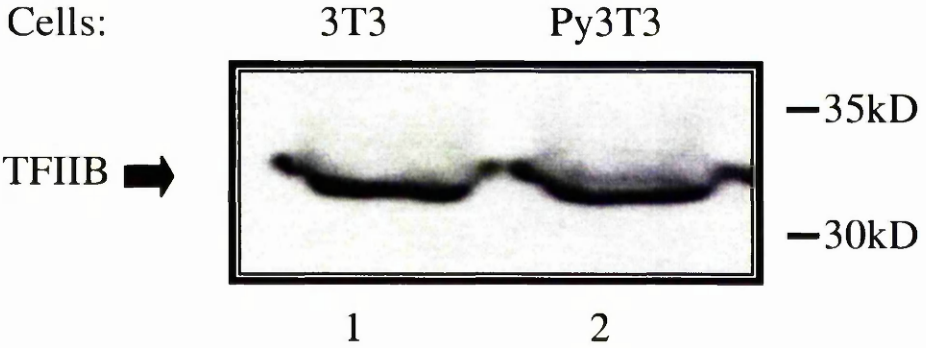
(A)


Cells:
3T3
Py3T3
TBP
35kD
1
2

(B)


Cells:
3T3
Py3T3
BRF
105kD
75kD
1
2

(C)


Cells:
3T3
Py3T3
TFIIB
35kD
30kD
1
2

These results provide evidence, not only of up-regulation of the B" subunit of TFIIIB by Polyomavirus but, moreover, provides additional and novel insight into the targeting of TFIIIB for deregulation following SV40 transformation.

4.2.6 Elevated B" transcripts in Py3T3, Cl38 and Cl49 cells

The increase in B" for Py3T3, Cl38 and Cl49 cells relative to the parental 3T3 cells, demonstrated by western analysis (Figure 4.5), was unequivocal. However, to determine the level at which B" becomes up-regulated, semi-quantitative RT-PCR analysis of the B" transcript was performed for each cell line. In agreement with the results obtained from the western blotting, a significant elevation in the transcript encoding B" was apparent in total RNA extracted from Py3T3, Cl38 and Cl49 cells, when compared with levels in 3T3 cells (Figure 4.6A, compare lanes 2, 3 and 4 with 1). This effect was specific, as no change was detected in the levels of mRNA encoding ARPP P0, which is synthesised by pol II (Figure 4.6B). The average relative increase in B" for each cell line, after normalisation against the ARPP P0 control, is displayed in panel C and highlights a 3.5-fold increase conferred by Polyomavirus. Furthermore, the more pronounced elevation in B" transcript levels observed for Cl38 cells than Cl49 cells is a significant finding (Figure 4.6C, compare columns 3 and 4), as Cl49 cells have a higher proliferation rate than that of Cl38 cells. Thus, the increased transcript level of B" in the Cl38 cells suggests that B" overexpression in the transformed cell lines is not simply a response to enhanced proliferation.

Figure 4.5

Py3T3, Cl38 and Cl49 cell extracts overexpress the B" subunit of TFIIIB

3T3 and Py3T3 cell extracts (lanes 1 and 2, respectively, panels A and B) or extracts prepared from 3T3, Cl38 and Cl49 cells (lanes 1, 2 and 3, respectively, panels C and D) were resolved on a SDS-7.8% polyacrylamide gel and analysed by western immunoblotting. Panels A and C show the blots probed with an anti-B" antibody, 2663-4, while panels B and D show the lower section of each blot probed with the anti-TFIIB antibody, C18.

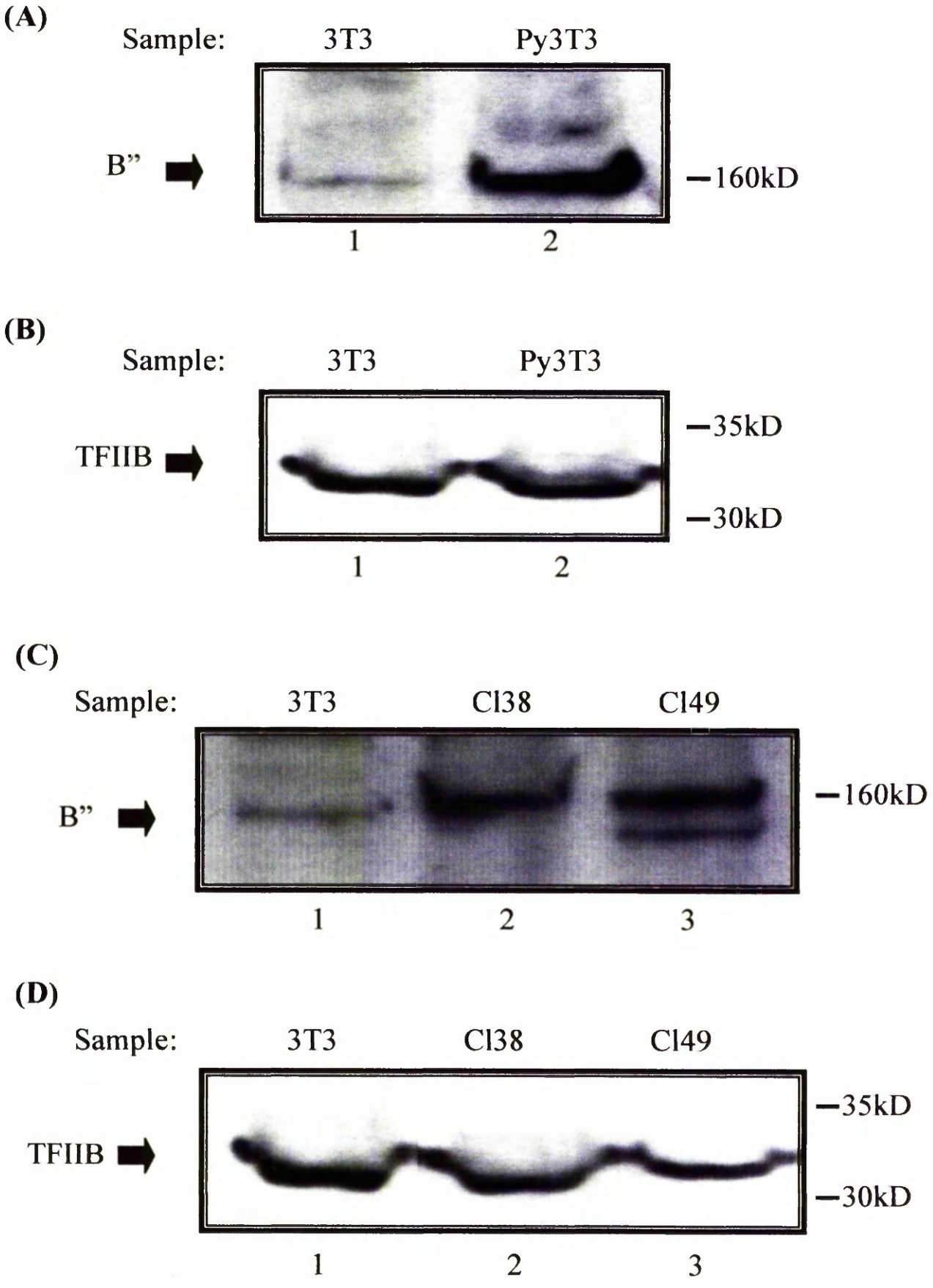
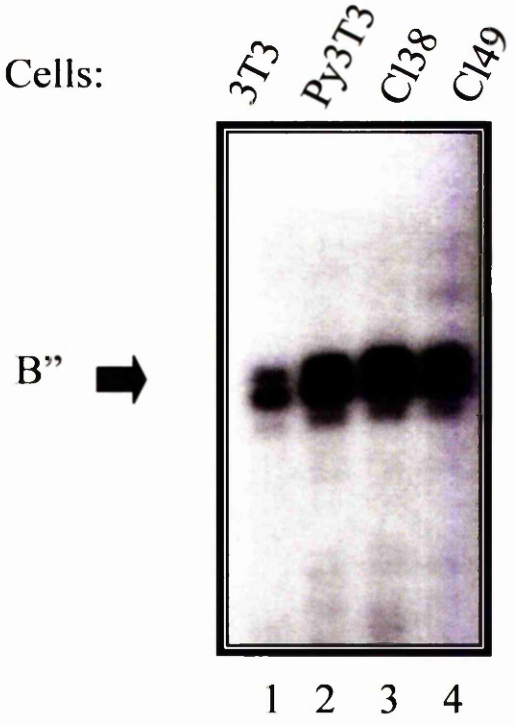

(A)
Sample:
3T3
Py3T3
B"
160kD
1
2
(B)
Sample:
3T3
Py3T3
TFIIB
35kD
30kD
1
2
(C)
Sample:
3T3
Cl38
Cl49
B"
160kD
1
2
3
(D)
Sample:
3T3
Cl38
Cl49
TFIIB
35kD
30kD
1
2
3

Figure 4.6

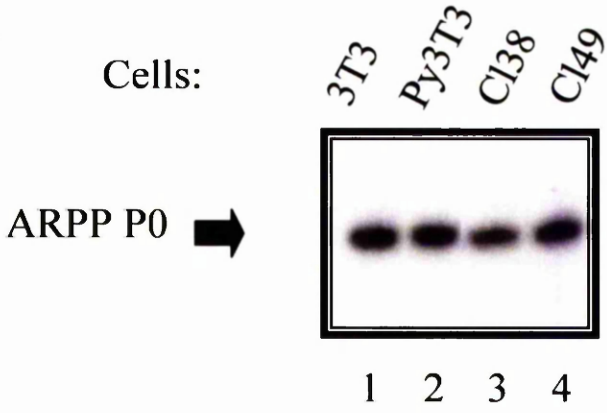
Overexpression of B" mRNA in Py3T3, Cl38 and Cl49 cells

cDNAs were generated by reverse transcription of RNA from 3T3, Py3T3, Cl38 and Cl49 (lanes 1, 2, 3 and 4, respectively, in each panel) cells and were PCR amplified using primers for B" (panel A) and ARPP P0 (panel B). Levels for each transcript for all four cell lines were quantitated by phosphoimaging (Fujix Bas 1000); values for B" were normalised against ARPP P0 and illustrated graphically (panel C). Values shown represent the mean of two experiments ± standard deviation, with the value obtained for the 3T3 cells being designated 1.

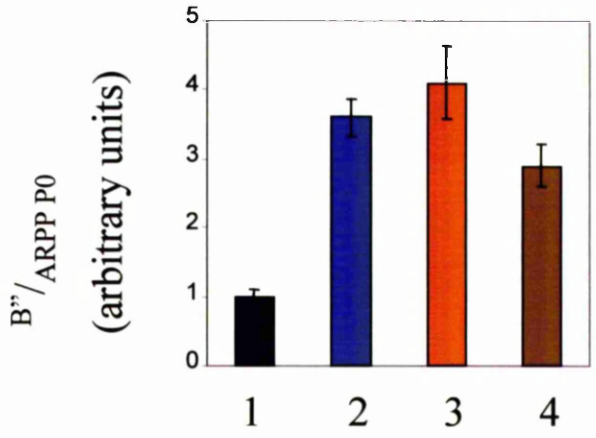
(A)


Cells:
3T3
Py3T3
Cl38
Cl49
B”
1 2 3 4

(B)


Cells:
3T3
Py3T3
Cl38
Cl49
ARPP P0
1 2 3 4

(C)


B”/ARPP P0 (arbitrary units)
0
1
2
3
4
5
1 2 3 4

4.2.7 The RB-BRF interaction is compromised in Py3T3 cells through targeting of RB by the large T antigen

Both the large T antigen of SV40 (DeCaprio et al., 1988) and Polyomavirus (Pilon et al., 1996) possess an LXCXE motif within their N-termini which allows them to interact with RB and its related family members. They specifically bind to the underphosphorylated form of RB (Khandjian and Tremblay, 1992; Ludlow et al., 1989), resulting in its inactivation. Indeed, it has been demonstrated that the immortalisation function of Polyomavirus and SV40 large T antigens is dependent on their binding site for RB, p107 and p130 (Larose et al., 1991; Tevethia et al., 1997).

Consequently, it was of interest to determine the degree of interaction between endogenous RB and TFIIIB, which is subject to RB-mediated repression, in order to establish if this interaction was compromised in Py3T3 cells. The amount of BRF co-immunoprecipitated from Py3T3 cell extracts by an anti-RB antibody was severely diminished relative to that seen for 3T3 cell extracts (Figure 4.7A, compare lanes 1 and 3). Extracts were similarly treated with an irrelevant control antibody against the TAF_I48 subunit of a pol I factor, SL1, which confirmed that the interaction between RB and BRF was specific, since BRF was not co-immunoprecipitated from either of the cell extracts using this antibody (Figure 4.7A, lanes 2 and 4).

Western analysis of RB protein levels in 3T3, Py3T3 and Pytsa3T3 cell extracts revealed that RB is similarly abundant in the different cell lines (Figure 4.7B, lanes 1, 2 and 3), providing evidence that the reduced interaction between RB and BRF observed in the Py3T3 cells was not a consequence of a lower RB expression level.

Complementary evidence, supporting the involvement of the large T antigen in this disrupted interaction, was obtained through a converse co-immunoprecipitation experiment addressing the interaction between endogenous RB and the large T antigen. RB is clearly co-immunoprecipitated from Py3T3 cell extract using an antibody against the Polyomavirus T antigens (Figure 4.7C, lane 3) and as expected, no RB was seen for the 3T3 cell extract (Figure 4.7C, lane 1). An additional cell line, Pytsa3T3 cells, which are transformed by Polyomavirus but defective for the function of large T, was used to demonstrate, through this mutation, that the interaction with RB was specific for the large T antigen. As expected, RB was not found to co-immunoprecipitate from extracts of the Pytsa3T3 cells (Figure 4.7C, lane 5). Again, extracts from each cell line were treated with the TAF_I48 antibody to provide a control for the specific interaction and in each case showed no presence of RB (Figure4.7C, lanes 2, 4 and 6).

Taken in conjunction with the previous results, these data support the evidence for inactivation of RB by Polyomavirus large T antigen and implicate the disruption of the interaction between endogenous BRF and RB in Polyomavirus-mediated activation of TFIIIB.

4.2.8 Large T antigen stimulates pol III transcription

The involvement of the large T antigen in transformation by Polyomavirus is paramount. In order to establish its effect specifically with regard to pol III transcriptional activation, large T was transfected into untransformed 3T3 cells and its

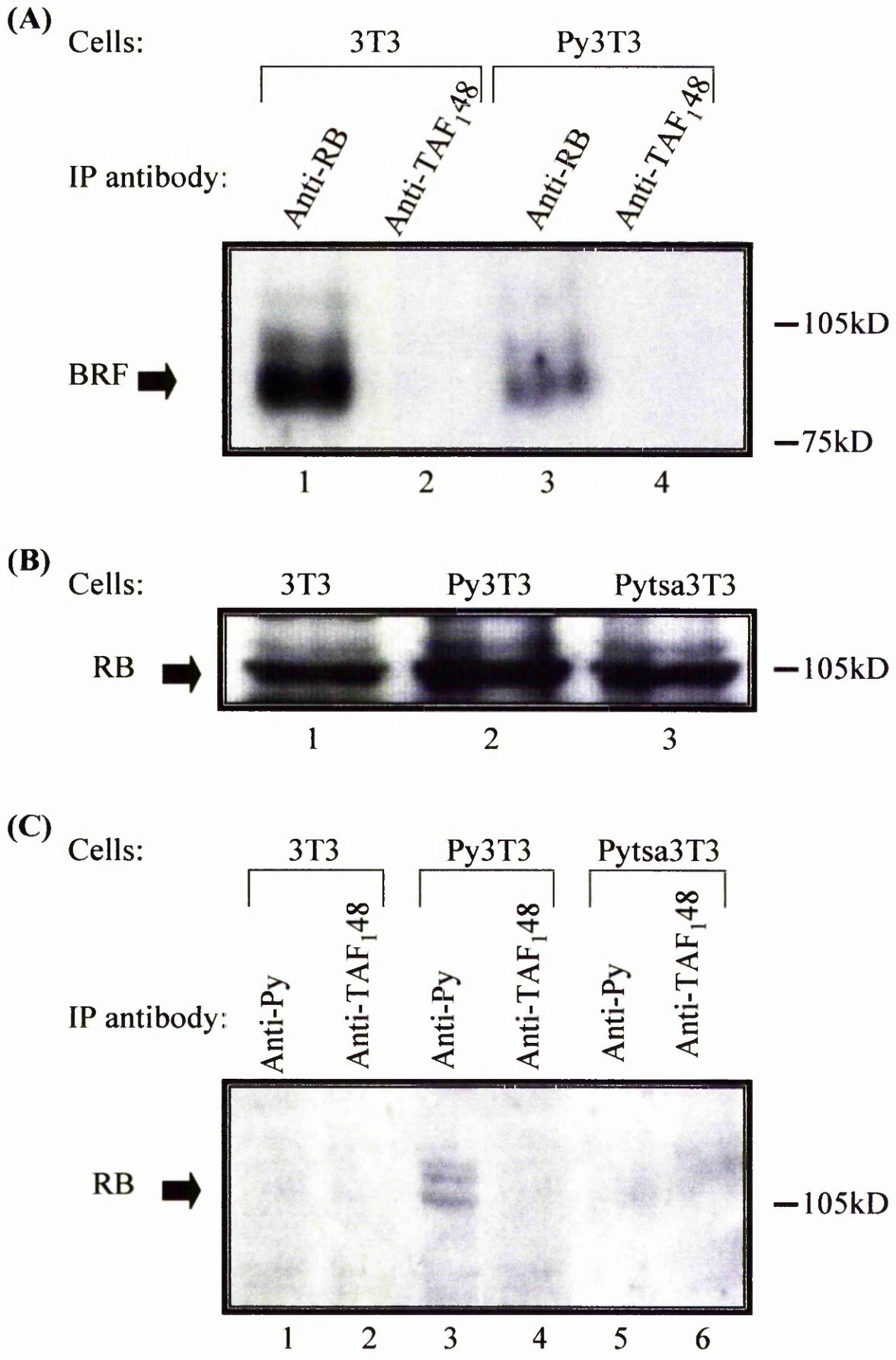
Figure 4.7

Interaction between RB and BRF is compromised in Py3T3 cells

Panel A shows whole cell extracts (150μg) prepared from 3T3 (lanes 1 and 2) and Py3T3 (lanes 3 and 4) cells, immunoprecipitated (IP) using the anti-RB antibody, C15 (lanes 1 and 3) or an anti-TAF_I48 antibody, M19 (lanes 2 and 4). The precipitated material was resolved on a SDS-7.8% polyacrylamide gel and the presence of BRF was determined by western analysis with the anti-BRF antibody, 128-4.

Panel B shows whole cell extracts (50μg) prepared from 3T3 (lane 1), Py3T3 (lane 2) and Pytsa3T3 (lane 3) cells resolved on a SDS-7.8% polyacrylamide gel and analysed by western immunoblotting using an anti-RB antibody, C15.

Whole cell extracts (150μg), shown in panel C, prepared from 3T3 (lanes 1 and 2), Py3T3 (lanes 3 and 4) and Pytsa3T3 (lanes 5 and 6) were immunoprecipitated using the F4 antibody against the Polyomavirus T antigens (lanes 1, 3 and 5) or an anti-TAF_I48 antibody, M19 (lanes 2, 4 and 6). Western blotting was performed using the anti-BRF antibody, 128-4, following electrophoretic separation of the precipitated material on a SDS-7.8% polyacrylamide gel.


(A)
Cells:
3T3
Py3T3
IP antibody:
Anti-RB
Anti-TAF$_I$48
Anti-RB
Anti-TAF$_I$48
BRF
105kD
75kD
1
2
3
4
(B)
Cells:
3T3
Py3T3
Pytsa3T3
RB
105kD
1
2
3
(C)
Cells:
3T3
Py3T3
Pytsa3T3
IP antibody:
Anti-Py
Anti-TAF$_I$48
Anti-Py
Anti-TAF$_I$48
Anti-Py
Anti-TAF$_I$48
RB
105kD
1
2
3
4
5
6

ability to stimulate transcription of the VA_1 pol III template analysed by primer extension. A dramatic stimulation of transcription over both the empty vector and NG59, a functionally dead mutant, was observed with 0.5μg of large T antigen DNA, which escalated further upon transfection of 1μg (Figure 4.8A, compare lanes 3 and 4 with lanes 1 and 2). Co-transfection of pCAT allowed transfection efficiency to be accounted for and confirmed a pol III-specific transcriptional stimulation (Figure 4.8B). Values for VA_1 normalised against CAT and presented graphically (Figure 4.8C), highlight a stimulation of pol III transcription in the region of 150-fold at the higher concentration of large T transfection. While clearly expressed at artificially high levels, the effect of the large T antigen on pol III transcription is striking and constitutes an important aspect of its deregulation.

4.2.9 Elevation of TFIIIB activity is severely compromised in the absence of the large T antigen

Given that the large T antigen demonstrated a compelling aptitude to stimulate pol III transcription (Figure 4.8) and in view of its ability to disrupt the interaction between RB and BRF (Figure 4.7), the elevation of TFIIIB activity noted in the Py3T3 extracts (Figure 4.3) could likely be construed as the release of TFIIIB from RB-repression through the action of large T binding and inactivating RB.

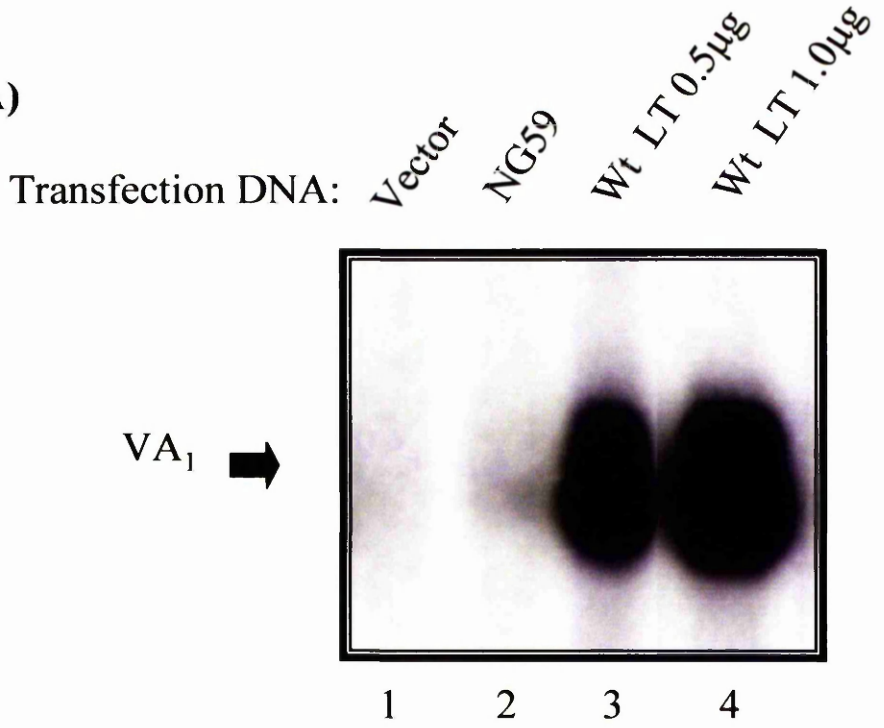
To confirm this hypothesis, further use was made of the Pytsa3T3 cells. A TFIIIB assay established that the 5.9-fold elevation of TFIIIB activity conferred by the wild-type Polyomavirus was severely impaired, with only a 2.2-fold elevation observed

Figure 4.8

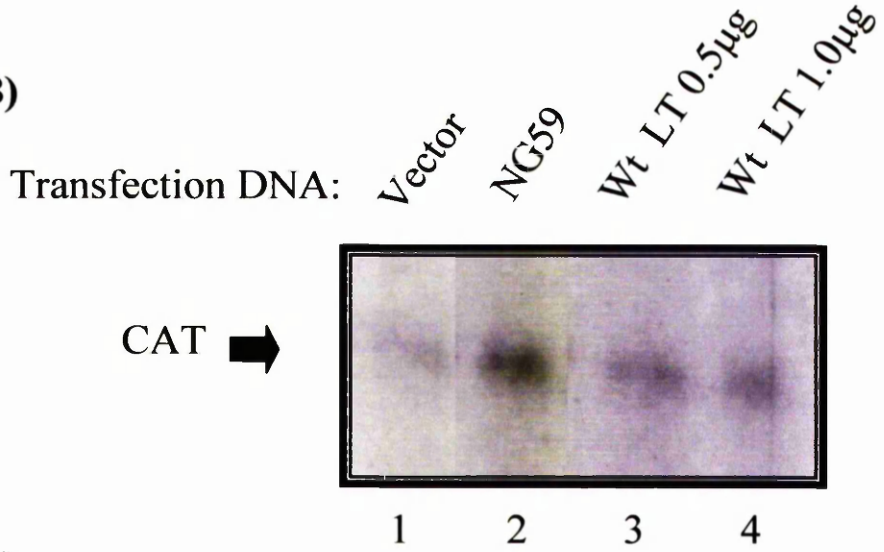
Large T antigen stimulates pol III transcription *in vivo*

Transient transfection of the Polyomavirus large T antigen into 3T3 cells using the Superfect method. 3T3 cells growing in 10% serum were transfected with pVA_1 (0.5μg), pCAT (0.5μg) and the relevant amount of the plasmid of interest made up to 3μg with "empty" pSV expression vector as follows: pSV alone (lane 1), pSV-NG59 (1μg, lane 2) or pSV-LT (0.5μg, lane 3 and 1μg, lane 4). VA_1 (panel A) and CAT (panel B) RNA levels were assayed by primer extension and then quantified by phosphoimaging (Fujix Bas 1000). Values shown in panel C are for VA_1 expression after normalisation to the levels of CAT RNA to correct for transfection efficiency; they are given relative to the value obtained with pSV vector alone (designated 1) and represent the mean of two experiments ± standard deviation.

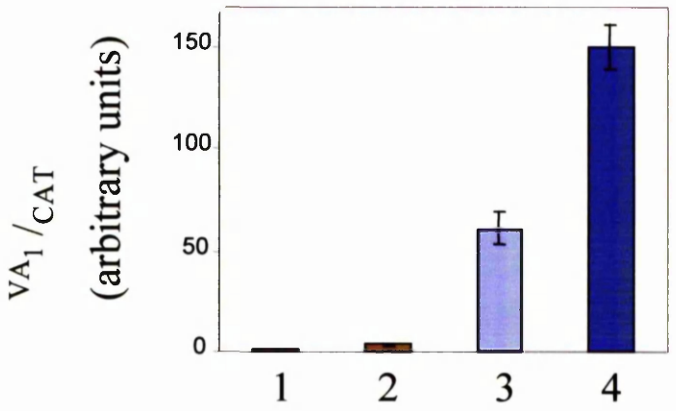
(A)


Transfection DNA:
Vector
NG59
Wt LT 0.5μg
Wt LT 1.0μg
VA1
1
2
3
4

(B)


Transfection DNA:
Vector
NG59
Wt LT 0.5μg
Wt LT 1.0μg
CAT
1
2
3
4

(C)


VA1 /CAT
(arbitrary units)
150
100
50
0
1
2
3
4

in the cells transformed by the mutant strain (Figure 4.9, compare lanes 3 and 4). This result implies that activation of TFIIIB is mediated through the action of the large T antigen and is consistent with the theory of release from repression by RB. However, it is significant that a slight elevation in TFIIIB activity still remains in the Pytsa3T3 cells, suggesting that TFIIIB activation is not wholly ascribed to large T function (Figure 4.9, compare lane 4 with lane 2). Western and RT-PCR analyses (Figure 4.5 and 4.6, respectively) showed that Py3T3 cells overexpress the B" subunit, which may explain this additional mode of TFIIIB activation, subject to it being a large T-independent effect.

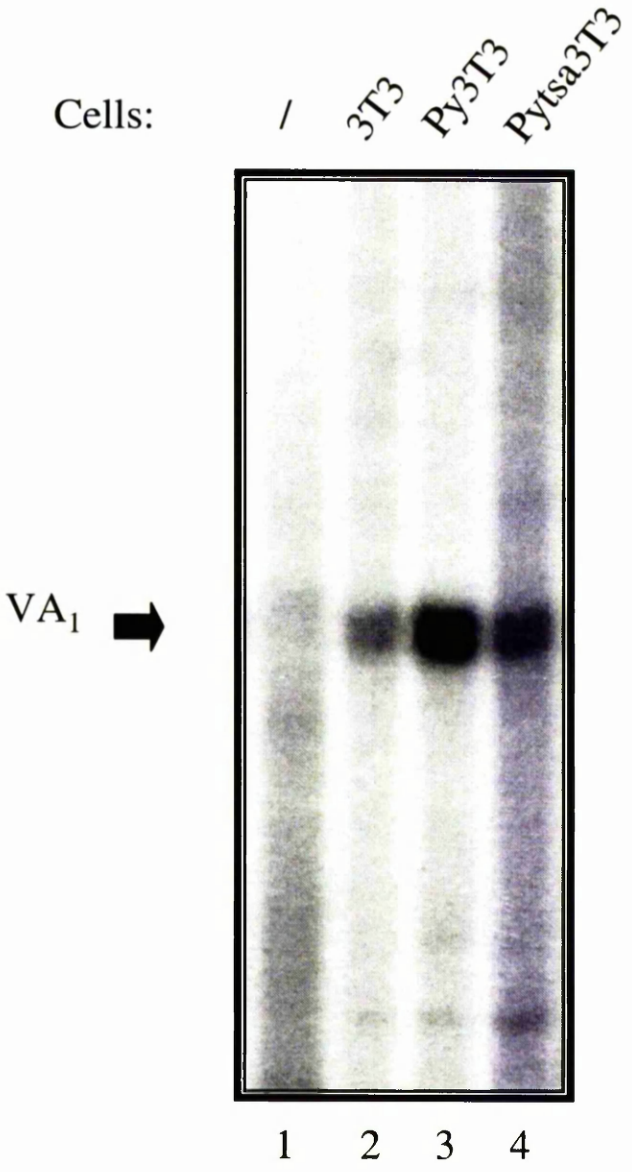
4.2.10 Pytsa3T3 cells overexpress the B" subunit of TFIIIB

Continuing in this line of investigation, Pytsa3T3 cell extracts were analysed by western blotting to substantiate the B" expression levels relative to those of 3T3 and Py3T3 cell extracts. Accordant to the theory of large T-independent overexpression of B", Pytsa3T3 cell extracts displayed significantly higher levels of B" than the untransformed 3T3 cells and equalled expression seen in wild-type Py3T3 cells (Figure 4.10A, compare lanes 3 and 4 with lane 2). Recombinant B" (Figure 4.10A, lane 1) provided a positive control for B" blotting and panel B (Figure 4.10), showing probing of the same blot for TFIIB, demonstrates equal protein loading and a B"-specific increase in the transformed cell lines.

Figure 4.9

Elevation of TFIIIB activity is LT-dependent

Relative levels of TFIIIB activity for 3T3 (lane 2), Py3T3 (lane 3) and Pytsa3T3 (lane 4) cells, as established by TFIIIB assay performed using 20μg of cell extracts and 250ng of pVA_1 template. Extracts were heat-treated to specifically inactive TFIIIC and TBP by a 15 minute incubation at exactly 47°C and analysed by *in vitro* transcription assay after the addition of TBP and PC-C (a TFIIIC-containing phosphocellulose-column fraction) required for reconstitution of pol III transcription. Lane 1 serves as a transcription control where reactions were carried out in the absence of cell extract.


Cells:
/
3T3
Py3T3
Pytsa3T3
VA1
1
2
3
4

This result, in conjunction with the previous results, indeed suggests that in addition to a large T-dependent release of TFIIIB from RB-mediated repression, TFIIIB activation by Polyomavirus is also a manifestation of B" overexpression.

4.2.11 The rate limiting factor in Polyomavirus-transformed cells shifts from TFIIIC to TFIIIB in the absence of functional large T antigen

Although both the polymerase and TFIIIB display elevated activity in Py3T3 cell extracts, it was of interest to establish which of the general pol III transcription factors were rate limiting. To address this question, addback experiments were performed using purified fractions of TFIIIB, TFIIIC and pol III. It has previously been reported that TFIIIB is a rate limiting factor in 3T3 cells (Scott et al., 2001) but not for VA_1 in Cl38 or Cl49 cells (White et al., 1990). Individually adding back purified TFIIIB to an unfractionated Py3T3 extract conferred little or no stimulation of pol III transcription (Figure 4.11A, compare lane 2 with lane 1), making this result compatible with the findings in Cl38 and Cl49 cells.

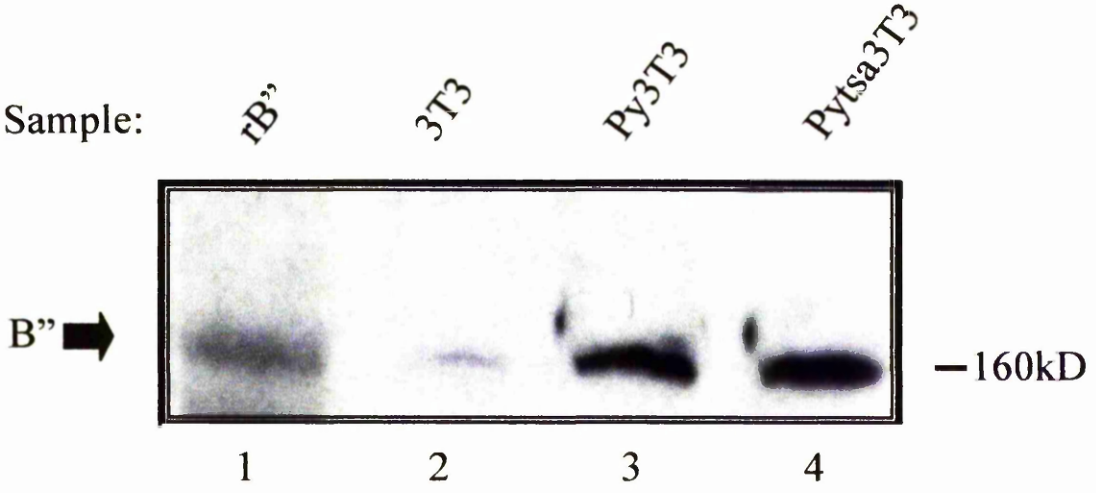
However, White *et al* also established that addition of PC-C, a crude phosphocellulose step fraction containing TFIIIC, resulted in stimulation of pol III transcription, identifying TFIIIC as the rate limiting factor in both the SV3T3 cell lines (White et al., 1990). Consistent with this observation, addition of the purified TFIIIC to a Py3T3 extract did confer a stimulation of pol III transcription of approximately 3-fold (Figure 4.11A, compare lane 3 with lane 1). However, addition of pol III did not

Figure 4.10

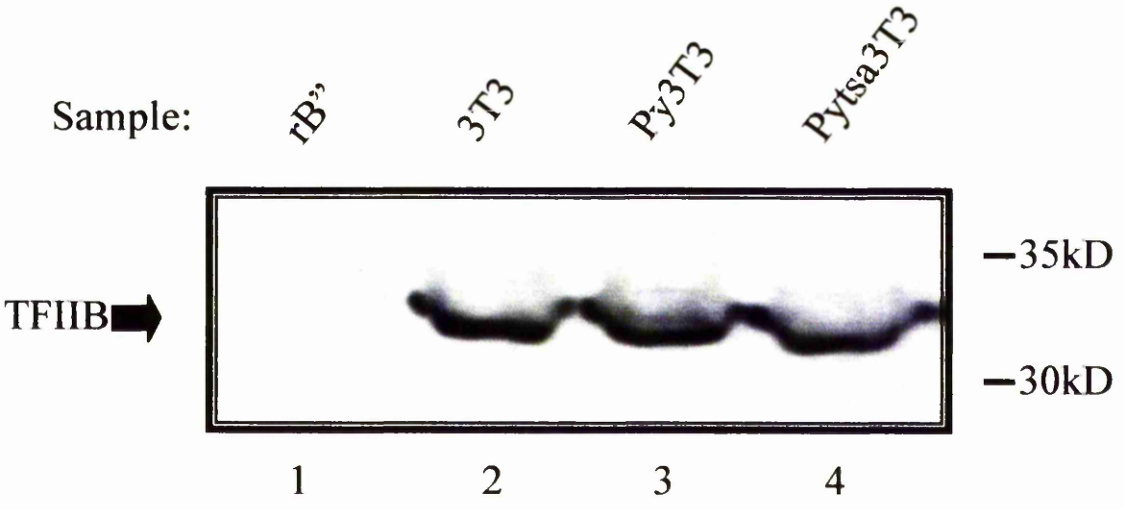
LT-defective Pytsa3T3 cells overexpress B" of TFIIIB

Whole cell extracts prepared from 3T3, Py3T3 and Pytsa3T3 cells (lanes 2, 3 and 4, respectively, in each panel) were resolved alongside recombinant B" (lane 1) on a SDS-7.8% polyacrylamide gel and analysed by western immunoblotting. Panels A and B show the same blot, cut and probed with an anti-B" antibody, 2663-4, or with the anti-TFIIB antibody, C18, respectively.

(A)


Sample:
rB"
3T3
Py3T3
Pytsa3T3
B"
160kD
1
2
3
4

(B)


Sample:
rB"
3T3
Py3T3
Pytsa3T3
TFIIB
35kD
30kD
1
2
3
4

result in elevation in transcription (Figure 4.11A, lane 4 relative to lane 1) and it can be concluded that TFIIIC is the rate limiting factor in the Py3T3 cell extracts.

Conversely, similar analysis using Pytsa3T3 cells identified TFIIIB as the principal rate limiting factor (Figure 4.11B, lane 2 compared with lanes 1, 3 and 4). TFIIIC remained limiting, still showing an increase in transcription. However, at 1.6-fold, it was substantially less than with TFIIIB, which produced a 3.9-fold elevation (Figure 4.11B, compare lanes 2 and 3 with lane 1). Transcription remained unstimulated by addition of pol III (Figure 4.11B, compare lane 4 with lane 1). Significantly, the shift from TFIIIC to TFIIIB as the factor predominantly rate limiting emphasises the proficiency of the large T antigen to deregulate TFIIIB activity. In Pytsa3T3 cells, where large T is defective and, consequently, unable to relieve TFIIIB from repression by RB, TFIIIB becomes limiting as TFIIIC levels increase; this would suggest a causal-link between the observations.

4.2.12 Model of large T antigen-mediated activation of TFIIIB

Previous studies and data presented in this chapter implicate the large T antigen as an efficient activator of TFIIIB activity. Figure 4.12 depicts a model for pol III transcriptional deregulation through release of TFIIIB from its RB-mediated repression. The interaction between RB and TFIIIB prevents TFIIIB binding to pol III and TFIIIC (Sutcliffe et al., 2000). Specific targeting of RB by the large T antigen causes its inactivation, resulting in release of TFIIIB that, consequently, makes it available for pol III transcription initiation.

Figure 4.11

TFIIIB becomes the most limiting factor in Pytsa3T3 cells

In vitro transcription was reconstituted using 20μg of Py3T3 (panel A) or Pytsa3T3 (panel B) cell extract and 250ng of VA_1 template. Reactions were supplemented with purified fractions of TFIIIB (lane 2 in each panel), TFIIIC (lane 3 in each panel) or pol III (lane 4 in each panel). Transcription reactions carried out in the absence of additional fractions are shown in lane 1 (panels A and B) to provide an indication of the basal transcription level.

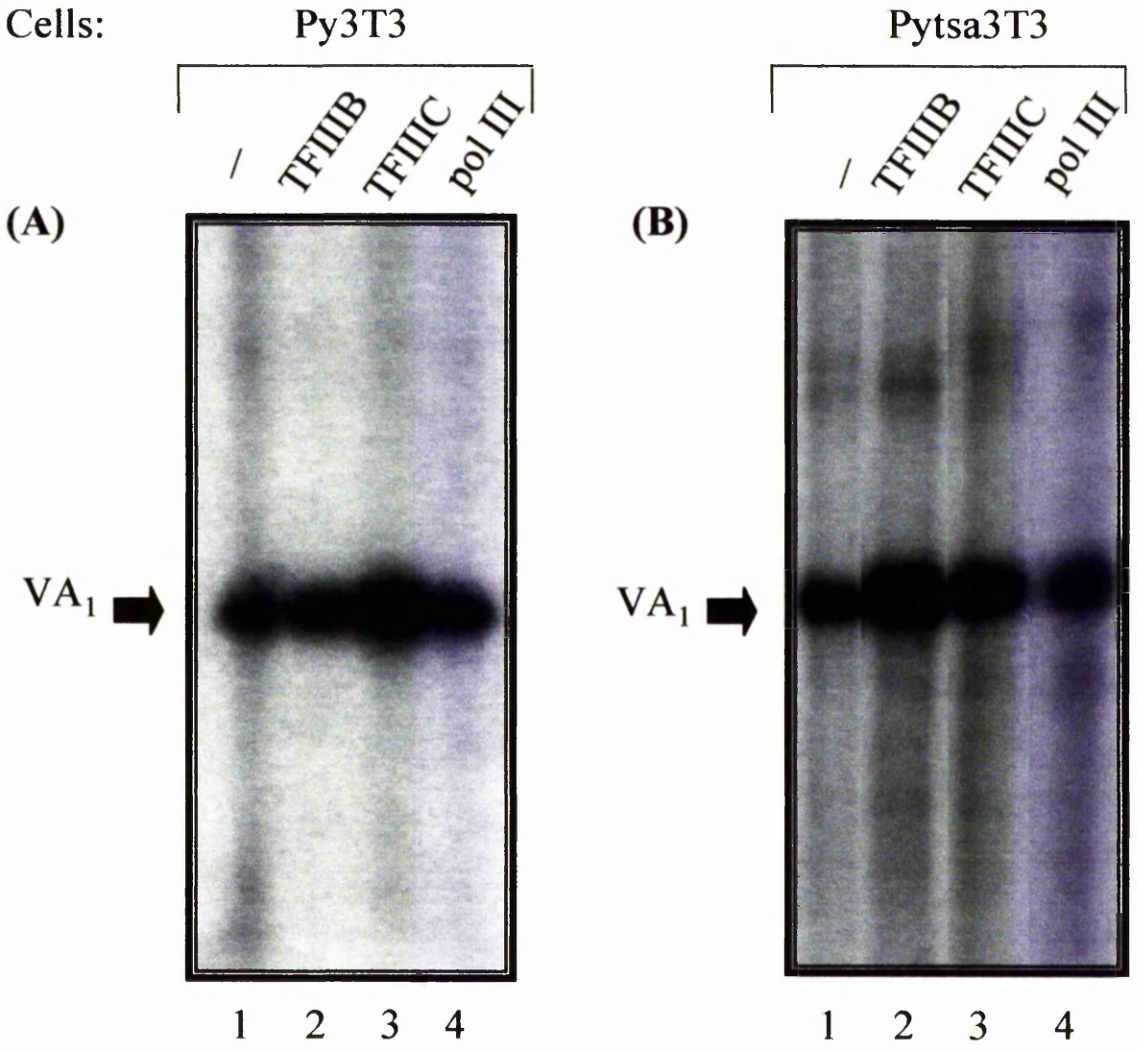
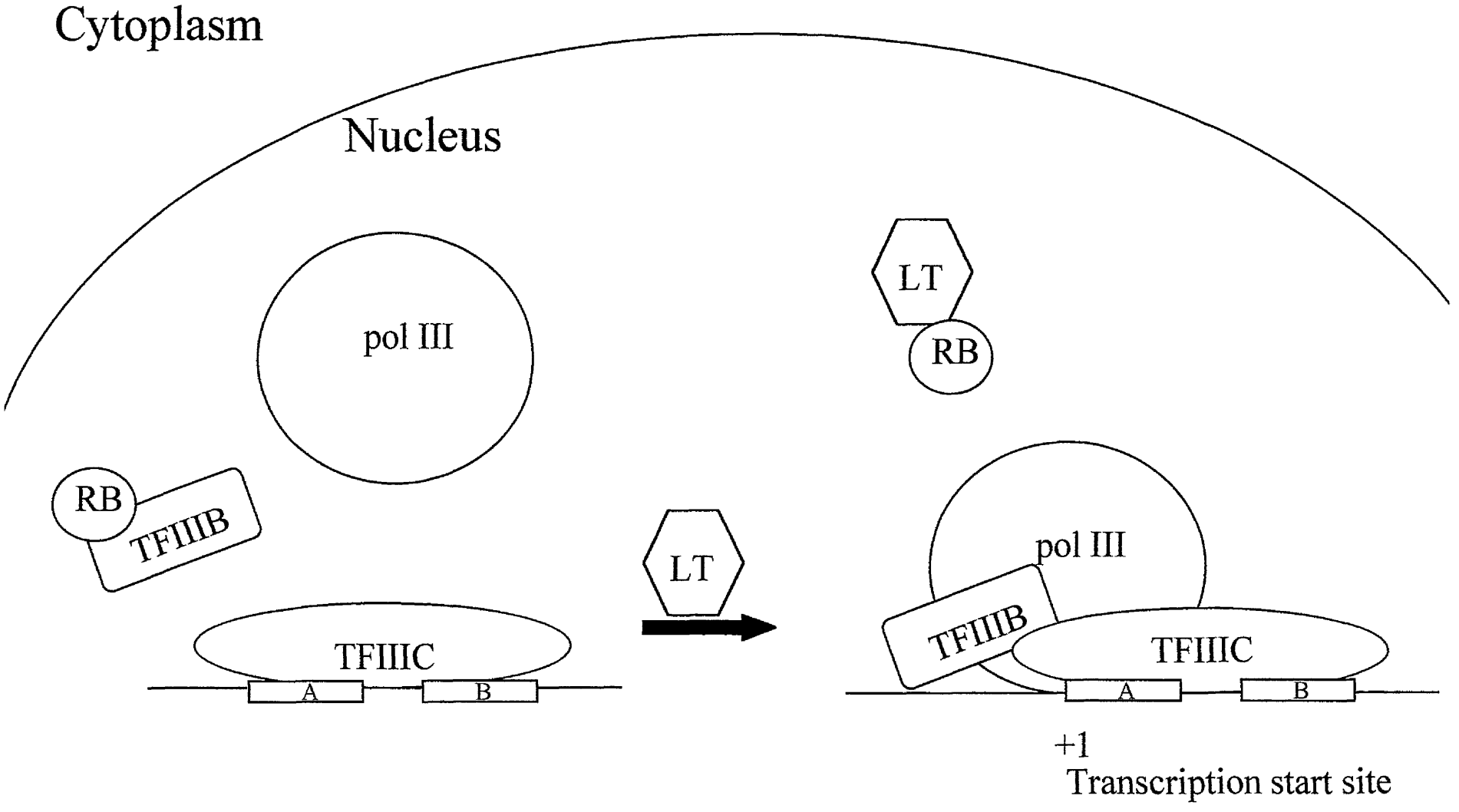

Cells:
Py3T3
Pytsa3T3
/
TFIIIB
TFIIIC
pol III
(A)
(B)
VA1
VA1
1 2 3 4
1 2 3 4

Figure 4.12

Model illustrating a LT-dependent mechanism of pol III transcriptional deregulation

The large T antigen of Polyomavirus targets RB, thereby releasing TFIIIB from its RB-mediated repression and making it available for pol III transcription initiation.


Cytoplasm
Nucleus
pol III
RB
TFIIIB
TFIIIC
A
B
LT
LT
RB
pol III
TFIIIB
TFIIIC
A
B
+1
Transcription start site

4.3 DISCUSSION

Although the deregulation of pol III transcription in tumour and transformed cells is principally through modulation of factors associated with the polymerase (Brown et al., 2000), results from random polymerisation assays established an enhanced level of actual polymerase activity in the region of 2-fold following transformation by Polyomavirus, which, furthermore, was reflected by an overexpression at the protein level of pol III subunits that were tested by western analysis.

That Polyomavirus specifically activates the polymerase presents a novel mechanism of deregulation, previously unreported in viral transformation. However, TFIIIB assays provided evidence of additional deregulation of a more commonly targeted transcription factor. The stimulation of TFIIIB activity conferred by Polyomavirus is a feature shared with its close relative, SV40 (Larminie et al., 1999). The observation from western blotting that this activation was not mediated through overexpression of the TBP and BRF subunits of TFIIIB was also consistent with previous findings (Larminie et al., 1999). Moreover, that the B" subunit of TFIIIB was clearly overexpressed in Py3T3 cells and two SV40-transformed cell lines, further extended this similarity. RT-PCR showed that in each case an increase in the B" mRNA is likely to account, at least in part, for the elevated levels of the B" polypeptides. These observations for TFIIIB, established by analyses at the activity, protein and transcript levels, show a notable correlation between the two transforming viruses.

Co-immunoprecipitation experiments supported previous studies documenting an interaction between the large T antigen and RB in Polyomavirus-transformed cells

(Pilon et al., 1996); it has been demonstrated here that this leads to a disruption of the RB-BRF interaction responsible for restraining TFIIIB activity. Furthermore, as would be predicted, Pytsa3T3 cells demonstrated, through their lack of functional large T antigen, the specific requirement of the large T antigen for interaction with RB.

The importance of the large T antigen in pol III transcriptional deregulation was emphasised through transfection into untransformed 3T3 cells, where it bestowed a marked stimulation. Additionally, TFIIIB assays utilising the large T defective Pytsa3T3 cells, established the large T antigen as the predominant activator of TFIIIB activity, which could be attributed, at least in part, to a partial release from repression by RB.

However, despite the efficacious action of the large T antigen on deregulation of pol III transcription, residual activation of TFIIIB activity was still observed in the Pytsa3T3 cells, suggesting that large T antigen activation of TFIIIB activity was complemented by an unrelated mechanism. As both Py3T3 and Pytsa3T3 cells displayed an overexpression of the B" subunit of TFIIIB, it could be construed that this overexpression may constitute the second mechanism of TFIIIB activation.

The data presented in this chapter provide compelling evidence for the activation of both pol III and TFIIIB by Polyomavirus and solicited the question of whether either of these, or another general pol III transcription factor, TFIIIC, was rate limiting for pol III transcription. Through addback experiments using purified fractions, TFIIIC was identified as the limiting factor in Py3T3 cells, in agreement with the finding of TFIIIC as the rate limiting factor in the cell lines Cl38 and Cl49 (White et al., 1990). Significantly, it was established that in Pytsa3T3 cells, where functional large T was

not present to stimulate TFIIIB, TFIIIB then became the rate limiting factor for pol III transcription.

Arguably, however, this may be a simplistic conclusion given that these experiments were performed on extracts from asynchronous cell populations, which may mask the complexity of the situation. Studies utilising synchronised HeLa cells have revealed that during different phases of the cell cycle, the limiting pol III factor alternates. Hence, extracts from cells harvested during early G_1 phase present TFIIIB activity as rate limiting for VA_1 expression, while in extracts of S- or G_2-phase HeLa cells, TFIIIC becomes the limiting factor (White et al., 1995a). Consequently, while both these factors are necessary throughout the cell cycle, either of them individually may influence transcription for only a discrete interval of the cell cycle, subject to it being the limiting factor. Thus, presuming a comparable situation exists in 3T3 cells, activation of both TFIIIB and TFIIIC would likely be required to allow sustained elevation of pol III transcription rate.

Nevertheless, these results collectively argue that Polyomavirus is capable of activating both the polymerase itself and the general transcription factor TFIIIB, and that in the case of the latter, this is ensured by the action of two distinct mechanisms.

Chapter 5

Overexpression of the pol III transcription factor TFIIIC2

5.1 INTRODUCTION

The multisubunit complex TFIIIC is a DNA-binding protein that has proved to be one of the largest and most complex transcription factors studied. In *S. cerevisiae*, TFIIIC comprises two globular domains, each approximately 300kD (Schultz et al., 1989). It is composed of six subunits, none of which have demonstrated the ability to bind specifically to DNA individually. Human TFIIIC appears to be somewhat different from its yeast counterpart. It demonstrates a lower overall stability and can be resolved into two components, TFIIIC1 and TFIIIC2, by ion exchange chromatography (Dean and Berk, 1987; Oettel et al., 1997; Wang and Roeder, 1996; Yoshinaga et al., 1987). Studies have documented that expression of 5S rRNA, VA_1 and tRNA genes require both components, while U6 and 7SK transcription requires TFIIIC1, although not TFIIIC2 (Lagna et al., 1994; Oettel et al., 1997; Yoon et al., 1995). Although TFIIIC1 is poorly characterised, human TFIIIC2 has been established as a five subunit complex consisting of polypeptides of 220, 110, 102, 90 and 63kD, designated α, β, γ, δ and ε, respectively (Sinn et al., 1995; Wang and Roeder, 1996; Yoshinaga et al., 1989). TFIIIC2 is responsible for the initial recognition of the A- and B-block sequences, which are core promoter elements found in type II promoters, serving then to recruit TFIIIC1 and TFIIIB (Dean and Berk,

1988). While the composition and exact function of TFIIIC1 remain elusive, a role has been established for it in stabilisation of TFIIIC2 binding to A- and B-boxes (Wang and Roeder, 1996). TFIIIC1 can bind independently to sequences downstream of the B-box that include the termination region, contributing at least in part, to the enhanced level of TFIIIC2 binding observed through their co-operative interactions (Wang and Roeder, 1996).

TFIIIC2 can be detected in at least two forms, distinguishable by their differential migrations in electrophoretic mobility shift assays (Hoeffler et al., 1988; Kovelman and Roeder, 1992; Sinn et al., 1995) and which are distinct in their abilities to support transcription (Kovelman and Roeder, 1992). Chromatographic fractionation of HeLa cells has identified that the low-mobility form is transcriptionally active, while the higher-mobility species is inactive for transcription, although it retains the ability to bind DNA (Kovelman and Roeder, 1992). The difference underlying these transcriptional inconsistencies can be attributed to the 110kD subunit, termed TFIIIC110; it is present in the active form, TFIIIC2a, but omitted from the inactive TFIIIC2b species (Kovelman and Roeder, 1992; Sinn et al., 1995). Notably, the slow-migrating species, TFIIIC2a, can be converted into the inactive TFIIIC2b form upon treatment with acid phosphatase (Hoeffler et al., 1988). Thus, the transcriptional activity of TFIIIC can be modulated through phosphorylation. Furthermore, *in vivo* labelling has demonstrated the phosphorylation of all five subunits of TFIIIC2 in HeLa cells (Shen et al., 1996).

UV crosslinking has shown that the 220kD component, TFIIIC220, is the DNA-binding subunit of the TFIIIC2 complex. Nevertheless, TFIIIC220 alone exhibits no specific B-block-binding activity (Shen et al., 1996). However, an N-terminal 83kD

fragment of the 220 subunit, comprising residues 1-732, can bind DNA specifically when in association with the 110 subunit, although this complex is unable to support transcription (Shen et al., 1996).

Intrinsic histone acetyltransferase (HAT) activity has been identified in TFIIIC110 and TFIIIC90 and strongly suggested in the TFIIIC220 subunit (Hsieh et al., 1999; Kundu et al., 1999). Elevated levels of TFIIIC2 have been demonstrated to reverse chromatin-mediated repression of a human tRNA gene and a partial inhibition of TFIIIC2 HAT activity correlates with a partial reduction in transcription from chromatin, although not naked DNA, templates (Kundu et al., 1999). The HAT activity of TFIIIC2 could, therefore, conceivably contribute to promoter accessibility to potentiate transcription, conferring an additional role to TFIIIC, over and above its function as an assembly factor for recruiting TFIIIB.

While TFIIIB is subject to strong regulation throughout the cell cycle (Scott, 2001; White, 1995; White, 1998b; White et al., 1995b), TFIIIC displays no evidence of cell cycle regulation. However, it has been observed in a murine system, that both TFIIIB and TFIIIC are less active in stationary-phase cells than they are during active growth (Tower and Sollner-Webb, 1988).

As a rudimentary factor in the pol III transcriptional machinery, there is a strong propensity for it to be a target for deregulation. HeLa cells infected with wild-type adenovirus display a substantial increase in TFIIIC2 activity (Hoeffler et al., 1988; Sinn et al., 1995). This is a manifestation of a selective increase in the level of the TFIIIC110 subunit, induced by the E1A oncoprotein, thereby raising the proportion of TFIIIC2 in the active TFIIIC2a form (Hoeffler et al., 1988; Sinn et al., 1995). Moreover, serum stimulation of these cells similarly results in an increase in the

abundance of TFIIIC110, although interestingly, the level of the DNA-binding subunit TFIIIC220 remains constant under these conditions (Sinn et al., 1995).

Another transforming virus that has revealed an ability to deregulate TFIIIC, is SV40. Elevation in the binding activity of TFIIIC2 has been documented in the SV40-transformed cell lines, SV3T3 Cl38 and SV3T3 Cl49 (White et al., 1990). Like adenovirus, SV40 is able to induce a significant increase in the level of TFIIIC110, thus converting inactive TFIIIC2b into the active TFIIIC2a form (Larminie et al., 1999; White et al., 1990). However, in contrast to adenovirus, the ability to activate TFIIIC2 is not restricted to the confines of TFIIIC110 elevation and SV40-transformed fibroblasts additionally show overexpression of TFIIIC220 (Larminie et al., 1999). Furthermore, the increased levels of TFIIIC2 subunits following SV40 transformation reflect an elevation of transcripts encoding these subunits. In the case of TFIIIC110, expression in Cl38 and Cl49 cell lines was seven- to eight-fold higher than in the corresponding untransformed parental cells (Larminie et al., 1999).

These examples of TFIIIC activation through viral transformation clearly establish it as an important target; however, perhaps more significantly, the first evidence of a pol III transcription factor, namely TFIIIC2, being overexpressed in tumours, has now been published (Winter et al., 2000). It was established that mRNAs encoding each of the TFIIIC2 subunits were overexpressed in human ovarian carcinomas. Given that there was little or no change in TFIIIC2 mRNA levels in actively cycling cells relative to growth-arrested cells in culture, deregulation seems unlikely to be a secondary response to rapid cell proliferation within the carcinomas. Instead, it suggests that it is a more specific characteristic of tumourigenesis, rather than a simple growth response (Winter et al., 2000).

Additionally, activation of TFIIIC2 has been observed in breast carcinomas. Such observations in malignant human cells *in vivo* confer important credibility to the mechanisms of deregulation established through studies using transformed cell lines.

This chapter provides evidence of TFIIIC2 activation following transformation by Polyomavirus. The intrinsic involvement of the large T antigen in activation of TFIIIB has already been discussed; however, potentially, activation of TFIIIC could have been another manifestation of large T function. Data presented in this chapter argue against a role for the large T antigen and suggest that activation of TFIIIC2 is mediated through a large T-independent mechanism.

5.2 RESULTS

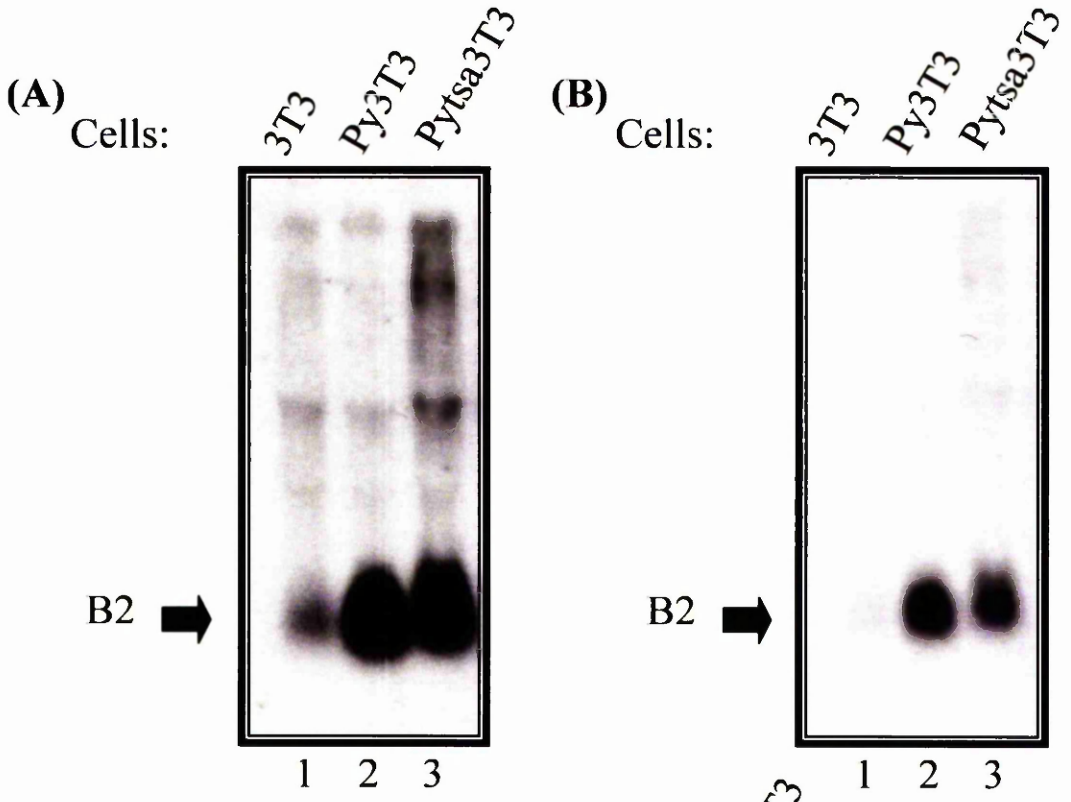
5.2.1 Partial B2 overexpression in the absence of the Polyomavirus large T antigen

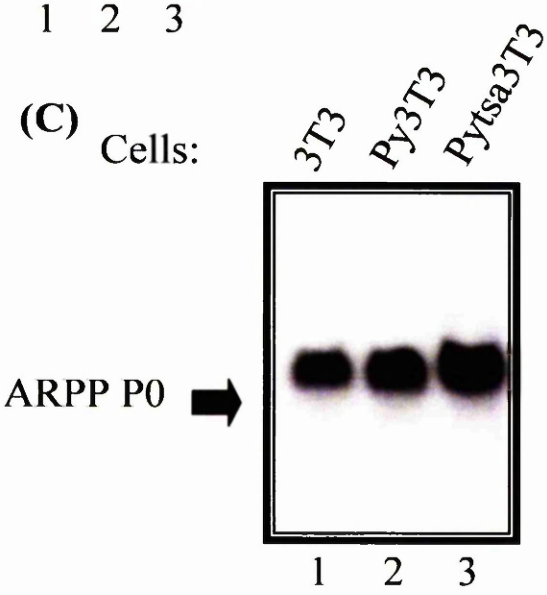
Chapter 4 identified the intrinsic involvement of the large T antigen in deregulation of TFIIIB activity. In order to establish if any pol III transcriptional deregulation persists in the absence of the large T antigen, Pytsa3T3 cells were utilised in northern analysis. Relative to the 3T3 cells, a marked overexpression of B2 transcripts was observed in the Pytsa3T3 cells (Figure 5.1A, compare lane 3 with lane 1). However, as is more clearly demonstrated by a shorter exposure of the same blot, these cells were unable to match the B2 overexpression observed for wild-type transformed Py3T3 cells (Figure 5.1B, compare lanes 2 and 3 with lane 1). Reprobing the blot for the pol II transcript ARPP P0 established that the B2 overexpression exhibited by these cell lines was specific (Figure 5.1C). Values for B2 levels were normalised against those for ARPP P0 and expressed graphically (Figure 5.1D), demonstrating an increase in B2 expression in the region of 11-fold for Py3T3 cells relative to the 3T3 cells and in the region of 4-fold in the case of the Pytsa3T3 cell line. Thus, while the large T antigen present in the wild-type transformed cells is clearly responsible for a substantial proportion of the pol III deregulation, this result provides strong evidence supporting the involvement of additional deregulatory mechanisms.

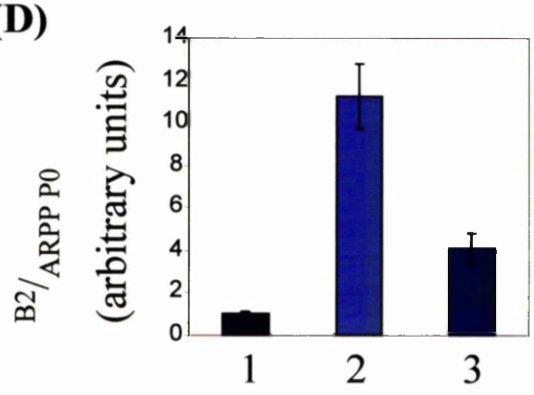
Figure 5.1

Partial overexpression of Pol III transcripts pertains in Pytsa3T3 cells

Total RNA (30μg) was extracted from 3T3 (lane 1), Py3T3 (lane 2) and Pytsa3T3 (lane3) cells and used for northern blot analysis. Panel A shows the blot probed with a B2 gene and a shorter exposure of the same blot is displayed in panel B. The blot was stripped and subsequently reprobed for the ARPP P0 gene (Panel C). The levels of B2 and ARPP P0 RNA from the northern analysis were quantitated by phosphoimaging (Fujix Bas 1000); B2 levels were normalised against levels for ARPP P0 and expressed as arbitrary units. Values shown represent the mean of three experiments ± standard deviation, with the value obtained for the 3T3 cells being designated 1, as illustrated in panel D.


(A)
Cells:
3T3
Py3T3
Pytsa3T3
B2
1 2 3
(B)
Cells:
3T3
Py3T3
Pytsa3T3
B2
1 2 3


(C)
Cells:
3T3
Py3T3
Pytsa3T3
ARPP P0
1 2 3


(D)
B2/ARPP P0 (arbitrary units)
14
12
10
8
6
4
2
0
1 2 3

5.2.2 Pol III transcription is partially deregulated in Pytsa3T3 cell extracts

The partial deregulation of pol III B2 expression levels observed in the Pytsa3T3 cells, as shown in figure 5.1, was supported by *in vitro* transcription assays. Figure 5.2 displays levels of transcription for the pol III VA_1 template and similarly demonstrates the partial activation of pol III transcription of 1.6-fold by the Pytsa3T3 cells over the 3T3 cells and the more prominent activation of 4.2-fold conferred by wild-type Polyomavirus.

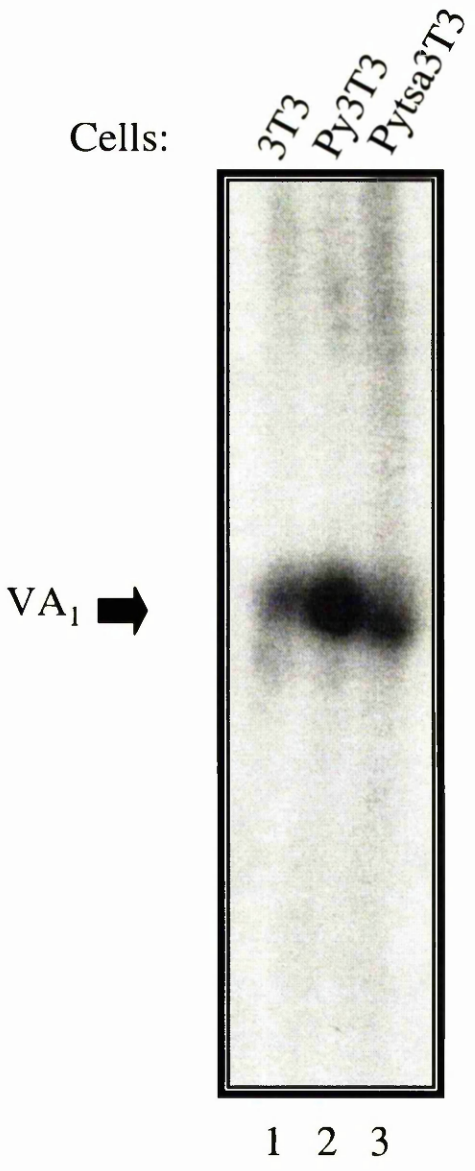
5.2.3 Py3T3 cell extracts display elevated TFIIIC activity

In addition to the activation of TFIIIB ascribed to the function of the large T antigen, previous results established both overexpression of B" in Pytsa3T3 cells and an activation of the polymerase itself following transformation. While these aspects must likely contribute some degree to the deregulation resulting from Polyomavirus transformation, the possibility remained that TFIIIC, as another fundamental transcription factor in the pol III machinery, could also be targeted for deregulation and could, therefore, account at some level for the deregulation persisting in the Pytsa3T3 cells. Precedent for this contention is given by the finding of elevated TFIIIC activity in SV3T3 Cl38 and SV3T3 Cl49 cells relative to the untransformed parental 3T3 cell line (White et al., 1990).

Figure 5.2

Pytsa3T3 cell extracts display partial deregulation of pol III transcription

In vitro transcription assay illustrating relative levels of pol III transcription for 3T3 (lane 1), Py3T3 (lane 2) and Pytsa3T3 (lane 3) cell extracts. Transcription reactions contained 250ng of pVA_1 template and 20μg of cell extract.


Cells:
3T3
Py3T3
Pytsa3T3
VA1
1 2 3

To address the possibility of TFIIIC2 activation, electrophoretic mobility shift assays were performed to identify the degree of TFIIIC2 DNA-binding activity in the cell lines. Levels of TFIIIC2 observed in the Py3T3 cell extracts were significantly more abundant than in extracts from 3T3 cells (Figure 5.3, compare lane 5 with lane 3). For both cell lines, the TFIIIC2 band was competed out by an excess of unlabelled B-block oligonucleotide (Figure 5.3, lane 2 and 4) but not by an equal amount of a non-specific control oligonucleotide (Figure 5.3, lanes 3 and 5), indicating that the bands were specific. A binding reaction performed in the absence of cell extract produced no band for TFIIIC2, as expected (Figure 5.3, lane 1).

5.2.4 TFIIIC activation is independent of the large T antigen

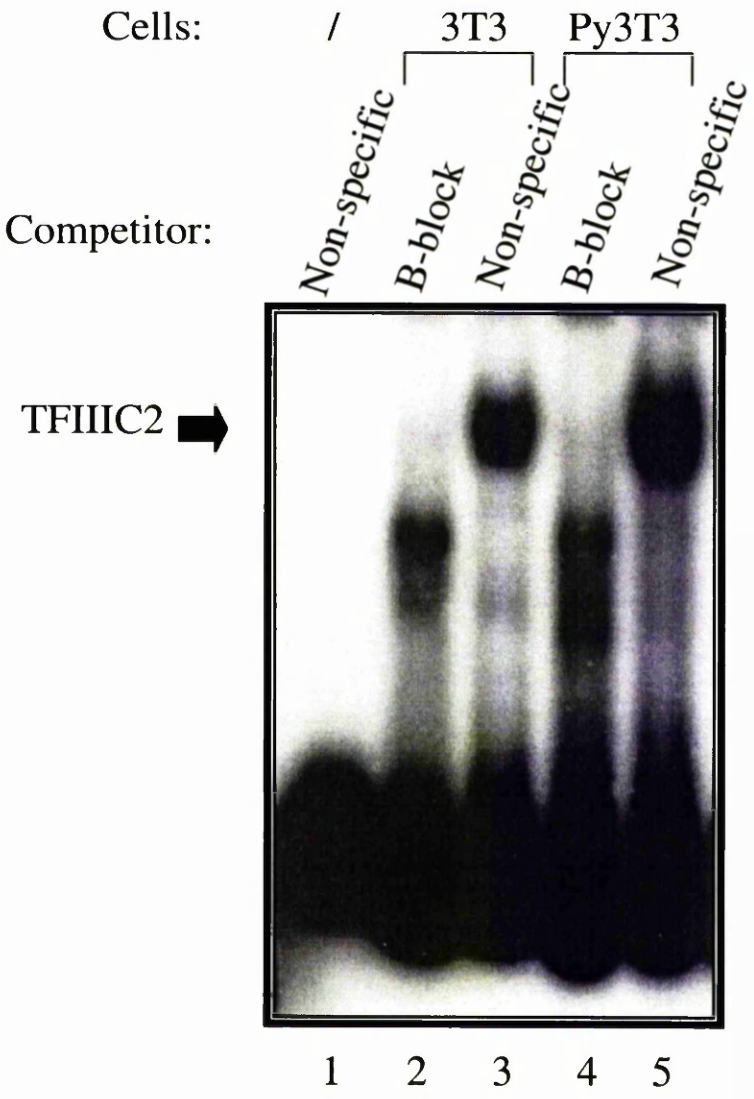
Similar DNA-binding assays were performed using Pytsa3T3 cell extracts to establish TFIIIC activity in the absence of the large T antigen of Polyomavirus and thus determine if activation of TFIIIC2 was attributed to the large T antigen. Py3T3 cells again exhibited elevated activity of TFIIIC2 relative to the untransformed parental cells; furthermore, Pytsa3T3 cells displayed similar TFIIIC activity (Figure 5.4A, compare lanes 3 and 4, respectively, with lane 2). Again, TFIIIC2 was absent in a reaction lacking cell extract (Figure 5.4A, lane 1).

The unrelated transcription factor Sp1 was similarly analysed as a control against a general loss of DNA-binding proteins during the preparation of 3T3 cell extracts or a general increase in transcription factors following Polyomavirus transformation.

Figure 5.3

Activation of TFIIIC2 is displayed in extracts from Py3T3 cells

Gel retardation assay for TFIIIC2 DNA-binding activities of parental and Polyomavirus-transformed cell lines. Reactions contained 0.5ng of labelled B-block oligonucleotide probe, 1μg of poly(dI.dC), no extract (lane 1), or 23μg of 3T3 (lanes 2 and 3) or Py3T3 (lanes 4 and 5) whole cell extract and 100ng of non-specific (lanes 1, 3 and 5) or specific B-block (lanes 2 and 4) competitor oligonucleotide.


Cells:
/
3T3
Py3T3
Competitor:
Non-specific
B-block
Non-specific
B-block
Non-specific
TFIIIC2
1
2
3
4
5

Although this control is weakened due to not being in probe excess, Sp1 levels appeared to remain constant (Figure 5.4B).

After quantification, TFIIIC2 values were delineated in the graph shown in figure 5.4C. It serves to highlight the difference between wild-type Polyomavirus-transformed Py3T3 cells and untransformed 3T3 cells in TFIIIC2 DNA-binding activity and, additionally, the ability of Pytsa3T3 cells to sustain this difference in the absence of the large T antigen.

5.2.5 TFIIIC2 transcripts are overexpressed in Polyomavirus-transformed cells

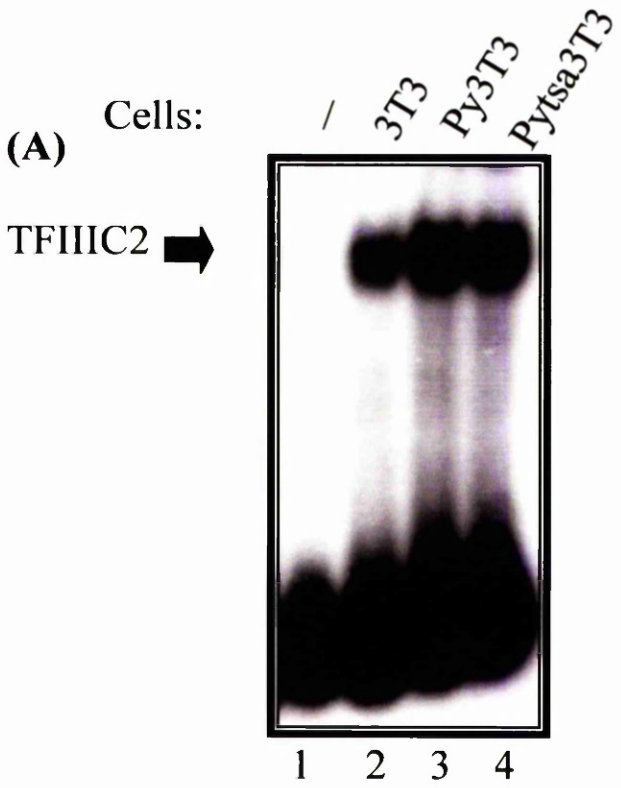
Previous studies have demonstrated that the activation of TFIIIC observed in SV40-transformed cell lines is accompanied by an overexpression of the transcripts encoding two of the principal components of TFIIIC2, TFIIIC220 and TFIIIC110 (Larminie et al., 1999). TFIIIC2 additionally comprises another three subunits (Kovelman and Roeder, 1992; Yoshinaga et al., 1989); however, at the time of the previous study, the cloning of cDNAs encoding these subunits had not been reported and, consequently, their expression levels following SV40 transformation were undetermined.

In order to establish whether the increased TFIIIC2 activity displayed by Polyomavirus-transformed cells showed a similar correlation with expression of TFIIIC2 subunits, semi-quantitative RT-PCR analyses were performed for each subunit utilising cDNAs prepared from 3T3 and Py3T3 cells. Additionally, given that

Figure 5.4

Polyomavirus activates TFIIIC2 in the absence of the LT antigen

TFIIIC2 DNA-binding assay using the B-block oligonucleotide as probe (panel A). Reactions contained 0.5ng of labelled B-block oligonucleotide, 1μg of poly(dI.dC), no extract (lane 1), or 23μg of 3T3 (lane 2), Py3T3 (lane 3) or Pytsa3T3 (lane 4) whole cell extract. Panel B displays DNA-binding activities of the three cell lines using Sp1 oligonucleotide as probe. Reactions contained 0.5ng of labelled Sp1 oligonucleotide, 1μg of poly(dI.dC), no extract (lane 1), or 23μg of 3T3 (lane 2), Py3T3 (lane 3) or Pytsa3T3 (lane 4) whole cell extract. Levels of TFIIIC2 DNA-binding activity were quantified by phosphoimaging (Fujix Bas 1000) and TFIIIC2 levels were expressed as arbitrary units, with the value obtained for the 3T3 cells being designated 1, as illustrated in panel C. The values displayed represent the mean of two experiments ± standard deviation.


(A)
Cells:
/
3T3
Py3T3
Pytsa3T3
TFIIIC2
1 2 3 4
(B)
Sp1
1 2 3 4
(C)
TFIIIC2/Sp1
(arbitrary units)
2
1.5
1
0.5
0
1 2 3 4

three of the subunits had not been previously analysed in Cl38 and Cl49 cells, these cell lines were included in analysis of the TFIIIC63, TFIIIC90 and TFIIIC102 subunits.

Consistent with the observations for TFIIIC220 and TFIIIC110 expression in Cl38 and Cl49 cells, Polyomavirus-transformed cells displayed an elevated level of these transcripts when compared with levels observed in the 3T3 cells (Figure 5.5, panels A and B, respectively, compare lanes 1 and 2). Similarly, Pytsa3T3 cells analysed for expression levels of the TFIIC220 and TFIIIC110 subunits displayed a comparable elevation relative to the untransformed 3T3 cells (Figure 5.5, panels A and B, respectively, compare lane 3 with lane 1). This was shown to be a specific phenomenon by analysis of pol II-transcribed ARPP P0, which showed equivalent transcript levels in the three cell lines (Figure 5.5C, compare lanes 1, 2 and 3).

Subsequent analyses of the TFIIIC63, TFIIIC90 and TFIIIC102 subunits provided evidence that indeed, transcripts of all five components of TFIIIC2 are overexpressed in the Py3T3 cells (Figure 5.5, panels D, E and F, respectively, compare lanes 1 and 2). Furthermore, this prevailing deregulation was reflected in the observations for Cl38 and Cl49 cells, which displayed even more striking increases in the mRNAs encoding these subunits (Figure 5.5, panels D, E and F, respectively, compare lanes 3 and 4 with lane 1). mRNAs encoding ARPP P0 in Cl38 and Cl49 cells are expressed at constant levels, demonstrating again, that these increases were specific (Figure 5.5G). Values obtained for each of the five subunits were normalised against the values for ARPP P0 in the case of each cell line. These are displayed in graphs H-L, which correspond to panels A, B and D-F, respectively. Each cell line activates subunits to a varying degree; however, with the exception of the TFIIIC102 transcript

levels observed in Cl49 cells, the relative increases for each subunit remain fairly constant, suggesting a degree of synchronised activation to ensure an overall increase in the level of functional TFIIIC2.

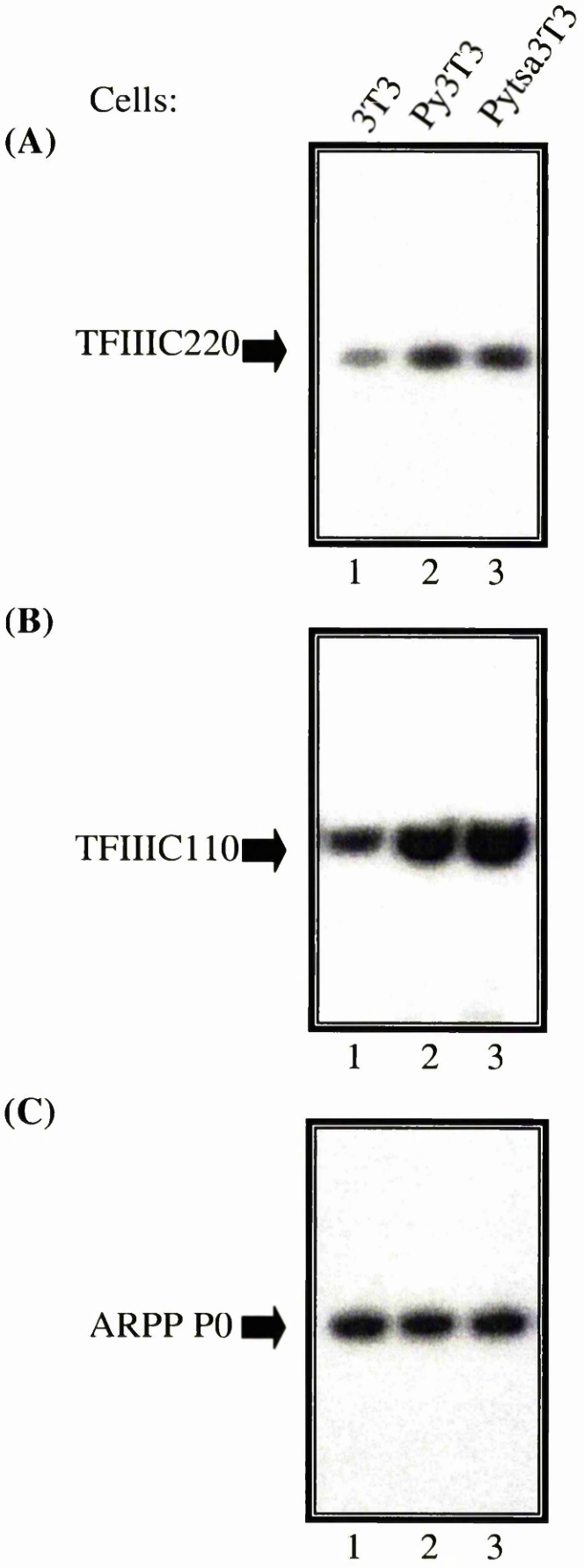
5.2.6 Py3T3 cells overexpress subunits of TFIIIC2

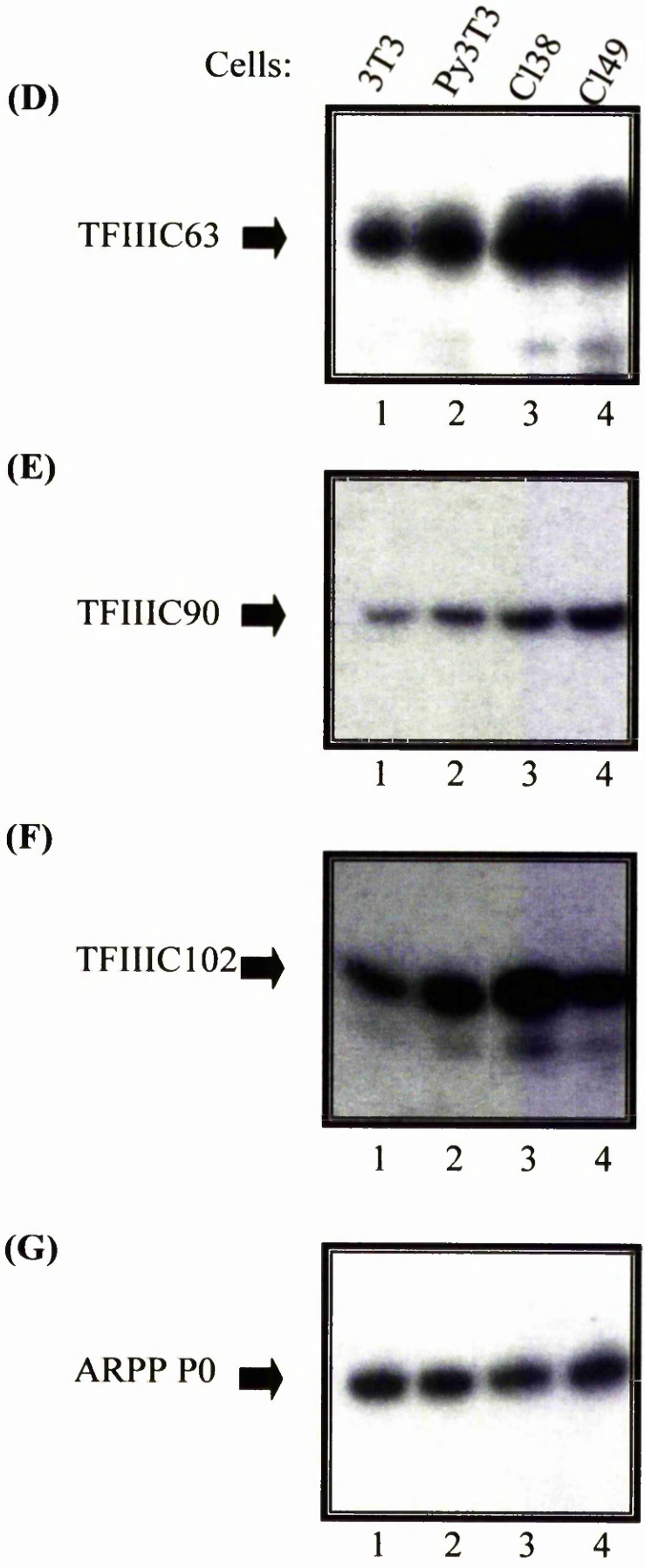
It is likely that an increase in TFIIIC2 subunits at the transcript level would confer, at least in part, an increase in expression at the protein level. Indeed, such a correlation has been seen for the TFIIIC220 and TFIIIC110 subunits in Cl38 and Cl49 cells (Larminie et al., 1999). Unfortunately, antibodies against all five subunits were not available, but western analysis, blotting for the TFIIIC220 and TFIIIC110 subunits, demonstrated that this relationship pertained in Py3T3 cells (Figure 5.6, panels A and B, respectively, compare lanes 1 and 2). The increases observed in both these subunits were confirmed in blots probed with alternative antibodies and were shown to be specific, as blotting for actin revealed similar expression levels in the two cell lines (Figure 5.6C). Although undetermined, it would seem likely that similar increases in abundance of the remaining TFIIIC2 subunits would also exist.

Figure 5.5

Overexpression of TFIIIC2 transcripts in Polyomavirus-transformed cells

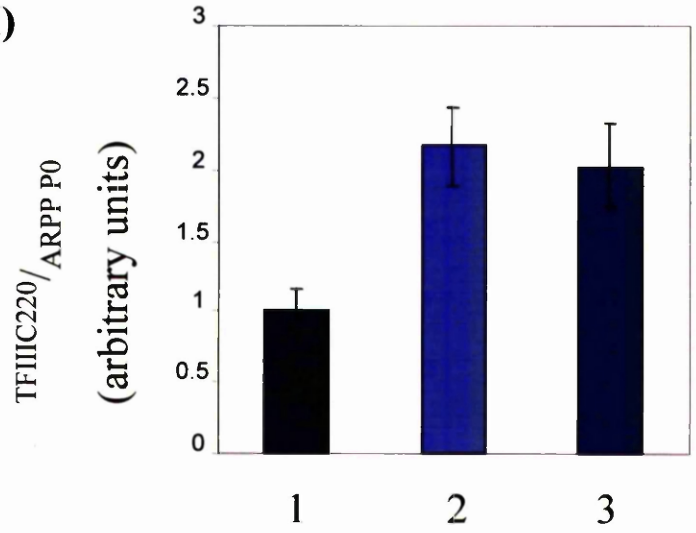
cDNAs were generated by reverse transcription of 3μg of RNA from 3T3 (lane 1, panels A-G), Py3T3 (lane 2, panels A-G), Pytsa3T3 (lane 3, panels A-C), Cl38 (lane 3, panels D-G) and Cl49 (lanes 4, panels D-G) cells and were PCR amplified using primers for TFIIIC220 (panel A), TFIIIC110 (panel B), TFIIIC63 (panel D), TFIIIC90 (panel E), TFIIIC102 (panel F), and ARPP P0 (panels C and G). Levels for the transcripts in the case of each cell line were quantitated by phosphoimaging (Fujix Bas 1000); values for the TFIIIC2 subunits were normalised against ARPP P0 and illustrated graphically (panels H-L). Values shown represent the mean of two experiments ± standard deviation, with the value obtained for the 3T3 cells being designated 1.


Cells:
3T3
Py3T3
Pytsa3T3
(A)
TFIIIC220
1 2 3
(B)
TFIIIC110
1 2 3
(C)
ARPP P0
1 2 3

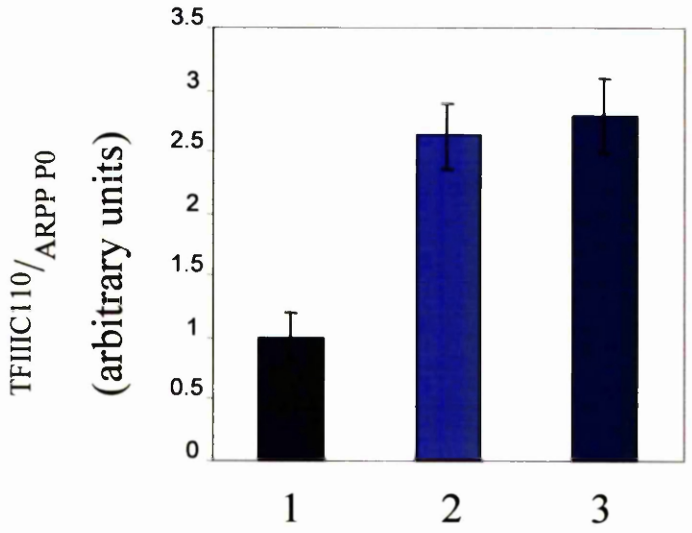

(D)
Cells:
3T3
Py3T3
Cl38
Cl49
TFIIIC63
1 2 3 4
(E)
TFIIIC90
1 2 3 4
(F)
TFIIIC102
1 2 3 4
(G)
ARPP P0
1 2 3 4

Cells: 3T3 Py3T3 Pytsa3T3

(H)



(I)



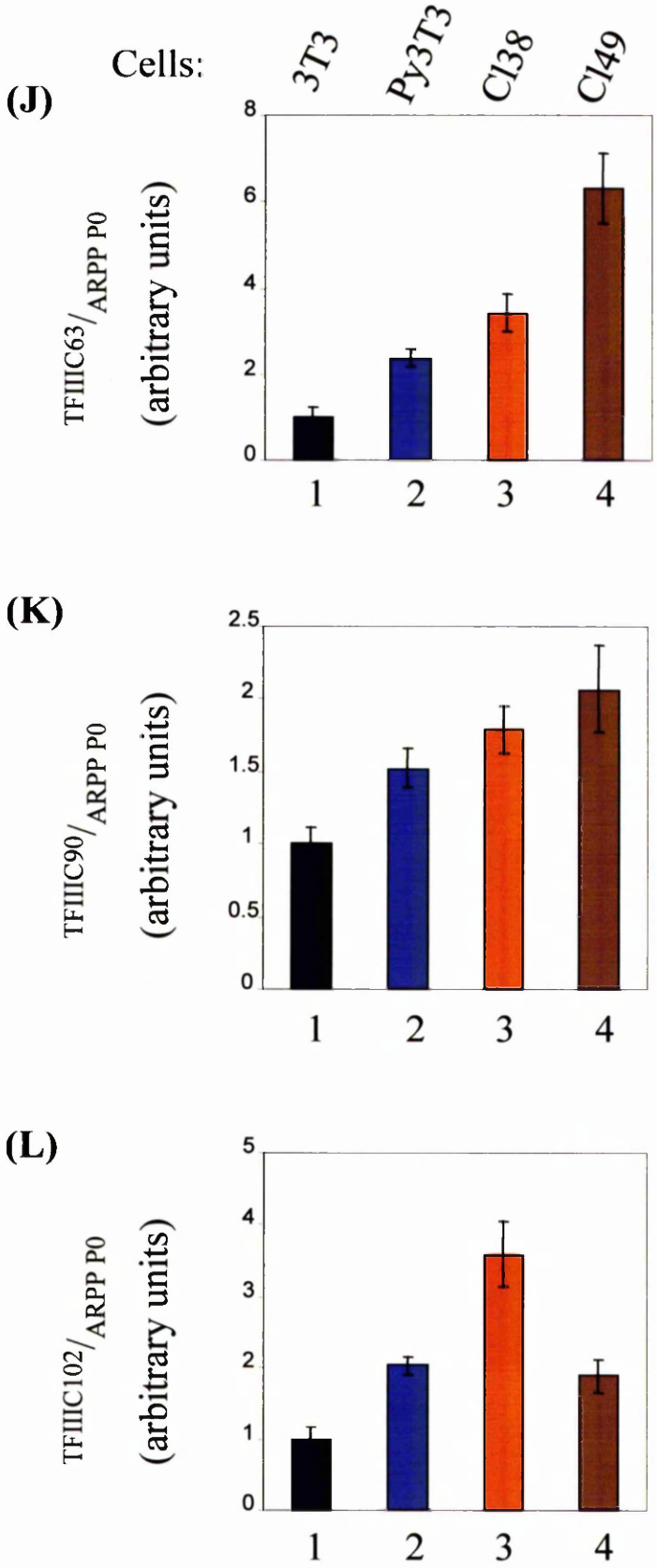
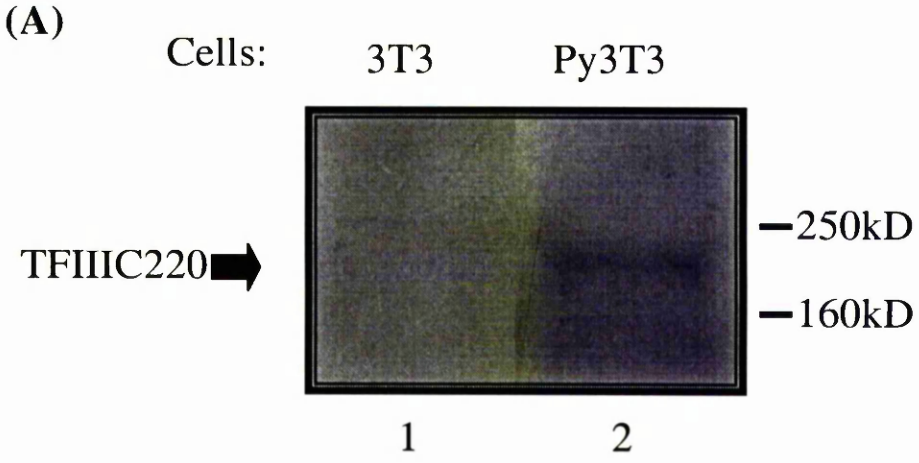

(J)
Cells:
3T3
Py3T3
Cl38
Cl49
TFIIIC63/ARPP P0
(arbitrary units)
8
6
4
2
0
1
2
3
4
(K)
TFIIIC90/ARPP P0
(arbitrary units)
2.5
2
1.5
1
0.5
0
1
2
3
4
(L)
TFIIIC102/ARPP P0
(arbitrary units)
5
4
3
2
1
0
1
2
3
4

Figure 5.6

Overexpression of TFIIIC2 components in Polyomavirus-transformed cells

Whole cell extracts prepared from 3T3 (lane 1 in each panel) and Py3T3 (lane 2 in each panel) cells were resolved on a SDS-7.8% polyacrylamide gel and analysed by western immunoblotting using either the Ab2E antibody raised against TFIIIC220 (panel A), an anti-TFIIIC110 antibody, T4 7220 (panel B) or an anti-actin antibody, C-11 (panel C). Panels A, B and C show the same blot, cut and probed with the respective antibodies.


(A)
Cells:
3T3
Py3T3
TFIIIC220
250kD
160kD
1
2
(B)
Cells:
3T3
Py3T3
TFIIIC110
105kD
1
2
(C)
Cells:
3T3
Py3T3
Actin
50kD
1
2

5.3 DISCUSSION

Results discussed previously have established the up-regulation of pol III transcription in Py3T3 cells. Analyses utilising Pytsa3T3 cells have demonstrated, through their lack of functional large T antigen, that the action of large T plays a pivotal role in the activation of the transcription factor TFIIIB. However, northern analysis using the Pytsa3T3 cells established that a significant degree of pol III deregulation remained in the absence of the large T antigen. Furthermore, this was reflected by *in vitro* transcription assays, similarly comparing levels of transcriptional activation in Pytsa3T3 cell extracts relative to 3T3 cells and wild-type Polyomavirus-transformed cells.

Both the polymerase and TFIIIB have been implicated in the deregulation of pol III transcription by Polyomavirus. However, despite the involvement of both these key pol III transcription factors, the potential for targeting of another principal transcription factor, TFIIIC, remained probable. The addback experiments shown in chapter 4 revealed that in Py3T3 cells, TFIIIC was rate limiting for pol III transcription. Consequently, it was of interest to establish whether Polyomavirus targeted TFIIIC for activation as an additional mode of deregulation. Moreover, TFIIIC2 deregulation has been demonstrated following transformation by oncogenic viruses such as adenovirus and the close relative of Polyomavirus, SV40.

Through gel retardation assays, it was established that this was also a characteristic of Polyomavirus-transformed cells, with Py3T3 cells exhibiting an elevation of TFIIIC2 DNA-binding activity in the region of 1.8-fold over levels displayed in 3T3 cell

extracts. Activation of TFIIIC2 in Py3T3 cells may seem contradictory to it being rate limiting for transcription. However, TFIIIC as a rate limiting factor is also an established feature of Cl38 and Cl49 cells, in which elevation of TFIIIC activity has already been established (White et al., 1990). Moreover, analyses employing the Pytsa3T3 cell line demonstrated that this activation of TFIIIC2 prevailed in the absence of the Polyomavirus large T antigen, implying the involvement of a large T-independent mechanism of activation.

Since it has been documented that whole cell extracts more accurately mimic *in vivo* regulation than do nuclear extracts (Hoeffler and Roeder, 1985) the binding assays utilised whole cell extracts to ensure that all the regulatory components were available and provide a more faithful reflection of the situation in the cells. Furthermore, control experiments, using alternative DNA-binding proteins to TFIIIC2, demonstrated that the differences observed between the cell lines was not symptomatic of a general increase in transcription factors following transformation by Polyomavirus.

Support for the activation of TFIIIC2 was conferred by RT-PCR analyses of the five components of TFIIIC2. In each case, a significant overexpression of transcripts encoding the subunits was observed in the Py3T3 cells relative to levels seen in the parental cell line. Furthermore, the observation of elevated TFIIIC220 and TFIIIC110 in the Pytsa3T3 cells provides additional evidence that activation of TFIIIC2 is not attributed to the function of the large T antigen. In addition, the subunits TFIIIC63, TFIIIC90 and TFIIIC102, previously untested in SV40-transformed cells, were shown to be up-regulated in Cl38 and Cl49 cells, making elevation of all five subunits a feature of SV40 transformational deregulation. Interestingly, while each transformed

cell line up-regulated the expression of subunit transcripts to varying degrees, the pattern displayed between the cell lines remained relatively consistent, supporting the logical argument that for an overall increase in functional TFIIIC2, subunits would likely have to be deregulated in a synchronised manner.

Established data has shown that following SV40 transformation, the TFIIIC220 and TFIIIC110 subunits of TFIIIC2 are overexpressed at the protein level and reflect, at least partially, the elevation of the transcripts encoding these components (Larminie et al., 1999). Western analysis of protein expression levels of the TFIIIC220 and TFIIIC110 subunits ensuing transformation by Polyomavirus demonstrated a congruous effect. Despite a lack of antibodies recognising the remaining TFIIIC2 subunits, it would appear likely that there is a correlation between transcript and protein expression that would be reflected in elevated protein levels of the TFIIIC63, TFIIIC90 and TFIIIC102 subunits in both SV40- and Polyomavirus-transformed cell lines.

Taken collectively, these results demonstrate deregulation of the pol III transcription factor TFIIIC2. This is seen in regard to both its DNA-binding activity, as established through gel retardation assays and, subsequently, in the levels of the five components comprising TFIIIC2, which was demonstrated by an increase in expression of transcripts through RT-PCR analysis, and at the level of protein expression, revealed by western blotting. Clearly these results establish TFIIIC as a target for activation by Polyomavirus and strongly implicate it in the general up-regulation of pol III transcription levels observed in Py3T3 cells, and moreover, in the deregulation that persists in the absence of the large T antigen.

Chapter 6

Signalling-mediated stimulation of pol III transcription

6.1 INTRODUCTION

In SV40, the principal oncoprotein, the large T antigen, is necessary and sufficient for cellular transformation (Marshall, 1991). The small t antigen aids the efficient transformation of several cell types (Watanabe et al., 1996), but essentially complements large T-mediated transformation in a supporting manner (Bikel et al., 1987). The small t antigen binds protein phosphatase 2A (PP2A) and is able to activate members of the mitogen-activated protein (MAP) kinase family (Sontag et al., 1993) and induce the cyclin D1 promoter (Watanabe et al., 1996).

In contrast, Polyomavirus produces an additional middle T antigen, which appears to take on characteristics of both the large and small T antigens of SV40. While, like SV40, the large T antigen of Polyomavirus binds and inactivates RB (Dyson et al., 1990) and the small t antigen associates with PP2A (Pallas et al., 1990), the middle T antigen replaces large T as the principal transforming oncoprotein and resembles the SV40 small t function through its ability to also target the PP2A cellular protein (Pallas et al., 1990; Treisman et al., 1981).

However, the middle T antigen exhibits a significantly more diverse capacity to interact with host cell proteins. Interactions have been documented with the Src tyrosine kinase family (Cheng et al., 1988; Courtneidge and Smith, 1983; Horak et al.,

1989; Kornbluth et al., 1987), potentiating subsequent interactions with the 85kD subunit of phosphatidylinositol-3 kinase (PI-3 kinase) (Courtneidge and Heber, 1987), Shc (Blaikie et al., 1997) and phospholipase C-γ1 (PLCγ1) (Su et al., 1995). Furthermore, phosphorylation of the middle T antigen on serine residues confers the ability to bind 14-3-3 proteins. 14-3-3 is an abundant, ubiquitously expressed and evolutionarily highly conserved protein family that regulates cell cycle checkpoints, proliferation, differentiation and apoptosis (Brunet et al., 1999; Piwnica-Worms, 1999). However, despite these functions, interactions between middle T and 14-3-3 proteins have little effect on transformation, but instead influence the type of tumour induced by transformation (Glover et al., 1999).

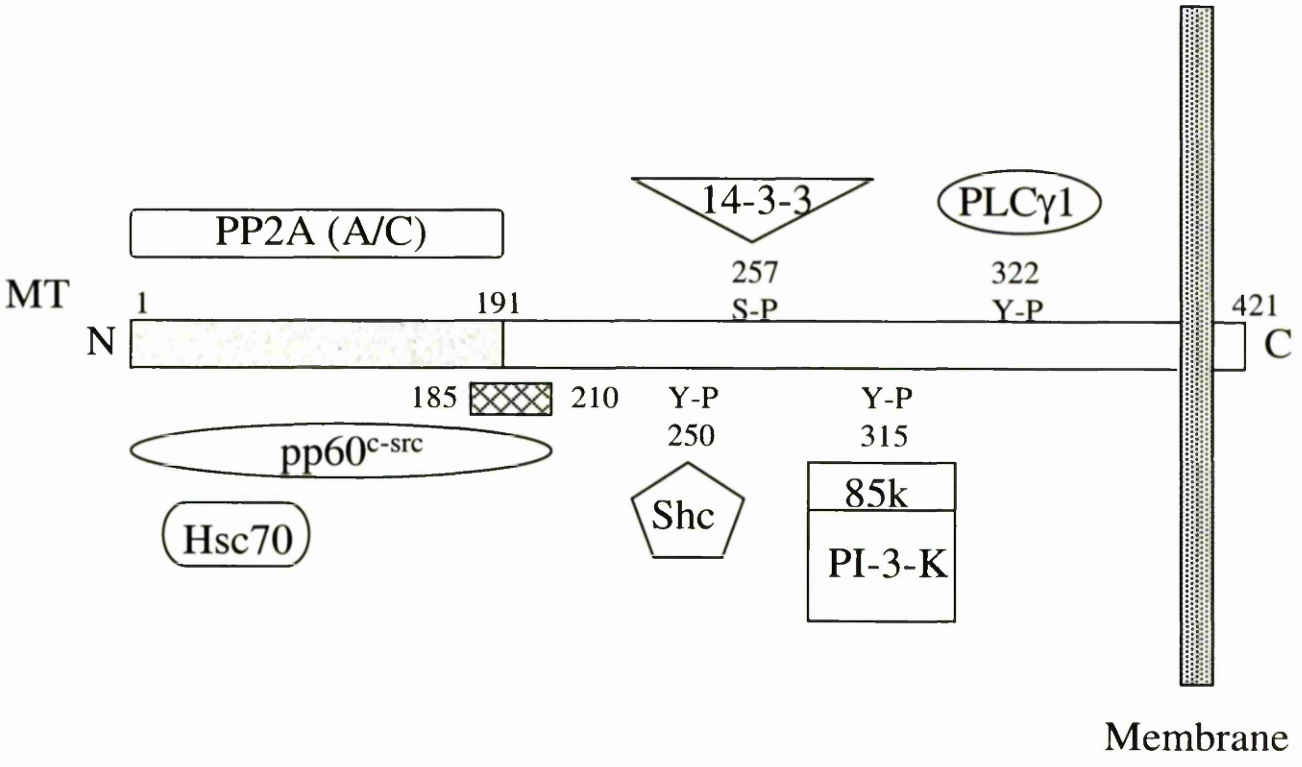
Mapping of interaction sites on the middle T antigen is summarised schematically in figure 6.1. These interactions are inextricably connected to the transforming ability of the middle T antigen (Armelin and Oliveira, 1996). Moreover, mutations blocking the PI-3 kinase or Shc interactions abrogate middle T antigen transformation ability (Urich et al., 1995).

Association of the middle T antigen with pp60c-src leads to its activation through interference with phosphorylation at Tyr 527, a site negatively regulating src kinase. The middle T antigen is, thus, able to abrogate mitosis-specific activation of pp60src, resulting in constitutive high kinase activity of the enzyme throughout all phases of the cell cycle (Kaech et al., 1991). Subsequent studies have suggested that complex interplay between Polyomavirus middle T antigen and the cellular regulatory network is cell cycle regulated. Indeed, interphase and mitotic cells express forms of the middle T antigen that vary in their degree of phosphorylation and are distinguishable through their differential migration on SDS/acrylamide gels (Perez et al., 1993). The

Figure 6.1

Schematic diagram of middle T antigen illustrating the regions involved in binding known cellular proteins

A linear representation of the Polyomavirus middle T antigen. The shaded area at the N terminus represents the 191 amino acids shared with the 195-amino acid small t antigen. Above and below, the regions required for binding the respective cellular proteins are indicated, together with the type of phosphorylated amino acid involved. The hatched box (residues 185-210) indicates the middle T sequences necessary to bind src-family kinases.


14-3-3
PLCγ1
PP2A (A/C)
257
S-P
322
Y-P
MT
1
191
421
N
C
185
210
Y-P
250
Y-P
315
pp60c-src
Shc
85k
PI-3-K
Hsc70
Membrane

middle T antigen is transiently phosphorylated during mitosis and mutation of one of two putative cdc2 phosphorylation sites (Thr 160) abolishes middle T antigen-mediated oncogenic transformation of cells in culture (Perez et al., 1993). It is suggested that phosphorylation of the middle T antigen by $p34^{cdc2}$, which is well established for transient phosphorylation of the c-src kinase during mitosis (Morgan et al., 1989; Shenoy et al., 1989), regulates the interaction of Polyomavirus with cellular targets implicated in growth regulation of normal cells (Perez et al., 1993).

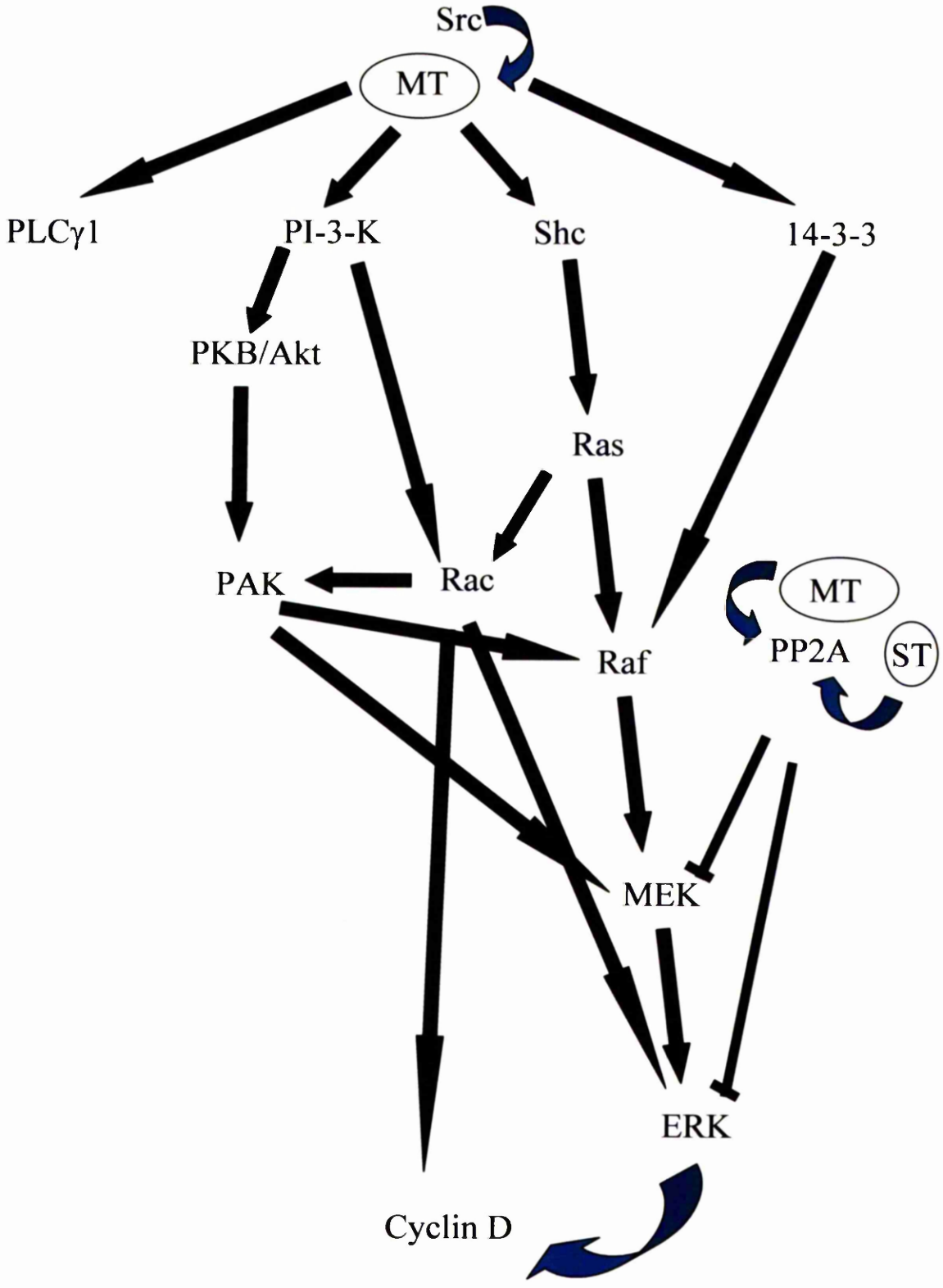
The activation of signalling pathways is complex and it appears that the requirement of individual components of the pathways is cell type-dependent. For example, while activation of PAK in COS7 cells is dependent upon PI-3 kinase-dependent activation of Rac (King et al., 1998), in Rat-1 cells, PAK activation occurs through a PI-3 kinase-mediated activation of another serine/threonine kinase, Akt, and independently of Rac (Tang et al., 2000). Consequently, the potential for varying requirements of other pathway components exists subject to cell type, or indeed, in response to different stimuli.

It has been established that the interactions of the middle T antigen with its target host proteins can result in activation of these signalling pathway components and the subsequent up-regulation of pathways leading to activation of transcription factors involved in cell cycle progression. Figure 6.2 illustrates some of the relationships that exist downstream of proteins that are known to interact with the middle and/or small T antigens. That all these components are involved in controlling pol III is doubtful and the investigation of some of the essential proteins is discussed within this chapter; however, the potential routes leading to transcriptional activation are clearly complex and numerous. Although middle and/or small T-mediated targeting of transcription

Figure 6.2

Illustration of middle T and small t antigen interactions with cellular signalling proteins and potential pathways activated

A representation of established signal transduction pathways and the interactions of middle and/or small T antigens with cellular proteins that could potentially activate them. Middle and/or small T antigen associations do not necessarily lead to activation of all the cellular proteins presented. Arrows between individual proteins indicate known signal activations but do not necessarily collectively identify full signal cascades.


Src
MT
PLCγ1
PI-3-K
Shc
14-3-3
PKB/Akt
Ras
PAK
Rac
MT
PP2A
ST
Raf
MEK
ERK
Cyclin D

factors of the pol III transcriptional machinery have not been documented, this capacity for transcriptional stimulation via signalling cascades presented a potential mechanism of pol III transcriptional deregulation.

This chapter presents evidence supporting previous studies documenting middle and/or small T antigen-mediated activation of proteins involved in signalling pathways and establishes an involvement for signal activation in the deregulation of pol III transcription. Specifically, the extracellular signal-regulated protein kinase (ERK) MAP kinase pathway has been strongly implicated as the target of converging signals mediated through the middle and/or small T antigens of Polyomavirus. Furthermore, the BRF component of TFIIIB, which possesses consensus sequences for potential ERK docking domains, has been shown to interact with ERK endogenously, providing a target of ERK activation that could contribute to the elevation of pol III transcription.

6.2 RESULTS

6.2.1 Middle T antigen stimulates pol III transcription *in vivo*

Unlike SV40, Polyomavirus encodes a middle T antigen in addition to the large and small T antigens. In SV40, the large T antigen is necessary and sufficient for full transformation (Marshall, 1991). In contrast, while the large T antigen of Polyomavirus is necessary for immortalisation of primary cells (Larose et al., 1991), expression of the middle T oncogene is sufficient to transform established fibroblast cell lines (Treisman et al., 1981). Effects of the large T antigen on pol III transcription have already been presented, but given that middle T is the principal transforming oncoprotein of Polyomavirus, it was consequently of interest to investigate whether the middle T antigen played an active role specifically with regard to the stimulation of pol III transcription.

To pursue this, untransformed parental 3T3 cells were transiently transfected with DNA encoding the middle T antigen. Through primer extension analysis of the levels of co-transfected VA_1, a clear and significant stimulation of pol III transcription was observed over levels produced by transfection of the empty vector (Figure 6.3A, compare lane 2 with lane 1). A comparable difference was also displayed relative to the functionally dead control NG59-transfected cells (Figure 6.3A, compare lanes 2 and 3). In addition to VA_1, CAT was similarly co-transfected and levels assessed by primer extension to control for transfection efficiency. The stimulation of VA_1 expression apparent in panel A is clearly not reflected in the expression levels of CAT (Figure 6.3B, compare lane 2 with lane 1). After quantification of VA_1 and CAT levels through phosphoimaging, VA_1 expression was normalised against CAT and

final values displayed graphically (Figure 6.3C), making apparent a pol III transcriptional stimulation in the region of 50-fold.

6.2.2 Specific inhibitors of proteins involved in cell signalling pathways do not compromise TFIIIC2 DNA-binding activity

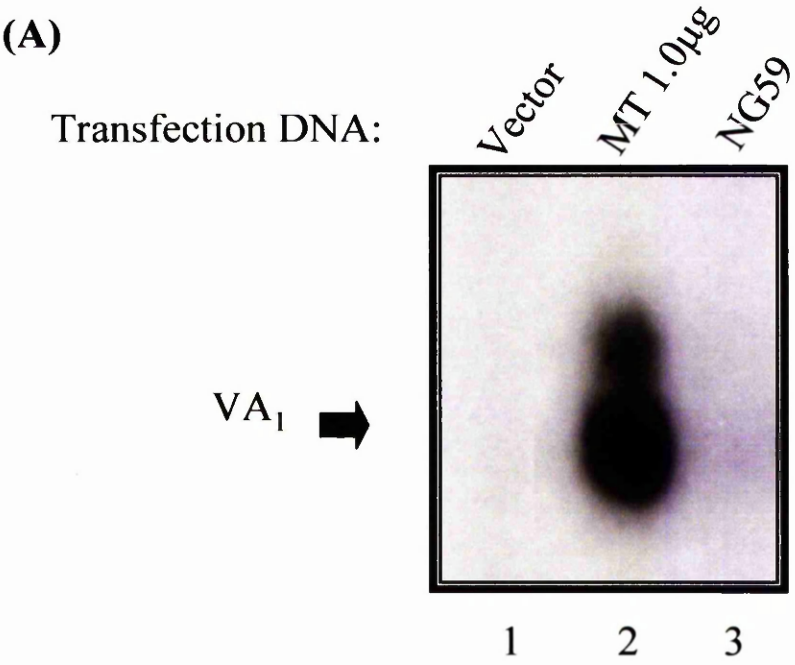
Middle T is able to induce phenotypic changes associated with malignant cell growth (Treisman et al., 1981) despite possessing no intrinsic enzymatic activity (Campbell et al., 1994). The middle T antigen binds exclusively to intracellular cytoplasmic membranes in a perinuclear location and to a lesser extent, at the plasma membrane (Dilworth et al., 1986) via a hydrophobic membrane anchor at the C-terminus of the protein (Dahl et al., 1992). These associations with cellular membranes are essential for oncogenic activity and interactions with target host proteins (Elliott et al., 1998). It is able to activate a number of mitogenic signal transduction pathways by associating with and modulating the activities of cellular proteins involved in control of cell proliferation (Elliott et al., 1998). Interactions with numerous cellular proteins have been documented, including pp60c-src (Courtneidge and Smith, 1983), PP2A (Walter et al., 1989) and 14-3-3 proteins (Pallas et al., 1994). Furthermore, phosphorylation of middle T by pp60c-src generates binding sites on middle T for an additional set of signalling proteins, including, PI-3 kinase (Courtneidge and Heber, 1987), Shc (Blaikie et al., 1997) and PLC-γ1 (Su et al., 1995). In addition, although the full function of the small t antigen of Polyomavirus remains elusive, like middle T, the small t antigen has been shown to bind PP2A (Pallas et al., 1990).

Figure 6.3

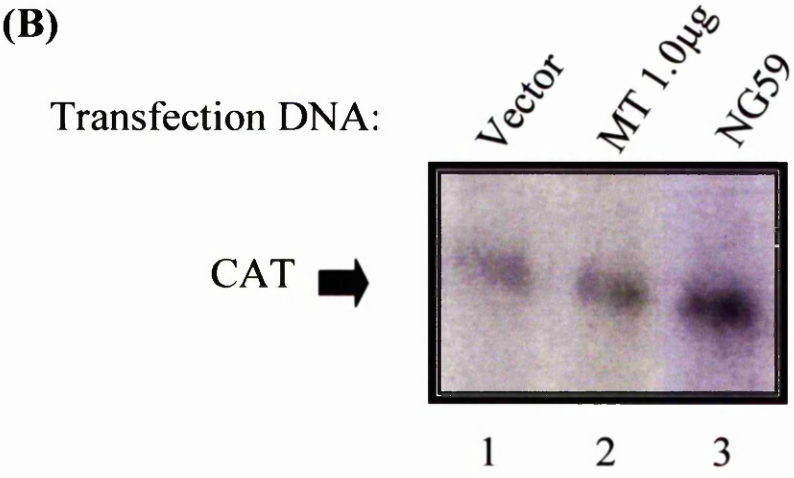
Middle T antigen stimulates pol III transcription *in vivo*

Transient transfection of the Polyomavirus middle T antigen into 3T3 cells using the Superfect method. 3T3 cells growing in 10% serum were transfected with pVA_1 (0.5μg), pCAT (0.5μg) and the relevant amount of the plasmid of interest made up to 3μg with "empty" pSV expression vector as follows: pSV alone (lane 1), pSV-MT (1μg, lane 2) or pSV-NG59 (1μg, lane 3). VA_1 (panel A) and CAT (panel B) RNA levels were assayed by primer extension and then quantified by phosphoimaging (Fujix Bas 1000). Values shown in panel C are for VA_1 expression after normalisation to the levels of CAT RNA to correct for transfection efficiency; they are given relative to the value obtained with pSV vector alone (designated 1) and represent the mean of two experiments ± standard deviation.

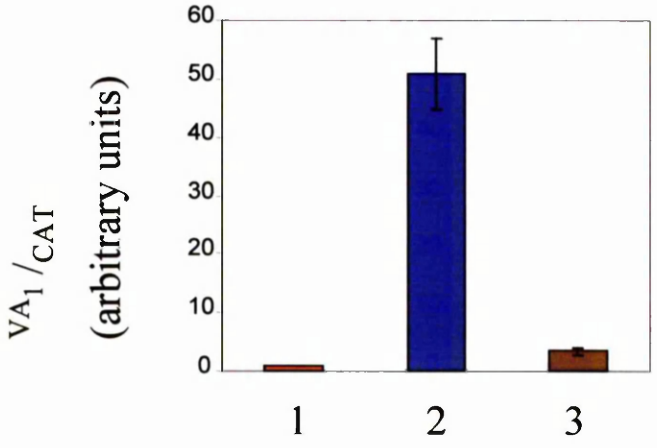
(A)


Transfection DNA:
Vector
MT 1.0μg
NG59
VA1
1
2
3

(B)


Transfection DNA:
Vector
MT 1.0μg
NG59
CAT
1
2
3

(C)


VA1 /CAT
(arbitrary units)
60
50
40
30
20
10
0
1
2
3

Since the use of Pytsa3T3 cells has shown that Polyomavirus activates TFIIIC2 in a large T antigen-independent manner, initial studies to identify a target of middle T or potentially, small t, were performed with regard to TFIIIC. TFIIIC activity is modulated by phosphorylation and was a potential target for activation by phosphorylation via one of these pathways. Extracts prepared from Py3T3 cells were pre-incubated with a range of specific inhibitors of cellular proteins involved in signalling pathways: Okadaic acid, which inhibits PP2A, Olomoucine, which inhibits cyclin-dependent kinases, U0126, which inhibits MEK, and Wortmannin, which is a potent inhibitor of PI-3 kinase. These extracts were subsequently analysed for TFIIIC2 DNA-binding activity by electrophoretic mobility shift assay, along with an untreated extract and extracts with the addition of an equivalent amount of DMSO as present in the inhibitors, to provide a control for a possible DMSO effect. None of these inhibitors, however, demonstrated any ability to influence the TFIIIC2 DNA-binding capacity in these cell extracts (Figure 6.4, compare lanes 3, 4, 6, 7, 9, 10, 11 and 12 with control lanes 2, 5 and 8). Lane 1 in figure 6.4 shows a binding reaction in the absence of cell extract and, consequently, presents no band for TFIIIC2.

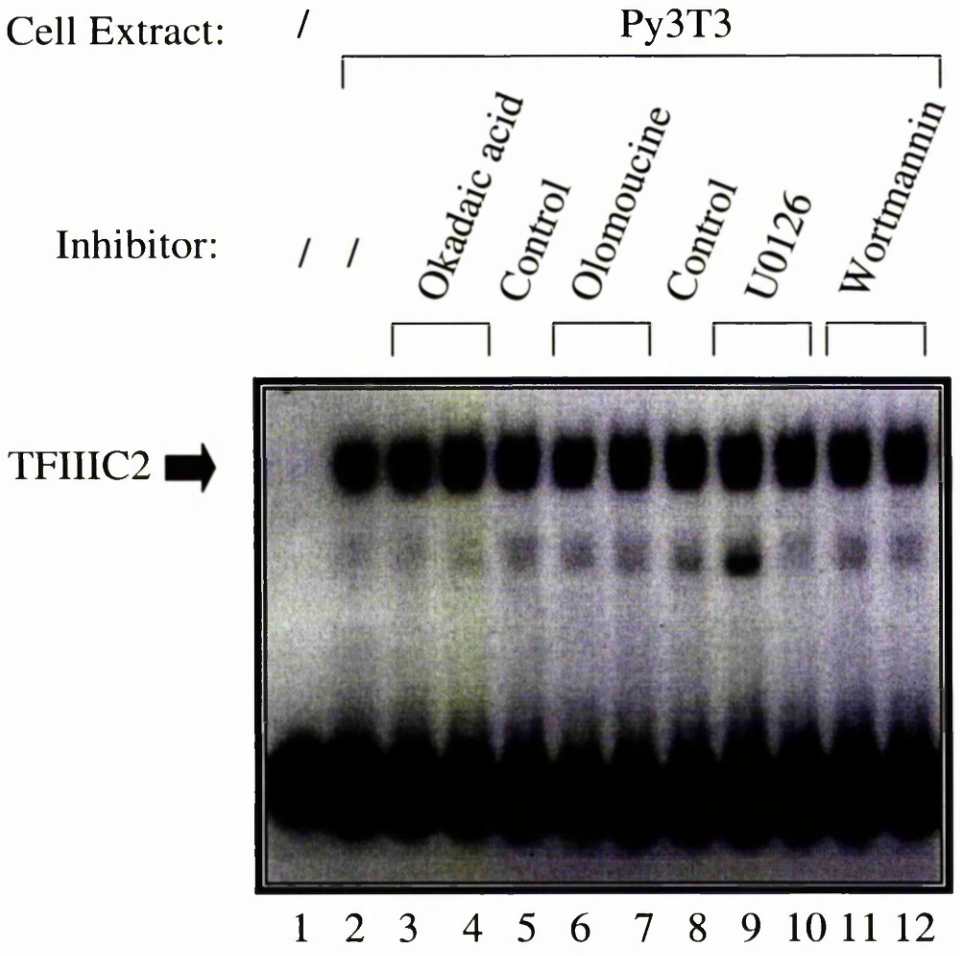
6.2.3 Cell extracts are uncompromised for TFIIIC2 DNA-binding activity in the presence of the general kinase inhibitor DMAP

Although the electrophoretic mobility shift assay presented in figure 6.4 demonstrated that TFIIIC2 binding ability was uncompromised by the presence of the specific inhibitors, the possibility remained that a signalling pathway was still involved. To confirm that TFIIIC2 DNA-binding activity was not being stimulated via a signalling

Figure 6.4

TFIIIC2 DNA-binding activity is not compromised by specific inhibitors of cell signalling pathways

TFIIIC2 DNA-binding assay using the B-block oligonucleotide as probe. Reactions contained 0.5ng of labelled B-block oligonucleotide, 1μg of poly(dI.dC), no extract (lane 1), 23μg of Py3T3 whole cell extract (lanes 2-12), a final concentration of 12% DMSO (lanes 3-12) and 10nM Okadaic acid (lanes 3 and 4), 200μM Olomoucine (lanes 6 and 7), 250μM U0126 (lanes 9 and 10) or 10μM Wortmannin (lanes 11 and 12). Extracts were pre-incubated with the respective inhibitors for 15 minutes at 30°C, prior to addition of the radiolabelled probe.


Cell Extract:
/
Py3T3
Inhibitor:
/
/
Okadaic acid
Control
Olomoucine
Control
U0126
Wortmannin
TFIIIC2
1 2 3 4 5 6 7 8 9 10 11 12

pathway undetected with the previous inhibitors, cell extracts were similarly analysed after pre-incubation with the general kinase inhibitor 6-Dimethylaminopurine (DMAP). As before, Py3T3 cell extracts displayed no effect upon treatment (Figure 6.5, compare lanes 4 and 5). Furthermore, TFIIIC2 DNA-binding activity in 3T3 and Pytsa3T3 cells equally revealed no response to the DMAP inhibitor (Figure 6.5, compare lanes 2 and 3, and lanes 6 and 7, respectively). Again, cell extracts for control binding reactions were in the presence of DMSO comparable to the amount present in the inhibitor stock.

6.2.4 Influence of Okadaic acid on pol III transcriptional activity

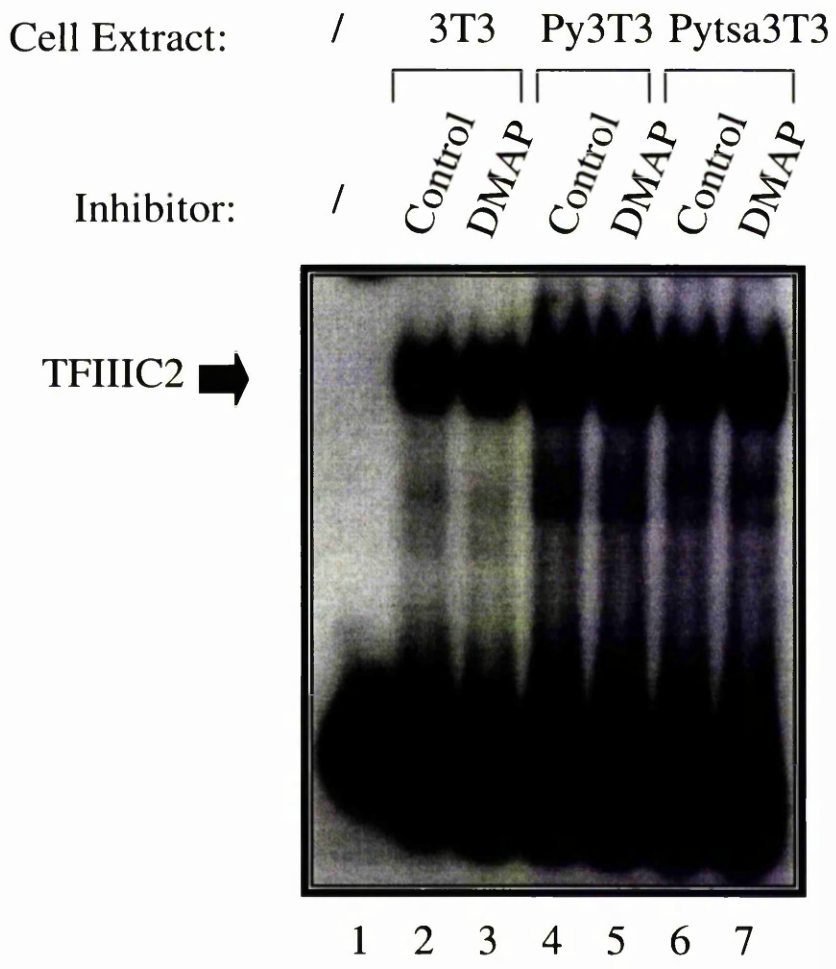
Despite TFIIIC2 DNA-binding activity remaining unaffected in the presence of the PP2A inhibitor Okadaic acid (Figure 6.4), it was of interest to determine whether Okadaic acid could exert an effect on overall pol III transcriptional activity.

PP2A is a serine-threonine phosphatase present in most cell types (Cohen, 1989) that has been implicated in the regulation of cell cycle progression, transcription, and DNA replication and translation (Mumby, 1995; Mumby and Walter, 1993; Shenolikar, 1994). It exists in multiple heterotrimeric forms comprising a common core structure of the 36kD catalytic C subunit and the 63kD A subunit, which associate with a mixture of regulatory proteins termed B subunits (Kamibayashi et al., 1991; Ruediger et al., 1992). The B subunit, which is replaced by middle or small T antigens, confers substrate specificity (Shenolikar, 1994) and localisation (Strack et al., 1998). Consequently, interactions between PP2A and the middle or small T

Figure 6.5

The general kinase inhibitor DMAP does not affect TFIIIC2 DNA-binding in 3T3, Py3T3 or Pytsa3T3 cell extracts

TFIIIC2 DNA-binding assay using the B-block oligonucleotide as probe. Reactions contained 0.5ng of labelled B-block oligonucleotide, 1μg of poly(dI.dC), no extract (lane 1), 23μg of 3T3 (lanes 2 and 3), Py3T3 (lanes 4 and 5) or Pytsa3T3 (lanes 6 and 7) whole cell extract, a final concentration of 12% DMSO (lanes 2-7) and 2.4mM DMAP (lanes 3, 5 and 7). Extracts were pre-incubated with the respective inhibitors for 15 minutes at 30°C prior to addition of the radiolabelled probe.


Cell Extract:
/
3T3
Py3T3
Pytsa3T3
Inhibitor:
/
Control
DMAP
Control
DMAP
Control
DMAP
TFIIIC2
1 2 3 4 5 6 7

antigens may lead to activation of signalling pathways, which in turn could potentially stimulate a pol III transcription factor(s).

Okadaic acid was titrated into 3T3 and Py3T3 cell extracts. These extracts were pre-incubated in the presence of the Okadaic acid before being analysed by *in vitro* transcription assay to determine their abilities to transcribe the pol III template VA_1. Relative to levels of VA_1 observed for the untreated cell extracts, a final concentration of 1μM, 100nM or 10nM Okadaic acid conferred no effect on transcriptional activity in either the 3T3 or Py3T3 cells (Figure 6.6, compare lanes 1 and 2 with lanes 3 and 4, 5 and 6 or 7 and 8, respectively). However, PP2A activity has been shown to activate, inactivate or redirect to new targets in a manner dependent on the regulatory subunit present (Pallas et al., 1990). Thus, replacement of this subunit by the middle and/or small T antigens, may simultaneously lead to both activation and inactivation of specific targets. Consequently, effects of inhibiting PP2A could potentially cancel out or, alternatively, may be masked by other deregulatory mechanisms. Hence, it is not possible to conclude, on the basis of this observation, which concerns only the situation *in vitro*, that PP2A does not influence pol III transcription in Polyomavirus-transformed cells.

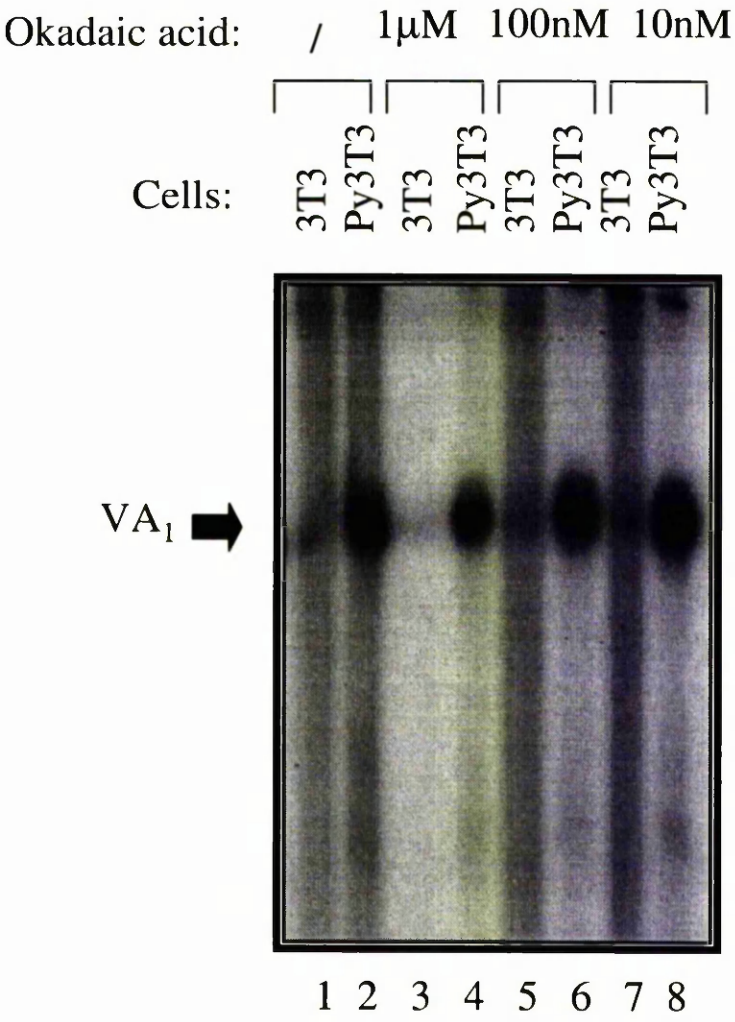
6.2.5 Pol III transcription in Py3T3 cells is compromised by the general kinase inhibitor DMAP

Although specific inhibition of PP2A produced no effect on transcription levels *in vitro*, the general kinase inhibitor DMAP was again used to broaden the range of

Figure 6.6

Okadaic acid does not reduce pol III transcription

In vitro transcription assay illustrating relative levels of pol III transcription for 3T3 (lanes 1, 3, 5 and 7) and Py3T3 (lanes 2, 4, 6 and 8) cell extracts in the absence (lanes 1 and 2) or presence of 1μM (lanes 3 and 4), 100nM (lanes 5 and 6) or 10nM (lanes 7 and 8) of Okadaic acid. Transcription reactions contained 250ng of pVA_1 template and 20μg of cell extract. Extracts were pre-incubated with the Okadaic acid for 10 minutes at 30°C prior to addition of the radioactive mix.


Okadaic acid:
/
1μM
100nM
10nM
Cells:
3T3
Py3T3
3T3
Py3T3
3T3
Py3T3
3T3
Py3T3
VA1
1 2 3 4 5 6 7 8

signalling pathways affected and, thus, test for their involvement. *In vitro* transcription assays were performed in duplicate using cell extracts prepared from 3T3 and Py3T3 cells pre-incubated in the presence of DMAP with a final concentration of 2.4mM. Transcriptional activity of Py3T3 cell extracts showed a marked reduction of 50%, relative to the untreated Py3T3 cells, when pre-treated with the DMAP inhibitor (Figure 6.7, compare lanes 4 and 6 with lane 2). In contrast, the 3T3 cell extracts failed to exhibit any effect of DMAP on transcriptional activity (Figure 6.7, compare lanes 3 and 5 with lane 1). Consequently, these data provide evidence for activation of pol III transcription in Py3T3 cells being mediated, in part, through phosphorylation.

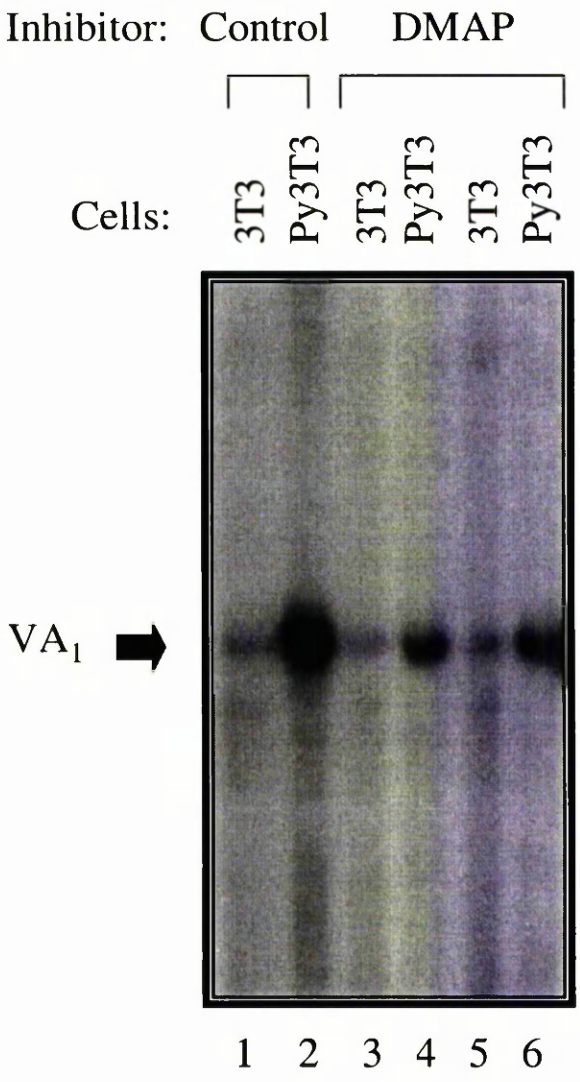
6.2.6 DMAP compromises transcriptional activity in Pytsa3T3 cells

As DMAP implicated a signalling pathway in activation of transcription by Polyomavirus, *in vitro* transcription analysis was carried out using the large T antigen-defective cell line Pytsa3T3. The large T antigen is localised to the nucleus by two nuclear localisation signals (NLSs) (Richardson et al., 1986) and is not associated with activation of signalling cascades. Consequently, as middle and small T antigens remain functional in these cells, loss of the large T antigen would not be expected to abrogate the effect conferred by the DMAP inhibitor. Indeed, Pytsa3T3 cell extracts similarly treated with a final concentration of 2.4mM DMAP demonstrated an equivalent reduction in transcriptional activity relative to the untreated cell extracts (Figure 6.8, compare lanes 2 and 3 with lane 1). This result

Figure 6.7

Pol III transcription in Py3T3 cells is compromised by the general kinase inhibitor DMAP

In vitro transcription assay illustrating relative levels of pol III transcription for extracts prepared from 3T3 (lanes 1, 3 and 5) and Py3T3 (lanes 2, 4 and 6) cells in the absence (lanes 1 and 2) or presence of 2.4mM DMAP (lanes 3, 4, 5 and 6). Transcription reactions contained 250ng of pVA_1 template and 20μg of cell extract. Extracts were pre-incubated with the inhibitor for 10 minutes at 30°C prior to addition of the radioactive mix.


Inhibitor: Control DMAP
Cells: 3T3 Py3T3 3T3 Py3T3 3T3 Py3T3
VA1
1 2 3 4 5 6

confirmed that stimulation of a signalling pathway involved in pol III transcriptional activation was not mediated through the action of the large T antigen.

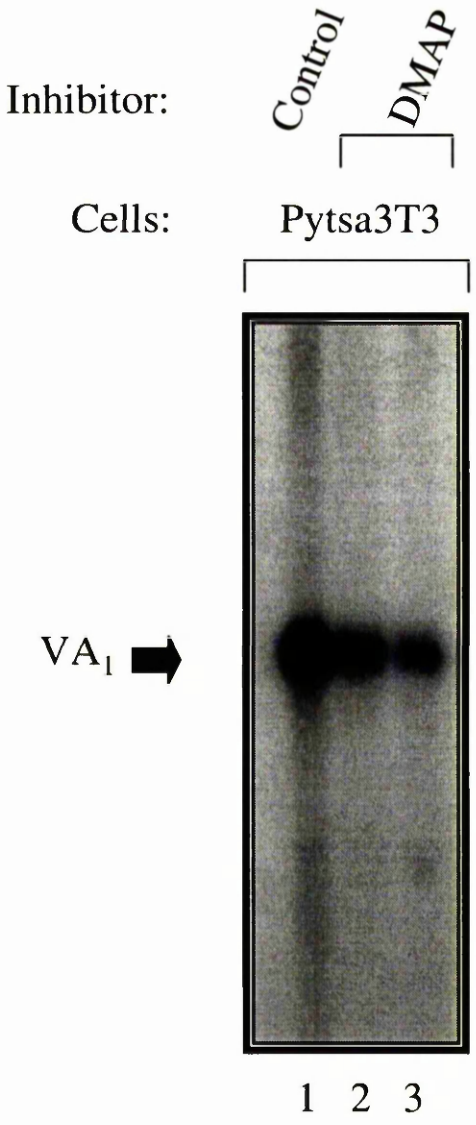
6.2.7 Pol III transcription is compromised by *in vivo* treatment with the PI-3 kinase inhibitor LY294002

In vitro transcription assays demonstrated the ability of the inhibitor DMAP to reduce pol III transcription activity in whole cell extracts of Py3T3 cells. Further studies to identify the involvement of specific pathways were performed *in vivo* to ensure that complete signalling pathways were active. Given that another of the cellular proteins known to interact with the middle T antigen is PI-3 kinase, a specific inhibitor of this protein, LY294002, was employed to assess the effect of its inactivation on possible downstream regulation of pol III transcription. 3T3 and Py3T3 cells were subjected to treatment with the PI-3 kinase inhibitor LY294002 and harvested at 1, 4 and 8 hour time points. Extracts prepared from these cells were subsequently analysed by *in vitro* transcription assay and in both 3T3 and Py3T3 cells a reduction in pol III transcription was noted after 4 hours of treatment, relative to the untreated cells (Figure 6.9, panels A and B, respectively, compare lane 3 with lanes 1 and 5). Despite transcription activity being partially restored by the 8 hour time point in both cell lines (Figure 6.9, panels A and B, respectively, compare lane 4 with lane 3), possibly through degradation of the LY294002 compound, this demonstrated that signals downstream of PI-3 kinase are involved in regulation of pol III transcription. However, notably, the relative effect conferred upon transcription observed in the 3T3 cells is equal, if not greater, than that displayed in the transformed Py3T3 cells,

Figure 6.8

Pytsa3T3 cell extracts display compromised pol III transcription in the presence of DMAP

In vitro transcription assay displaying levels of pol III transcription for Pytsa3T3 cell extracts (all lanes) in the absence (lane 1) or presence of 2.4mM DMAP (lanes 2 and 3). Transcription reactions contained 250ng of pVA_1 template and 20μg of cell extract. Extracts were pre-incubated with the inhibitor for 10 minutes at 30°C prior to addition of the radioactive mix.


Inhibitor:
Control
DMAP
Cells:
Pytsa3T3
VA1
1 2 3

suggesting that the middle and/or small T antigens of Polyomavirus do not principally exert their effects through activation of signals downstream of PI-3 kinase.

It should be noted that panel A displays a longer exposure than panel B, and 3T3 transcription levels should not be compared relative to the Py3T3 experiment shown in panel B.

6.2.8 Cells treated *in vivo* with LY294002 display inhibition of the PI-3 kinase signalling pathway

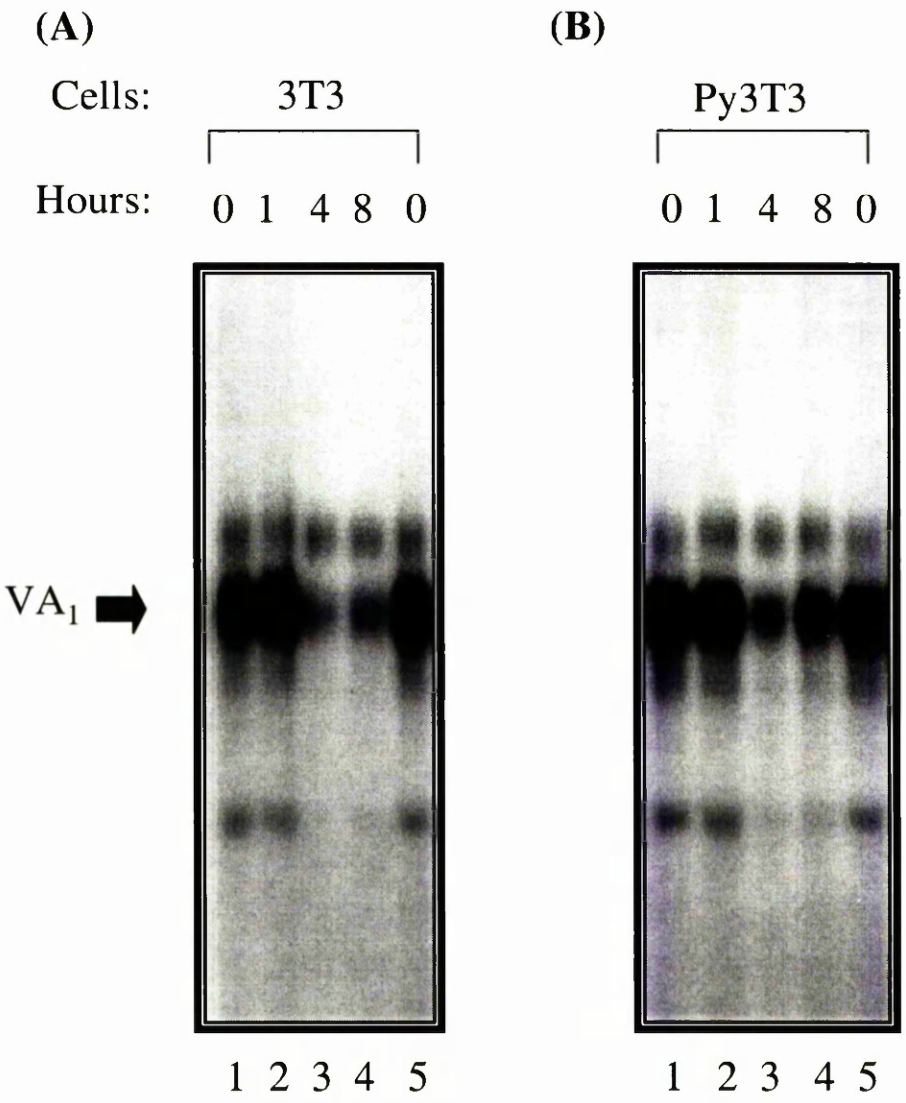
In support of observations noted in figure 6.9, the cell extracts harvested over the three time points, following *in vivo* treatment with LY294002 and used for *in vitro* transcription assays, were subsequently analysed by western blotting. This served to confirm that both cell lines were responding to the inhibitor and thus establish that the effects observed previously were consistent with an inhibition of signals downstream of PI-3 kinase.

p70S6 kinase, which acts downstream of PI-3 kinase, is responsible for the phosphorylation and activation of the p70S6 ribosomal protein. Western analysis revealed a shift in mobility of p70S6 kinase in cell extracts prepared from both the LY294002-treated 3T3 and Py3T3 cell lines, demonstrating a change in phosphorylation status (Figure 6.10A, compare lanes 2, 3 and 4 with lane 1 and lanes 6, 7 and 8 with lane 5, respectively). Similarly, Phas1, another protein modulated through signals downstream of PI-3 kinase, also displayed dephosphorylation in the extracts from 3T3 and Py3T3 cells treated with LY294002, as the lower mobility band

Figure 6.9

In vivo treatment with the PI-3-kinase inhibitor LY294002 compromises pol III transcription in 3T3 and Py3T3 cells

In vitro transcription assay demonstrating effect of *in vivo* treatment with LY294002 on pol III transcription. 3T3 (panel A) and Py3T3 (panel B) cells cultured in 10% serum were exposed to 0.1% DMSO alone for 1 hour (lanes 1 and 5, in each panel) or 0.1% DMSO and a final concentration of 10μM LY294002 for 1, 4 or 8 hours (lanes 2-4, respectively, in each panel). 20μg of cell extract prepared from each treatment was analysed for ability to transcribe 250ng of pVA_1 template.


(A)
(B)
Cells:
3T3
Py3T3
Hours:
0 1 4 8 0
0 1 4 8 0
VA1
1 2 3 4 5
1 2 3 4 5

representing the phosphorylated form was absent after the 4 hour time point (Figure 6.10B, compare lanes 3 and 4 with lanes 1 and 2, and lanes 7 and 8 relative to lanes 5 and 6, respectively). Indeed, both p70S6 kinase and Phas1 had been dephosphorylated after 4 hours, although this effect was displayed after 1 hour for p70S6 kinase, suggesting it was more sensitive to this signal inhibition. The changes in phosphorylation of these downstream proteins confirms the response of the two cell lines to inhibition by LY294002 and are consistent with the reduced transcriptional activity demonstrated in figure 6.9 being a manifestation of PI-3 kinase inhibition.

6.2.9 *In vivo* treatment with the PI-3 kinase inhibitor LY294002 compromises expression of B2 transcripts

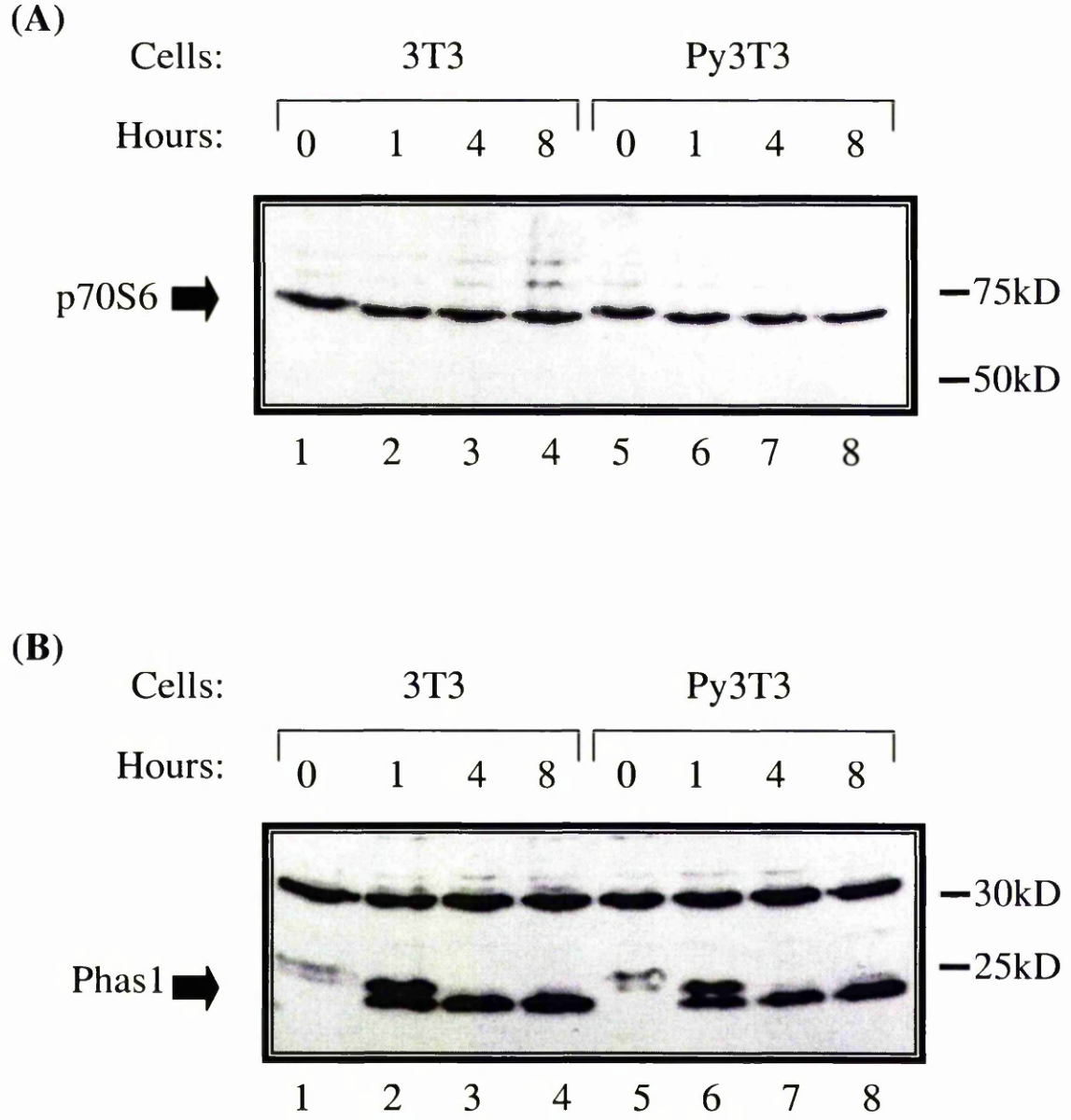
Cells treated with LY294002 in parallel with those used for preparation of whole cell extracts were harvested at the same 1, 4 and 8 hour time points and were subsequently used for preparation of total RNA. Northern analysis was then carried out to ascertain levels of B2 expression in these LY294002-treated cells relative to levels in RNA extracted from untreated cells. Effects of LY294002 on B2 levels *in vivo* appeared to be slower to manifest than the effects on *in vitro* transcription of VA_1 and the most significant decreases, in both the cell lines, were observed after 8 hours of treatment (Figure 6.11A, compare lane 4 with lanes 1, 2 and 3, and lane 8 with lanes 5, 6 and 7). Given that B2 expression analysed through northern blotting is indicative of steady state levels, the variation in time for the most marked effect to LY294002 treatment could be attributed to the time required for RNA turnover after transcription has been inhibited.

Figure 6.10

Cells treated *in vivo* with LY294002 display inhibition of the PI-3 kinase signalling pathway

Whole cell extracts prepared from 3T3 (lanes 1-4 in each panel) and Py3T3 (lanes 5-8 in each panel) cells cultured in 10% serum and subjected to 0.1% DMSO alone for 1 hour (lanes 1 and 5 in each panel) or 0.1% DMSO and a final LY294002 concentration of 10μM for 1, 4 or 8 hours (lanes 2, 3 and 4 or lanes 6, 7 and 8, respectively, in each panel) were resolved on a SDS-7.8% polyacrylamide gel and analysed by western immunoblotting using either an antibody against p70S6 kinase C18 (panel A) or the anit-Phas1 antibody R-113 (panel B).

Panels A and B show the same blot, cut and probed with the respective antibodies.


(A)
Cells:
3T3
Py3T3
Hours:
0 1 4 8 0 1 4 8
p70S6
75kD
50kD
1 2 3 4 5 6 7 8
(B)
Cells:
3T3
Py3T3
Hours:
0 1 4 8 0 1 4 8
Phas1
30kD
25kD
1 2 3 4 5 6 7 8

Stripping and reprobing the blot for ARPP P0 demonstrated that while levels of B2 expression decreased after LY294002 treatment, ARPP P0 was unaffected under these conditions and the effects of treatment were specific for the pol III B2 transcripts (Figure 6.11B). Values were obtained by phosphoimaging for both B2 and ARPP P0 expression levels and the graph presented in panel C shows B2 levels normalised against the ARPP P0. Again, in agreement with the observations of *in vitro* transcriptional activity (Figure 6.9), the 3T3 cells display a comparable, if not greater, response to LY294002 treatment. This suggests that while signals downstream of PI-3 kinase may be required for activation of pol III transcription, it is not necessarily a major pathway contributing to deregulation following Polyomavirus transformation.

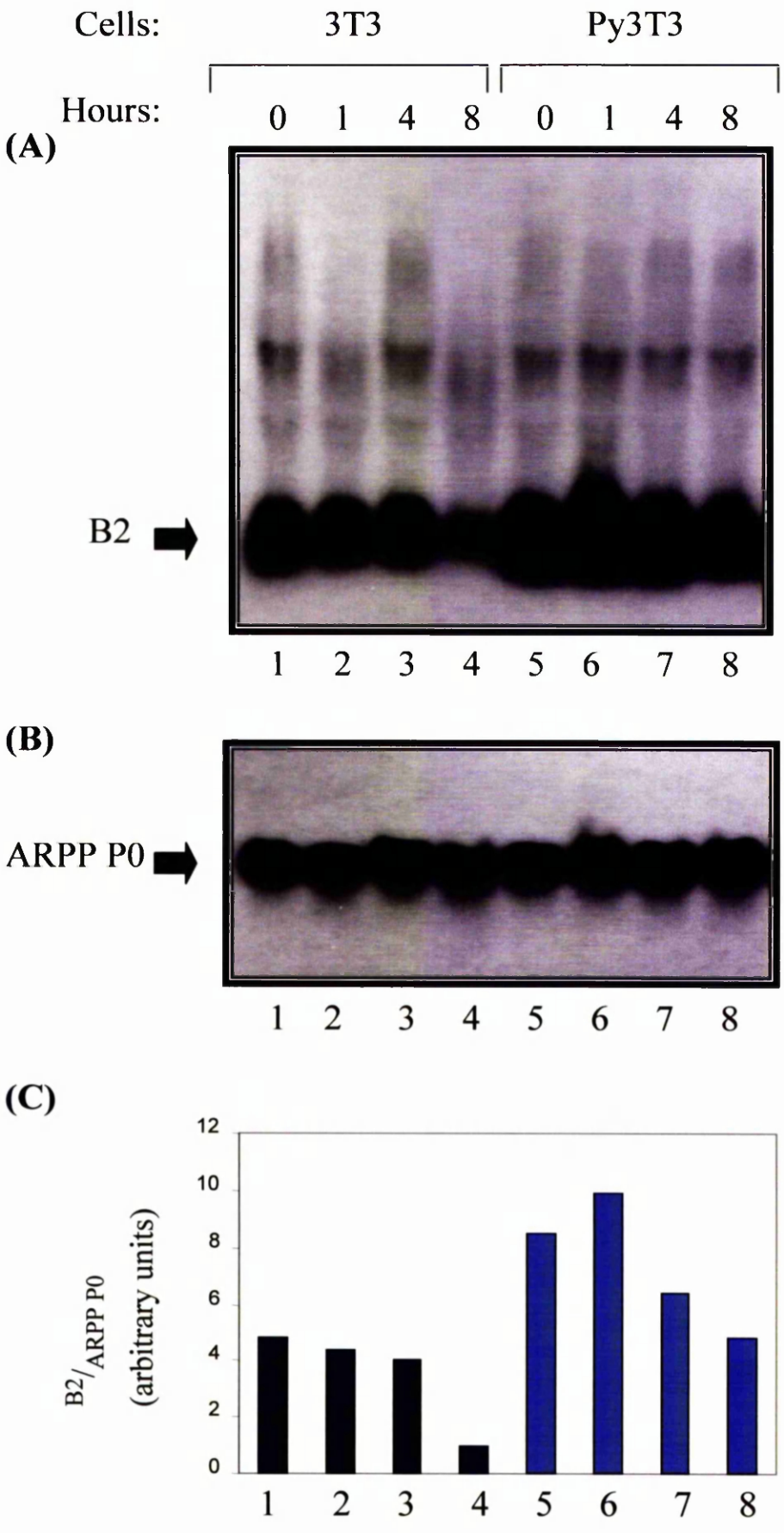
6.2.10 Py3T3 cells display a more significant reduction in pol III transcription than 3T3 cells following treatment with the MEK inhibitor U0126

The set of experiments determining the effect of *in vivo* treatment with the LY294002 compound suggested that the PI-3 kinase pathway is not contributing to the up-regulation of pol III transcription by Polyomavirus. Hence, similar *in vivo* treatment of 3T3 and Py3T3 cells was also performed with the U0126 compound, which specifically inhibits MEK1 and MEK2. In this instance, cells were incubated in the presence of U0126 for 1 hour before harvesting and whole cell extracts prepared. The treated Py3T3 cell extracts display a striking reduction relative to untreated extracts in their ability to transcribe the pol III template VA_1 after this short treatment (Figure

Figure 6.11

Expression of B2 transcripts is compromised by *in vivo* treatment with the PI-3-kinase inhibitor LY294002

Total RNA (30μg) was extracted from 3T3 (lanes 1-4 in each panel) and Py3T3 (lanes 5-8 in each panel) cells cultured in 10% serum and exposed to 0.1% DMSO alone for 1 hour (lanes 1 and 5 in each panel) or 0.1% DMSO and a final LY294002 concentration of 10μM for 1, 4 or 8 hours (lanes 2, 3 and 4 or 6, 7 and 8, respectively, in each panel) and used for northern blot analysis. Panel A shows the blot probed with a B2 gene. The blot was stripped and subsequently reprobed for the ARPP P0 gene (Panel B). The levels of B2 and ARPP P0 RNA from the northern analysis were quantitated by phosphoimaging (Fujix Bas 1000); B2 levels were normalised against levels for ARPP P0 and expressed as arbitrary units, with the lowest value obtained for the 3T3 cells being designated 1, as illustrated in panel C.


Cells:
3T3
Py3T3
Hours:
0 1 4 8 0 1 4 8
(A)
B2
1 2 3 4 5 6 7 8
(B)
ARPP P0
1 2 3 4 5 6 7 8
(C)
B2/ARPP P0 (arbitrary units)
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8

6.12, compare lanes 3 and 4). The 3T3 cells also display a response to U0126, but it is significantly less substantial than that conferred in Py3T3 cells (Figure 6.12, compare lanes 1 and 2). The observation of a response in 3T3 cells would suggest that signals downstream of MEK1 and MEK2 are implicated in pol III transcriptional regulation in untransformed cells; however, that the Py3T3 cells display such a significant reduction in transcriptional activity following treatment with U0126 strongly suggests that this pathway is a key target of Polyomavirus for deregulation in order to stimulate pol III transcription.

Although a variation in the time required for cellular responses to be manifested through treatment with the LY294002 and U0126 inhibitors might be expected, it is notable that the effects of U0126 are expressed far more rapidly.

6.2.11 The MAP kinase signalling pathway is inhibited by *in vivo* treatment of cells with U0126

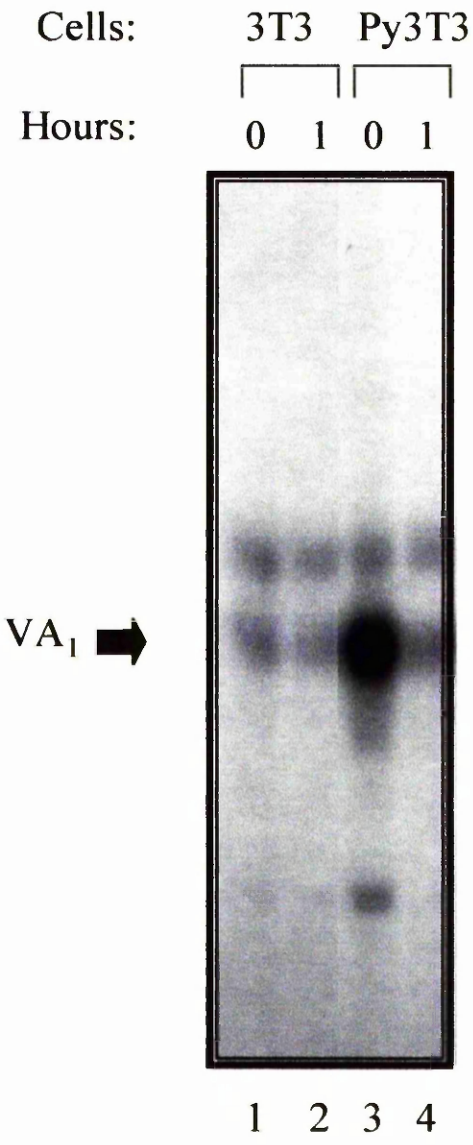
Cell extracts prepared from the U0126-treated Py3T3 cells and used in figure 6.12, were subsequently assayed for levels of total ERK and the active phosphorylated forms of ERK, in order to confirm that targets downstream of MEK1 and MEK2 were indeed responding to the U0126 inhibitor.

Western analysis of the total ERK expression levels in Py3T3 cells showed no response after 1hour treatment with U0126 (Figure 6.13A, compare lane 2 with lane 1). However, the same Py3T3 cell extract analysed for levels of the active forms of ERK clearly displayed a loss of the active phosphorylated forms following U0126

Figure 6.12

In vivo treatment with the MEK inhibitor U0126 confers a more substantial level of inhibition of pol III transcription in Py3T3 than in 3T3 cells

In vitro transcription assay demonstrating effect of *in vivo* treatment with U0126 on pol III transcription. 3T3 (lanes 1 and 2) and Py3T3 (lanes 3 and 4) cells were cultured in 10% serum and subjected to treatment for 1 hour with 0.1% DMSO alone (lanes 1 and 3) or 0.1% DMSO and U0126 inhibitor with a final concentration of 10μM (lanes 2 and 4). Transcription reactions contained 20μg of whole cell extract and 250ng of pVA_1 template.


Cells:
3T3
Py3T3
Hours:
0
1
0
1
VA1
1
2
3
4

treatment (Figure 6.13B, compare lanes 1 and 2). These results provide clear evidence that *in vivo* treatment with U0126 for 1 hour abolishes the phosphorylation of targets downstream of MEK1 and MEK2, which are responsible for ERK activation. These data support the contention that the reduction in transcriptional activity following U0126 treatment is a consequence of inhibiting signals downstream of MEK1 and MEK2.

6.2.12 Active forms of ERK, but not total ERK, are more abundant in Py3T3 cells than 3T3 cells

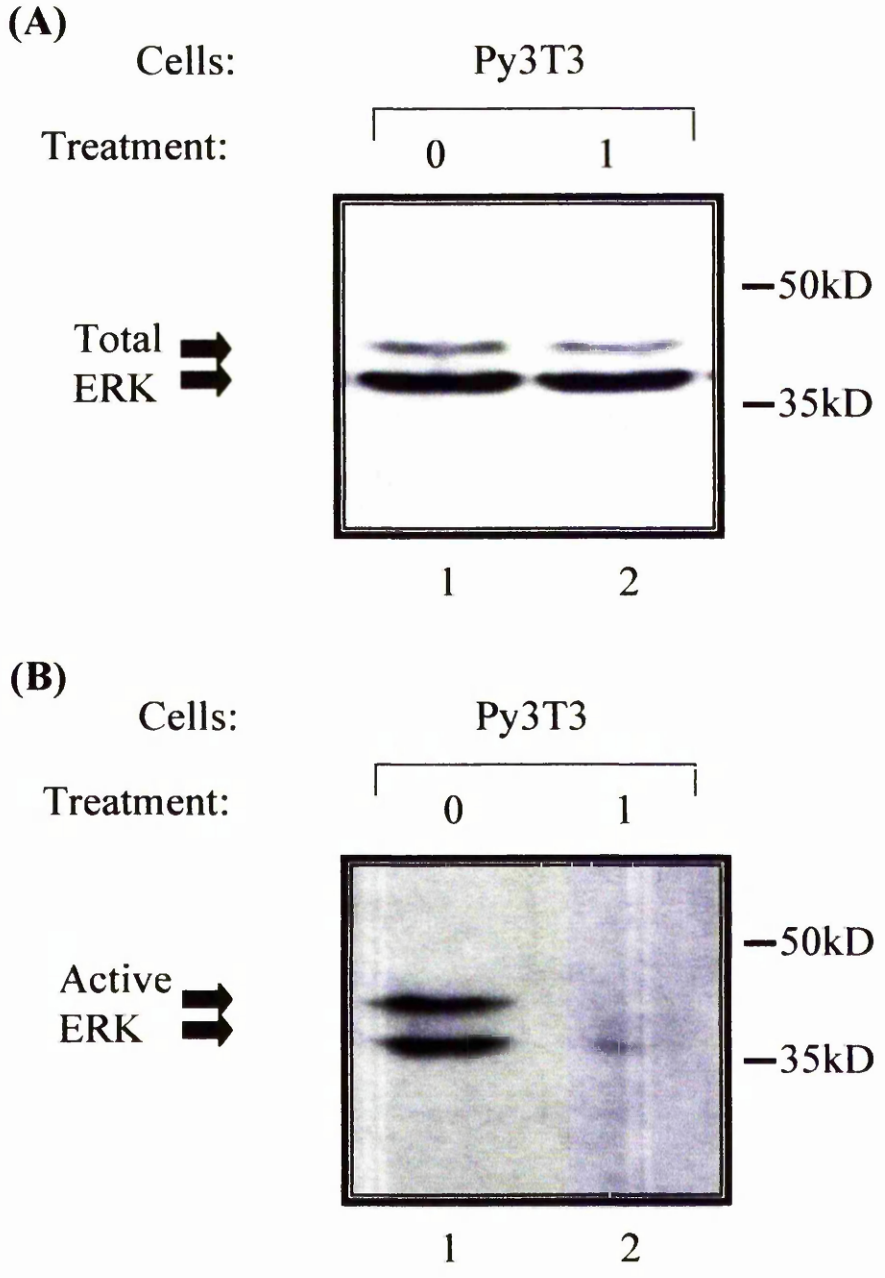
Having established an involvement of the MAP kinase signalling pathway, it was of interest to determine the levels of ERK expression in untreated extracts as used in previous assays. Western analysis of two sets of 3T3 and Py3T3 whole cell extracts demonstrated that 3T3 cells possessed higher levels of total ERK than the Polyomavirus-transformed Py3T3 cells (Figure 6.14A, compare lanes 1 and 2 and lanes 3 and 4, respectively). Furthermore, it was notable that SV40-transformed Cl49 cells expressed ERK at a level equivalent to 3T3 cell extracts (Figure 6.14A, compare lanes 6 and 7).

However, more significantly, 3T3 and Py3T3 cell extracts blotted for the active forms of ERK showed a striking reversal in expression levels, with Py3T3 cells displaying a far more substantial level of the active forms than the untransformed 3T3 cells (Figure 6.14B, compare lanes 1 and 2 and lanes 3 and 4, respectively). This striking pattern of expression suggests that despite 3T3 cells expressing a higher level of total ERK,

Figure 6.13

Cells treated *in vivo* with U0126 display inhibition of the MAPK signalling pathway

Whole cell extracts prepared from Py3T3 cells (lanes 1 and 2 in each panel) cultured in 10% serum and subjected to 0.1% DMSO alone (lane 1 in each panel) or 0.1% DMSO and a final U0126 concentration of 10μM for 1 hour (lane 2 in each panel) were resolved on a SDS-7.8% polyacrylamide gel and analysed by western immunoblotting using either an antibody against total ERK, anti-p44/42 MAPK (panel A) or a phospho-ERK antibody against active ERK (panel B).


(A)
Cells:
Py3T3
Treatment:
0
1
Total
ERK
50kD
35kD
1
2
(B)
Cells:
Py3T3
Treatment:
0
1
Active
ERK
50kD
35kD
1
2

ERK expressed in Py3T3 cells is predominantly in the active forms and that there is more active ERK in Py3T3 than in the 3T3 cells. Taken together, these results demonstrate that Polyomavirus targets ERK for activation.

6.2.13 ERK-immunodepleted Py3T3 cell extracts display reduced pol III transcription

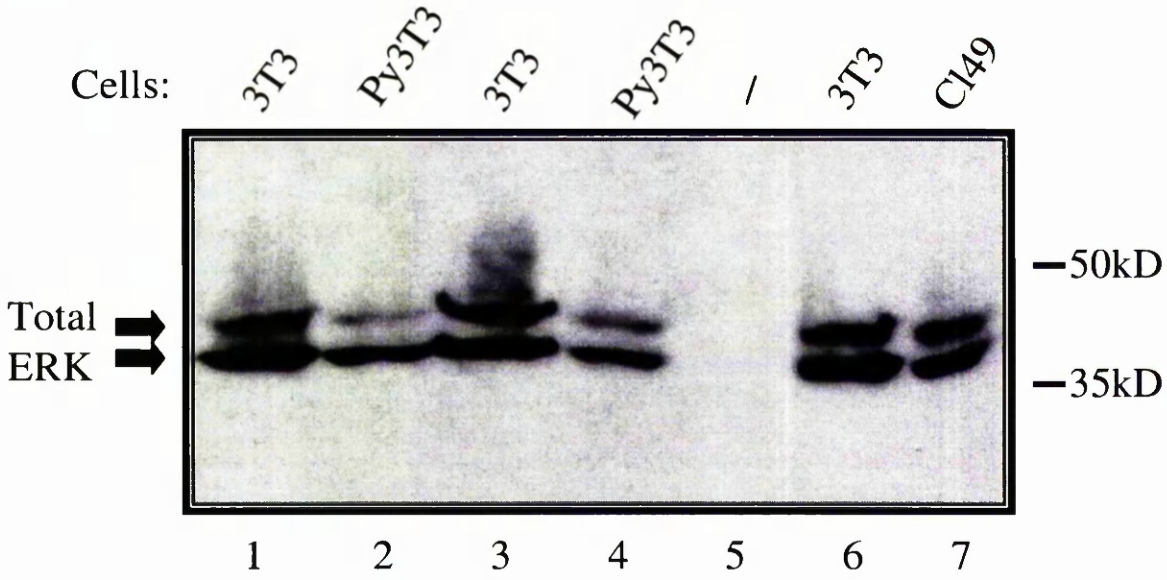
The observation that levels of phosphorylated ERK were more abundant in the Py3T3 cells (Figure 6.14) is significant and suggests the possibility that activation of ERK could be involved in deregulation of pol III transcription by Polyomavirus. To confirm an influence of ERK on pol III transcription, cell extracts prepared from 3T3 and Py3T3 cells were immunodepleted of ERK using an anti-p44/42 antibody. The transcriptional activities of these extracts were compared to extracts immunodepleted with antibodies against the E7 oncoprotein of HPV, the T antigens of Polyomavirus or the essential TFIIIB component TBP. *In vitro* transcription assays revealed that in Py3T3 cell extracts depleted of ERK, transcription was substantially reduced relative to extracts immunodepleted with the irrelevant control antibody against E7 (Figure 6.15B, compare lane 3 with lane 1). TBP-depleted extracts showed an even more marked reduction in transcription, as would be expected by removal of this essential transcription factor (Figure 6.15B, compare lane 4 with lanes 1 and 3). In contrast, immunodepletion of the Polyomavirus T antigens produced no effect on pol III transcription (Figure 6.15B, compare lanes 1 and 2). Immunodepleting the large T antigen would not be expected to compromise transcription if it co-depletes the RB to which it is bound. In the extracts prepared from 3T3 cells, immunodepletion of TBP

Figure 6.14

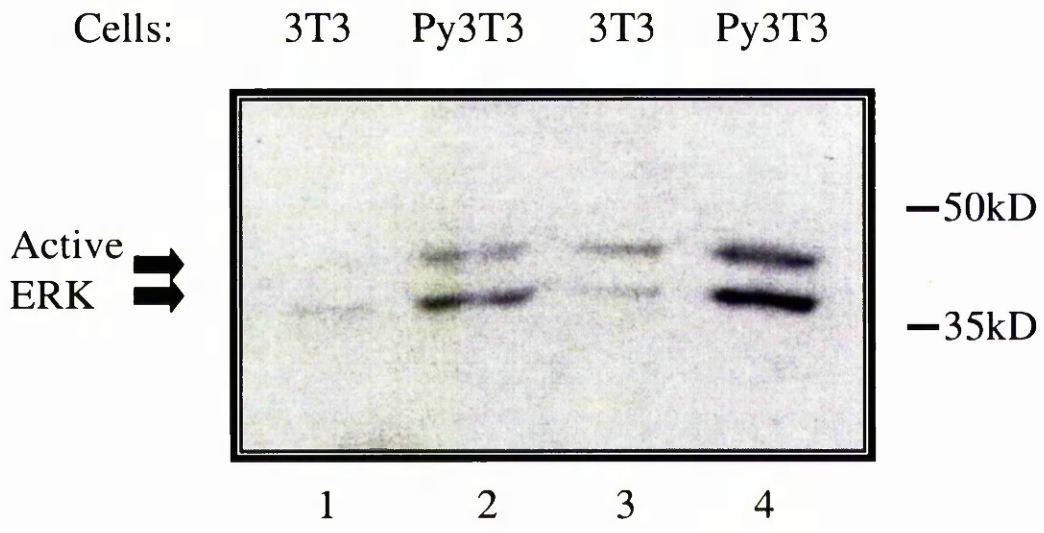
Py3T3 cells display lower levels of total ERK but elevated expression of the active forms when compared with 3T3 cells

Whole cell extracts prepared from 3T3 (lanes 1, 3 and 6, panel A and lanes 1 and 3, panel B), Py3T3 (lane 2 and 4 in each panel) and SV3T3 Cl49 (lane 7, panel A) cells were resolved on a SDS-7.8% polyacrylamide gel and analysed by western immunoblotting using either an anti-p44/42 MAPK antibody against total ERK (panel A) or a phospho-ERK antibody against only the active forms of ERK (panel B).

(A)


Cells:
3T3
Py3T3
3T3
Py3T3
/
3T3
Cl49
Total ERK
50kD
35kD
1
2
3
4
5
6
7

(B)


Cells:
3T3
Py3T3
3T3
Py3T3
Active ERK
50kD
35kD
1
2
3
4

also produced a decrease in transcriptional activity when compared to irrelevant control immunodepletions with E7 and Polyomavirus T antigens (Figure 6.15A, compare lane 4 with lanes 1 and 2). However, in contrast to Py3T3 cell extracts, depletion of ERK conferred no effect on pol III transcription of the VA_1 template (Figure 6.15A, compare lane 3 with lanes 1 and 2). These data support the contention that ERK plays a role in stimulating pol III transcription after Polyomavirus transformation that exceeds its normal role in untransformed 3T3 cells.

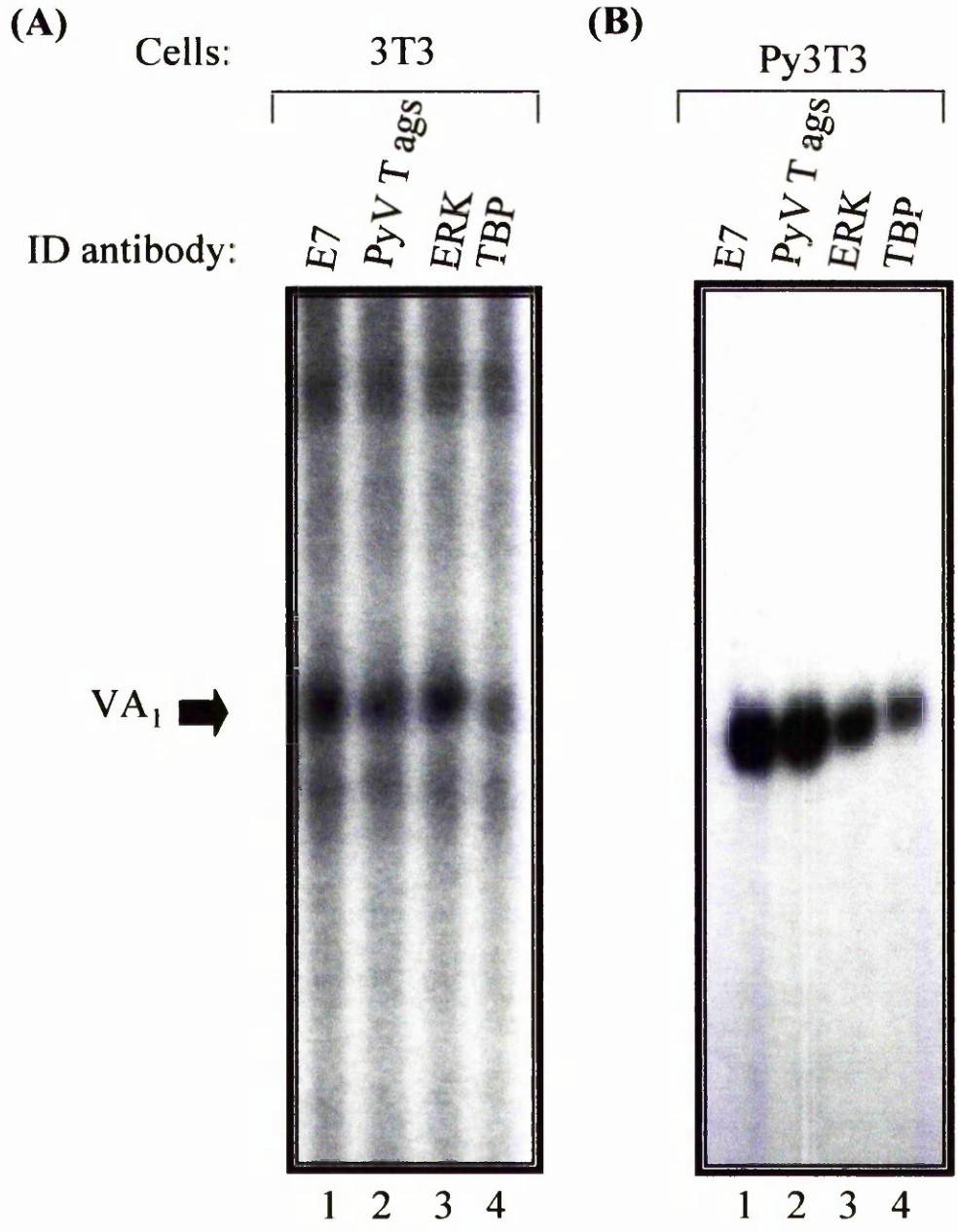
6.2.14 Pol III transcription is inhibited by an ERK substrate competitor peptide

Evidence to provide further credibility to the argument for ERK involvement in pol III transcription was attained through titration of an ERK substrate competitor peptide into 3T3 and Py3T3 cell extracts and subsequent analysis of transcriptional activity. In contrast to the ERK-depletion experiment (Figure 6. 15), 3T3 cells display a reduced transcriptional activity at 30μg of ERK peptide, although it was only marginal (Figure 6.16A, compare lane 4 with lanes 1, 2 and 3). However, Py3T3 extracts show a more apparent reduction with increasing amounts of ERK peptide, starting from 20μg (Figure 6.16B, compare lanes 3 and 4 with lanes 1 and 2). Notably, a control titration of an irrelevant competitor PKA peptide conferred no effect on transcriptional activity in either the 3T3 or Py3T3 cell extracts, demonstrating that the effect of the ERK peptide was specific (Figure 6.16, panels A and B, respectively, compare lanes 5-8). Additionally, it should be noted that analysis

Figure 6.15

ERK-immunodepleted Py3T3 cell extracts displayed reduced pol III transcription

Whole cell extracts (145µg) prepared from 3T3 (panel A) and Py3T3 (panel B) cells were immunodepleted (ID) using the anti-E7 antibody TVG710Y (lane 1 in each panel), an antibody against the T antigens of Polyomavirus F4 (lane 2 in each panel), the anti-ERK antibody p44/42 MAPK (lane 3 in each panel) or an anti-TBP antibody MTBP-6 (lane 4 in each panel). 20µg of each of the immunodepleted extracts were then analysed by *in vitro* transcription assay for their ability to transcribe 250ng of VA_1 template.


(A)
Cells:
3T3
(B)
Py3T3
E7
PyV T ags
ERK
TBP
ID antibody:
VA1
1 2 3 4
1 2 3 4

of the 3T3 cells was not performed in conjunction with the Py3T3 cells and panel A displays a longer exposure of the 3T3 experiment; consequently, panels A and B should not be compared for overall transcription levels. However, transcription levels in response to increasing ERK peptide are presented graphically as a percentage of the control lane for each cell type in figure 6.16C.

6.2.15 Endogenous interaction between ERK and the BRF component of TFIIIB

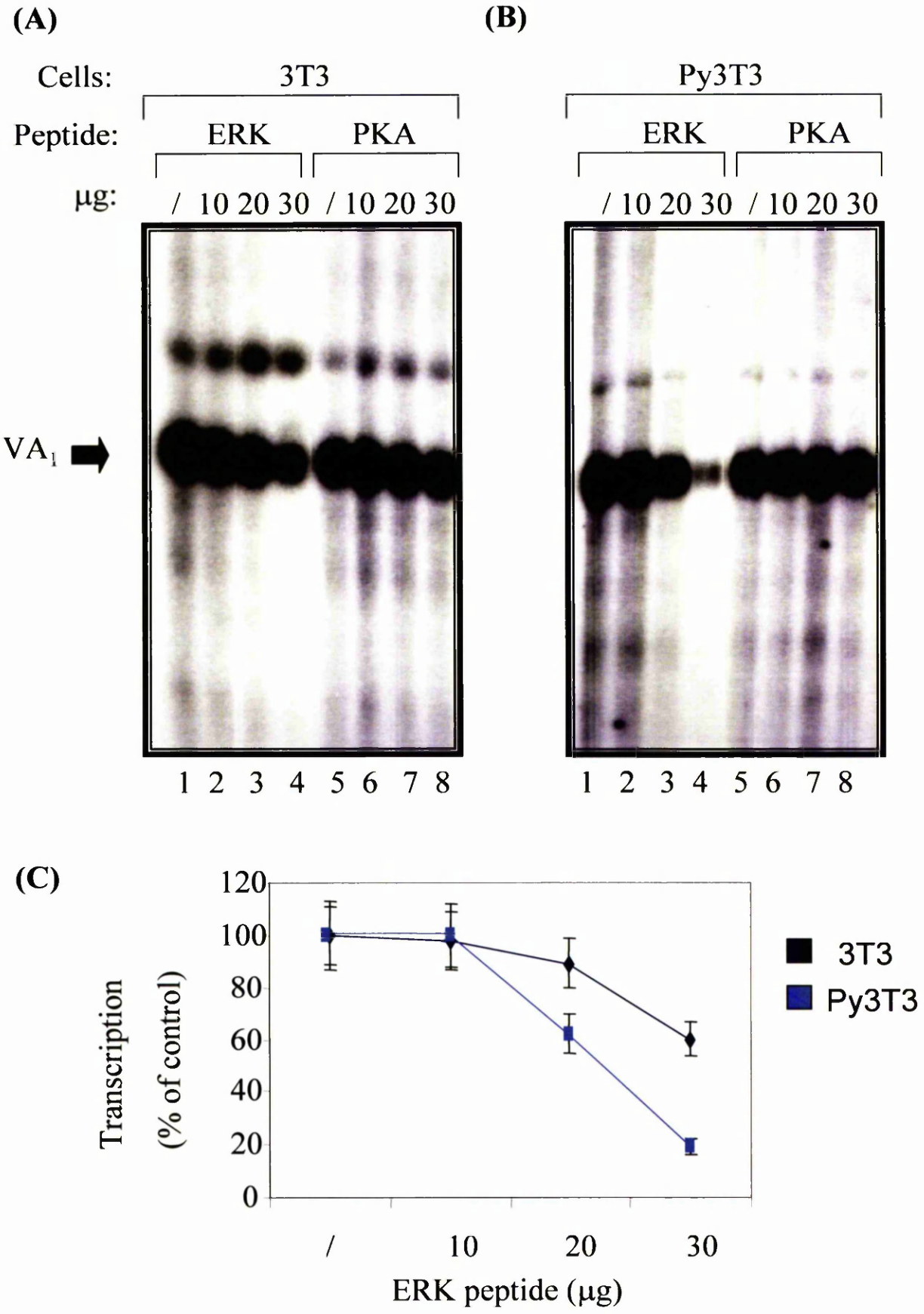
For ERK to confer an effect on pol III transcription, a target within the basal pol III transcriptional machinery would be required. In view of the fact that signalling pathways appeared not to influence TFIIIC2 DNA-binding activity (Figures 6.4 and 6.5), the possibility existed that the target of ERK was TFIIIB. Data reinforcing this theory was provided by co-immunoprecipitation experiments demonstrating an endogenous interaction between ERK and the BRF component of TFIIIB, in both 3T3 and Py3T3 cell extracts (Figure 6.17, lanes 2 and 5, respectively).

Control immunoprecipitation using an irrelevant antibody against Phas1 revealed that the BRF interaction with ERK was specific (Figure 6.17, lanes 1 and 4). As expected, immunoprecipitation of the TBP subunit of TFIIIB co-immunoprecipitated BRF, providing a positive control for BRF interactions (Figure 6.17, lane 3). A greater amount of BRF is co-immunoprecipitated with ERK from 3T3 cells than Py3T3 cells (Figure 6.17, compare lanes 2 and 5). However, assuming that binding of BRF is not

Figure 6.16

ERK peptide competitor inhibits pol III transcription

Titration of an ERK substrate peptide into *in vitro* transcription assays displaying effects on transcription of the pol III template VA_1. Whole cell extracts prepared from 3T3 (panel A) and Py3T3 (panel B) cells were pre-incubated for 10 minutes at 30°C in the absence (lanes 1 and 5 in each panel) or presence of 10, 20 or 30μg of either the ERK peptide (lanes 2-4, respectively, in each panel) or a control PKA peptide (lanes 6-8, respectively, in each panel). Transcription reactions contained 20μg of whole cell extract and 250ng of pVA_1 template. Transcription levels in response to increasing ERK peptide are summarised relative to the control (lane 1, panels A and B) ± standard deviation for each cell line (panel C).

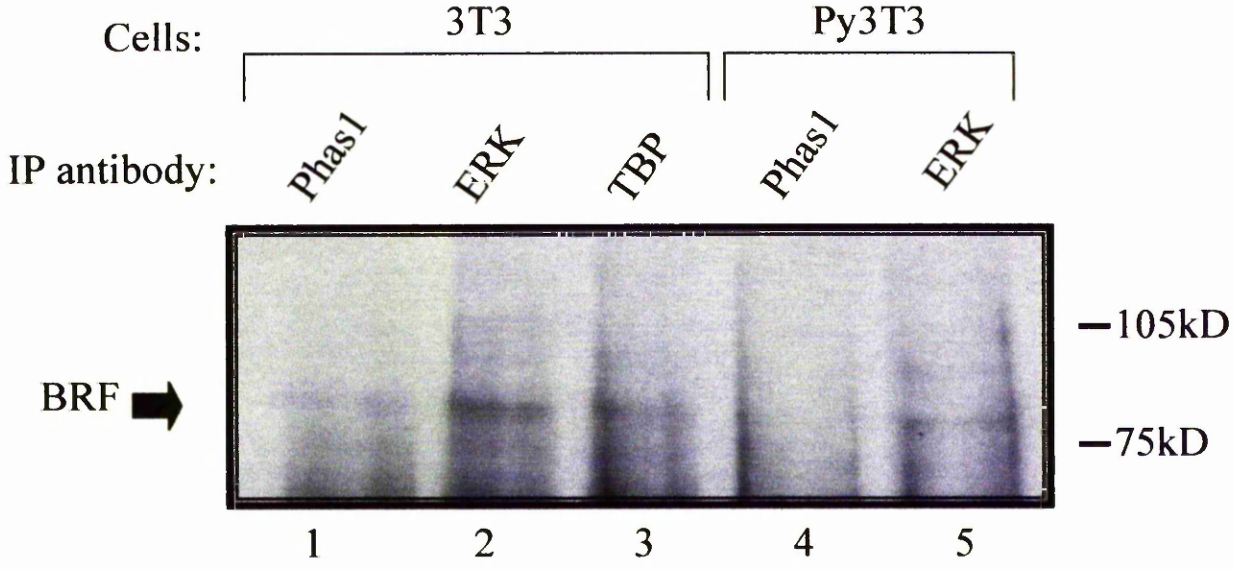

(A)
(B)
Cells:
3T3
Py3T3
Peptide:
ERK
PKA
µg:
/ 10 20 30 / 10 20 30
VA1
1 2 3 4 5 6 7 8
(C)
Transcription (% of control)
120
100
80
60
40
20
0
/
10
20
30
ERK peptide (µg)
3T3
Py3T3

dependent on activation of ERK, this would be expected, given that 3T3 cell extracts express a greater abundance of total ERK.

Figure 6.17

Endogenous interaction between ERK and the BRF component of TFIIIB

Whole cell extracts (150μg) prepared from 3T3 (lanes 1, 2 and 3) and Py3T3 (lanes 4 and 5) cells immunoprecipitated (IP) using the anti-Phas1 antibody R-113 (lanes 1 and 4), the anti-ERK antibody K-23 (lanes 2 and 5) or an antibody against TBP SL-1 (lane 3). The precipitated material was resolved on a SDS-7.8% polyacrylamide gel and the presence of BRF was determined by western analysis with the anti-BRF antibody 128-4.


Cells:
3T3
Py3T3
IP antibody:
Phas1
ERK
TBP
Phas1
ERK
BRF
105kD
75kD
1
2
3
4
5

6.3 DISCUSSION

Although the large T antigen of Polyomavirus plays an important role in the deregulation of pol III transcription, the middle T antigen has been established as the principal transforming oncoprotein (Treisman et al., 1981) and the small t antigen has been implicated in promoting cell cycle progression in a manner dependent on its binding of protein phosphatase 2A (PP2A) (Mullane et al., 1998). Consequently, roles for the middle, small or both T antigens in deregulation of pol III transcription seemed plausible.

Transient transfection into untransformed 3T3 cells demonstrated that expression of the Polyomavirus middle T antigen conferred a substantial elevation in pol III transcription, providing a compelling argument in favour of its involvement.

However, in contrast to the nuclear large T antigen, middle T is cytoplasmic and associates with intracellular membranes (Dilworth et al., 1986). Consequently, middle T antigen functions have been ascribed to its ability to bind and activate numerous cytoplasmic cellular proteins (Elliott et al., 1998), leading to activation of a variety of signalling pathways that culminate with growth and proliferation, even in the absence of growth factors (Armelin et al., 1985). Moreover, it has been shown that middle T activates genes encoding various transcription factors (Rameh and Armelin, 1991), like Fos (Talmage and Listerud, 1994) and Jun (Schonthal et al., 1992). These studies presented the possibility that middle T could stimulate pol III transcription via a signalling pathway, targeting one of the basal pol III transcription factors. Given that the use of Pytsa3T3 cells has shown that Polyomavirus activates

TFIIIC2 in a large T antigen-independent manner, initial investigation for a middle and/or small T antigen target was focussed on this fundamental transcription factor.

The binding of transcription factors to promoter sites can be regulated directly or indirectly by protein phosphorylation (Whitmarsh and Davis, 2000). Thus, in order to establish whether inhibition of a set of specific cellular proteins involved in signal transduction conferred an effect on TFIIIC2 DNA-binding activity, cell extracts prepared from Py3T3 cells were pre-incubated with Okadaic acid, Olomoucine, U0126 and Wortmannin, specific inhibitors of PP2A, cyclin-dependent kinases, MEK and PI-3 kinase, respectively. Results demonstrated no effect on TFIIIC2 DNA-binding activity in the presence of these inhibitors and, furthermore, similar analyses using the general kinase inhibitor DMAP supported the exemption of TFIIIC2 DNA-binding activity from signal transduction inhibition, at least *in vitro*.

Although the DNA-binding activity of TFIIIC2 proved not to be a target for activated signalling pathways, influences on other aspects of pol III transcription remained likely. Of particular interest was the effect on overall transcriptional activity of inhibiting PP2A. Interactions with PP2A have been documented with not only the middle T antigen of Polyomavirus, but also the small t antigens of both Polyomavirus and SV40 (Pallas et al., 1990). Since these oncoproteins replace the regulatory B subunit of PP2A, which is responsible for substrate specificity (Shenolikar, 1994) and localisation (Strack et al., 1998), interactions with these oncoproteins could produce a variety of responses in PP2A activity, including activation, inactivation or redirection of PP2A to new targets, possibilities that have been shown not to be mutually exclusive (Pallas et al., 1990).

PP2A has been established as active *in vitro* against a number of enzymes involved in metabolic pathways (Cohen, 1989). Consequently, in order to ascertain whether oncoprotein-bound PP2A influences pol III transcription and obtain an indication of whether it predominantly activates or inactivates any targets, extracts of 3T3 and Py3T3 cells were incubated in the presence of the potent PP2A inhibitor Okadaic acid and transcriptional activity analysed by *in vitro* transcription assays. Transcriptional activity of both the 3T3 and Py3T3 cell extracts displayed little or no change in the presence of the inhibitor, relative to their respective untreated cell extracts.

While inhibiting PP2A may indeed not confer an effect on pol III transcription, it is conceivable that activating and inactivating effects were cancelled out. Alternatively, slight effects may have been masked by other phosphatases, if any redundancy exists between them and PP2A. Moreover, although a response to Okadaic acid treatment would have established an involvement of a signalling pathway, it is not possible to conclude from these data that PP2A interactions with Polyomavirus middle and/or small T antigens do not contribute to pol III transcriptional activation *in vivo*.

In order to test for an involvement of signalling pathways in stimulation of pol III transcription, cell extracts were treated with the general kinase inhibitor DMAP and used for *in vitro* transcription assays. 3T3 extracts remained uncompromised by the DMAP treatment, while a 50% inhibition of pol III transcription was observed for the treated Py3T3 cell extracts, strongly implicating the recruitment of a signalling cascade by Polyomavirus.

Support for this was conferred by an equivalent inhibition of pol III transcription in the large T-defective cell line, Pytsa3T3, indicating the recruitment of a signalling pathway for pol III transcriptional deregulation, an effect that is large T-independent.

Although DMAP generated a clear response *in vitro*, some further studies were performed *in vivo* to ensure activity of full signal pathways. In addition to PP2A, another of the cellular proteins bound by middle T is PI-3 kinase. Cells treated for four hours with the PI-3 kinase inhibitor LY294002 displayed a significant reduction in transcriptional activity. This was observed in the Py3T3 cells but, significantly, to a greater degree in the 3T3 cells. This suggests that although PI-3 kinase may be involved in pol III transcriptional regulation, Polyomavirus may not significantly up-regulate this pathway.

Confirmation that the LY294002 was inhibiting this pathway was achieved through western analysis of substrate proteins downstream of PI-3 kinase, which revealed dephosphorylation in the presence of the LY294002 inhibitor. Similarly, northern analysis results were consistent with the *in vitro* transcription assay observations and demonstrated an inhibition of B2 transcript levels in both cell lines following inactivation of the PI-3 kinase downstream signals.

Studies with SV40 have demonstrated that through binding and inactivating PP2A, the small t antigen of SV40 stimulates the MAP kinase pathway by preventing PP2A-mediated dephosphorylation of ERK1 and MEK in the signal cascade, resulting in their constitutive activation (Sontag et al., 1993). Furthermore, middle T antigen of Polyomavirus is able to bind and activate Shc, leading in turn to Shc binding and activating the Ras protein. Consequently, this also leads to activation of the MAP kinase signal pathway (Dilworth et al., 1994; Rozakis-Adckock et al., 1992). Given that activated MAP kinases can translocate to the nucleus, where they phosphorylate transcription factors that regulate expression of genes pivotal to cell proliferation (Hill

and Treisman, 1995), such activation might potentially target pol III transcription factors.

Consequently, *in vivo* treatment in the presence of the MEK inhibitor U0126 was carried out and extracts prepared after a treatment time of 1 hour were used for analysis by *in vitro* transcription assay. Results conveyed an extreme inhibition of pol III transcription in the Py3T3 cells after U0126 treatment. Conversely, while the untransformed 3T3 cells also display inhibition of transcription by U0126 treatment, the effect was limited and significantly less substantial than that observed in the Py3T3 cells. Again, inhibition of the pathway via inactivation of MEK was ratified by western analyses demonstrating the dephosphorylation of downstream targets following treatment. Thus, this provided evidence of activation of pol III transcription mediated through signals blocked by MEK inactivation.

With a clearly established involvement in activation of pol III transcription, it was of interest to determine the relative abundance of the total and active forms of ERK in the untransformed and Polyomavirus-transformed cell lines. A striking pattern of expression was observed, with total levels of ERK in 3T3 cells exceeding levels displayed in Py3T3 cells, but active forms significantly more abundant in Py3T3 cells than the parental cells. These results demonstrate that although less abundant in Py3T3 cells, a significantly greater proportion of ERK present is in the active forms. This is consistent with ERK activation through up-regulation of the MAP kinase signalling pathway.

Subsequent analyses demonstrated reduced transcriptional activity in ERK-depleted Py3T3 cell extracts, an observation not pertaining to 3T3 cells. Furthermore, a marked inhibition of transcription in Py3T3 cell extracts pre-incubated in the presence

of an ERK substrate competitor peptide was only weakly reflected in 3T3 cell extracts. A potential target for ERK was strongly suggested by the discovery of an endogenous interaction between ERK and the BRF component of TFIIIB.

Studies have documented that middle T binding to Shc results in a binding site for the SH2 domain of the adapter molecule Grb2 (Campbell et al., 1994; Dilworth et al., 1994). This in turn recruits, through its SH3 domains, the guanine nucleotide exchange factor mSos into the middle T complex and this re-location of mSos to a membrane site can be sufficient to activate the MAP kinase pathway (Aronheim et al., 1994; Schlessinger, 1993). Furthermore, SV40 small t antigen can promote receptor-independent activation of the MAP kinase pathway through its inhibition of PP2A (Sontag et al., 1993). However, a study by Urich *et al* documented the requirement for PI-3 kinase-mediated signals in addition to those mediated through Ras for middle T-activation of the MAP kinase pathway, stating that inhibition of PI-3 kinase blocked relocalisation of ERK1 (Urich et al., 1995). They also reported that cells expressing middle T antigen mutants unable to bind Shc or PI-3 kinase showed neither activation nor nuclear translocation of MAP kinases and suggested that both pathways feed into the MAP kinase cascade (Urich et al., 1995).

Subsequent studies by the same group then established an essential role of the Rho family GTPases in cell transformation by middle T, where they demonstrated c-fos promoter activation by two Ras-initiated signalling cascades, Raf-dependent and –independent, with both pathways requiring functional Rac (Urich et al., 1997). Furthermore, the serine/threonine kinase PAK has emerged as a molecule that can link Rac and Ras signalling by converging on the ERK MAP kinase pathway (Bar-Sagi and Hall, 2000). It is widely reported that overexpression of a Rho GTPase

cannot, in itself, lead to ERK activation; however, dominant-negative Rac can block Ras-dependent ERK activation in 293 cells (Frost et al., 1996)

Hence, previous studies identified essential interactions of PI-3 kinase and Shc with middle T, the requirement of Rac and the convergence of activated signals on the ERK pathway. Data established in this chapter are in agreement with these previous findings. Inhibition of PI-3 kinase in Py3T3 cells showed only a moderate inhibition of pol III transcription, while that conferred by inactivation of MEK1 and MEK2 was significantly more substantial. Furthermore, levels of active ERK were clearly elevated in Py3T3 cells. This would be consistent with merging of the PI-3 kinase- and Shc-mediated pathways upstream of MEK and the subsequent activation of the ERK pathway, which may be augmented by the binding of the middle and/or small T antigens to PP2A. Thus, it appears that middle and/or small T antigen(s) of Polyomavirus target the ERK MAP kinase signalling pathway for constitutive activation, allowing in turn, activation of a pol III transcription factor(s); perhaps TFIIIB, via an interaction between ERK and BRF. However, TFIIIB is not substantially activated in Pytsa3T3 cells. Binding of ERK to BRF may position it in the transcription complex, where it can phosphorylate other targets, such as TFIIIC or pol III.

Chapter 7

Discussion

It has long been recognised that pol III transcription is stimulated in response to DNA and RNA tumour viruses, in addition to a host of other carcinogens and environmental stimuli. Continuing studies are unravelling the mechanisms involved in viral transformation, with substantial progress made in the understanding of transformation by SV40, which leads to a marked increase in the expression of pol III transcripts (Carey et al., 1986; Larminie et al., 1999; Scott et al., 1983; Singh et al., 1985; White et al., 1990). Two mechanisms have been identified that contribute to this effect (Larminie et al., 1999). A major restraint on pol III transcription in untransformed fibroblasts is provided by RB and its relatives p107 and p130, which bind and repress TFIIIB (Larminie et al., 1997; Larminie et al., 1999; Scott, 2001; Sutcliffe et al., 2000; Sutcliffe et al., 1999). The large T antigen of SV40 is able to bind and neutralise RB, p107 and p130, thereby releasing TFIIIB from this control and allowing a substantial increase in its transcriptional activity (Larminie et al., 1999). A second and apparently unrelated mechanism that accompanies SV40 transformation involves the overexpression of TFIIIC2; EMSAs showed elevated TFIIIC2 activity, while RT-PCR and western blotting revealed that this correlates with the overproduction of TFIIIC220 and TFIIIC110 mRNA and protein (Larminie et al., 1999; White et al., 1990). This current study has confirmed that the remaining three subunits of TFIIIC2 are similarly overexpressed in SV40-transformed cells at the mRNA level. Although this might have been anticipated, since the five subunits of

TFIIIC2 are assumed to function in a stoichiometric complex, one study has reported that TFIIIC110 is induced selectively by adenovirus infection (Sinn et al., 1995). In contrast to the increases in TFIIIC2 subunits, there is little or no change in the levels of TBP and BRF components of TFIIIB (Larminie et al., 1999). Since TFIIIB is also believed to be a stoichiometric complex, it would have been expected that its third essential subunit would also remain constant following SV40 transformation. However, as first demonstrated in this study, B" is clearly overexpressed at both the mRNA and protein levels in SV3T3 cells, providing an additional and unanticipated instance of how the pol III transcriptional machinery can be affected by a virus. Transformation by SV40 therefore involves at least three distinct changes in the basal pol III factors, which combine to allow unusually high expression of class III genes.

The principal focus, however, of this study has been analysing the effects of Polyomavirus on pol III transcription and components of the pol III machinery, identifying specific effects conferred by the individual oncoproteins that it expresses. Polyomavirus, another papovavirus, is closely related to SV40 and yet differs in several important ways (Tooze, 1980). It has been demonstrated here that the Polyomavirus large T antigen releases TFIIIB from repression by RB and that Polyomavirus-transformed fibroblasts overexpress mRNAs encoding all five subunits of TFIIIC2, as well as the B" subunit of TFIIIB, but not TBP or BRF. Thus, these three mechanisms of deregulation are shared by different members of the papovavirus family. Given that other types of DNA tumour virus are also able to activate pol III transcription, it will be of interest to determine the extent to which they employ similar deregulatory mechanisms.

RB is a common target for transforming viruses and it has previously been demonstrated that both adenovirus and human papillomavirus (HPV) can stimulate pol III activity by expressing oncoproteins that target and neutralise RB (Larminie et al., 1999; Sutcliffe et al., 1999; White et al., 1996). Indeed, inactivation of RB may be the most common mechanism for inducing pol III transcription in transformed cells (Brown et al., 2000). Induction of TFIIIC2 may also prove to be a strategy that is commonly exploited by viruses, since early template commitment assays provided evidence that the concentration of this factor increases when HeLa cells are infected with adenovirus (Yoshinaga et al., 1986). A more recent study, however, found that TFIIIC220 is not induced in adenovirus-infected HeLa cells, although TFIIIC110 levels increase markedly; the remaining TFIIIC2 subunits and the components of TFIIIB were not examined in that study (Sinn et al., 1995) and their expression levels following adenovirus transformation remain undetermined. The latter report clearly differs from the effects observed following SV40- and Polyomavirus-mediated transformation, but TFIIIC220 levels may already be very high in uninfected HeLa cells, which are transformed by HPV; furthermore, infection may elicit a different TFIIIC2 response to transformation. Establishing the response of the pol III transcriptional machinery to malignant transformation by HPV will be of significant interest.

The overexpression of TFIIIC2 is a clinically important phenomenon, since it has been found to occur in human cancers. Thus, a study of nine ovarian epithelial carcinomas revealed abnormally high TFIIIC2 activity in each of the tumours when compared with untransformed ovarian tissue from the same individuals (Winter et al., 2000). This effect correlated with a specific increase in the levels of all five mRNAs encoding the subunits of TFIIIC2 (Winter et al., 2000). Since ovarian cancer is not

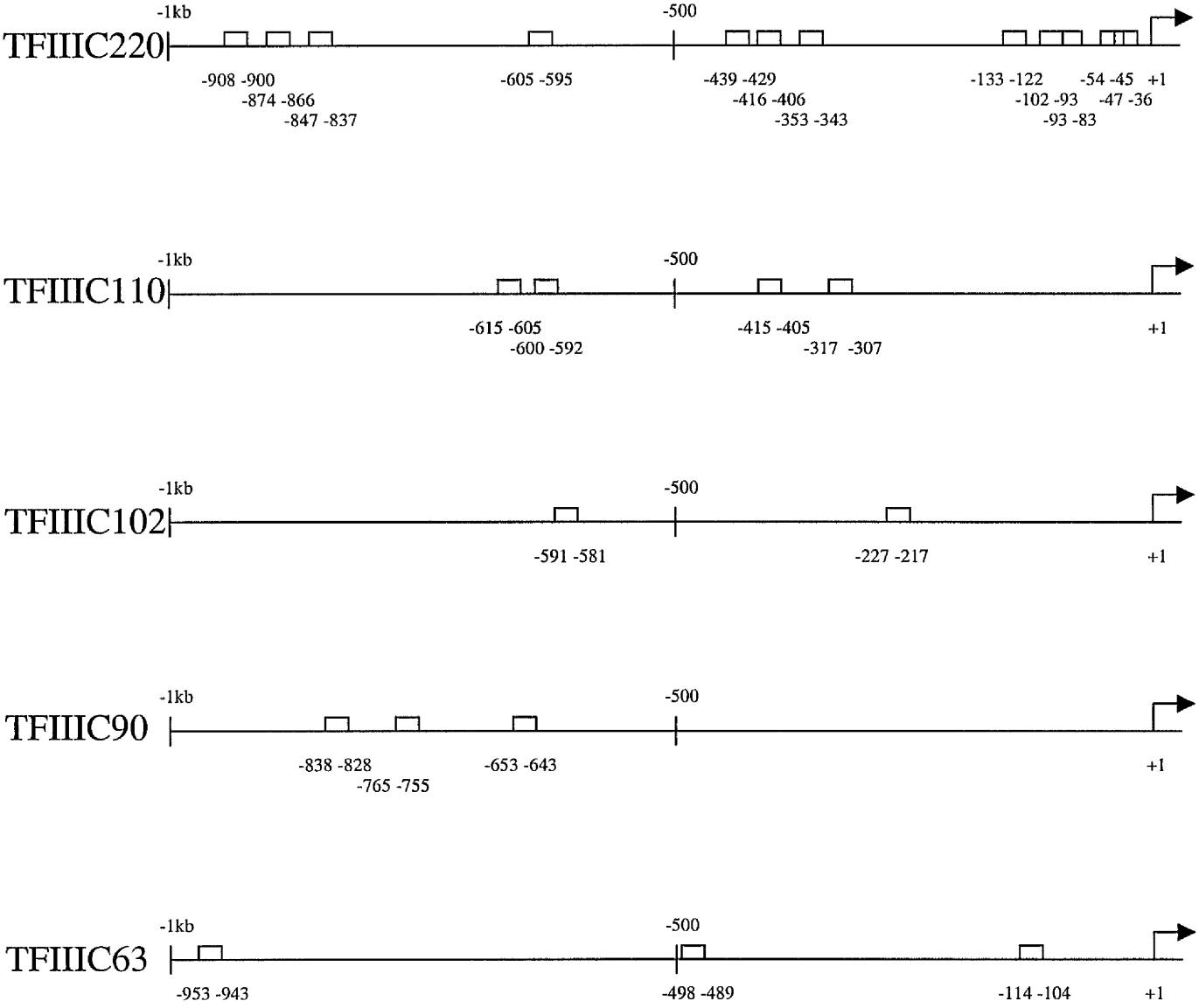
believed to be associated with tumour viruses (Gallion et al., 1995), it seems that TFIIIC2 expression can respond to distinct types of oncogenic signal. The use of papovavirus-transformed cell lines first uncovered this feature of pol III regulation (Larminie et al., 1999; White et al., 1990), hence, it is of notable significance that these model systems have proved again to be directly relevant to the situation in human disease. Consequently, their employment in investigating further the mechanisms responsible for inducing TFIIIC2 during carcinogenesis will be important.

In the three types of tumour cell analysed, namely SV3T3, Py3T3 and ovarian carcinomas, the five transcripts encoding the components of TFIIIC2 are all induced together. This co-ordinate induction under distinct circumstances suggests that the genes encoding these subunits might have common promoter or enhancer sequences that allow their co-regulation. This would seem logical, since the five subunits are believed to function stoichiometrically. With regard to this contention, analysis of sequences of the TFIIIC2 subunit promoters has revealed, in each case, that a number of potential AP-1 sites are present, as illustrated in figure 7.1. Hence, the possibility exists that through activation of these sites, expression of these subunits could potentially be co-ordinated. Furthermore, that oncoproteins of Polyomavirus have been shown to activate the c-fos and c-jun proto-oncogenes, members of the AP-1 family, strengthens the appeal of this hypothesis. Thus, future studies of the effects of AP-1 activation on the co-ordinate expression of TFIIIC2 subunits could provide significant insight to the mechanisms surrounding TFIIIC2 activation following transformation. Indeed, unpublished observations demonstrate that AP-1 DNA-binding activity is elevated in Py3T3 cells when compared to the untransformed parental 3T3 cell line. However, studies with HeLa extracts have suggested the

Figure 7.1

Schematic diagram of the relative positions of potential AP-1 binding sites within TFIIIC2 promoter regions

Linear representation of each of the TFIIIC2 subunit promoters. The boxes represent the potential AP-1 binding sites located in each promoter region, with their respective positions relative to the transcription start site (+1) indicated below.


TFIIIC220
-1kb
-500
-908 -900
-874 -866
-847 -837
-605 -595
-439 -429
-416 -406
-353 -343
-133 -122
-102 -93
-93 -83
-54 -45
-47 -36
+1
TFIIIC110
-1kb
-500
-615 -605
-600 -592
-415 -405
-317 -307
+1
TFIIIC102
-1kb
-500
-591 -581
-227 -217
+1
TFIIIC90
-1kb
-500
-838 -828
-765 -755
-653 -643
+1
TFIIIC63
-1kb
-500
-953 -943
-498 -489
-114 -104
+1

existence of an inactive TFIIIC2 complex that specifically lacks the TFIIIC110 subunit (Hoeffler et al., 1988; Kovelman and Roeder, 1992; Sinn et al., 1995). This lead to an alternative model, in which the selective induction of the TFIIIC110 subunit might convert pre-existing inactive complexes into functionally competent TFIIIC2 (Hoeffler et al., 1988; Kovelman and Roeder, 1992; Sinn et al., 1995). In regard to this model, it is notable that TFIIIC110 is more strongly induced by papovavirus transformation than the other components of the complex, especially in the SV3T3 cells (Larminie et al., 1999). This, however, was not the case in ovarian cancers (Winter et al., 2000), suggesting that both these models may contribute to the deregulation of TFIIIC2, but to varying extents depending on the cell type in question.

The induction of B" reported here has not been observed previously, principally on account of the fact that mammalian B" has only recently been identified (Schramm et al., 2000). This observation, however, is somewhat surprising given that the TBP and BRF components of TFIIIB are not overexpressed in SV3T3 or Py3T3 cells. Nevertheless, the level of B" mRNA and protein is clearly elevated in all three papovavirus-transformed cell lines analysed in this study. As in yeast, mammalian B" has a relatively low affinity for the TBP/BRF subcomplex (Schramm et al., 2000). Thus, increasing the level of B" may serve to promote its assembly into functional TFIIIB complexes by mass action. Indeed, direct assays of TFIIIB activity have demonstrated that activity remains partially elevated in Pytsa3T3 cells. While this cell line is defective for expression of the large T antigen of Polyomavirus, and thus unable to relieve RB-mediated repression of TFIIIB activity, it does continue to overexpress B" at a comparable level to the wild-type Py3T3 cells. This indicates not only that overexpression of B" is large T-independent, but moreover, that the remaining elevation of TFIIIB activity observed in the absence of the large T antigen

may be explained by the overexpression of B". Consequently, it appears that two independent mechanisms may be involved in the activation of TFIIIB that contribute to the deregulation of pol III transcription that accompanies Polyomavirus transformation of fibroblasts. However, the relative importance of the different pathways utilised by Polyomavirus to act on pol III may vary according to cell type and induction of B" might contribute to a greater or lesser extent in, for example, the endotheliomas that Polyomavirus induces in transgenic mice. Similarly, its impact on the stimulation of TFIIIB activity may also be subject to the particular class III gene being expressed.

Another finding from this study was the strong activation of a pol III reporter induced by the Polyomavirus middle T antigen *in vivo*. The middle T antigen is generated by alternative splicing of the viral early transcript and has no equivalent in SV40 (Tooze, 1980). Nevertheless, it is the principal transforming oncoprotein of Polyomavirus, being necessary and sufficient to induce morphological transformation and alterations in the growth properties of established cell lines (Raptis et al., 1985). It is able to achieve this through association with signal transducers, such as members of the Src family, phosphatidylinositol-3 kinase and the Shc protein that activates the Ras pathway (Messerschmitt et al., 1997; Urich et al., 1995). The middle T antigen is located outside the nucleus and its stimulatory effect on pol III transcription appears to be conferred through its activation of signalling cascades. In this regard, the action of the middle T antigen is reminiscent of the situation with the X oncoprotein of hepatitis B virus (HBV), which stimulates pol III transcription through activation of the Ras/Raf-1 signal transduction cascade (Wang et al., 1997). Indeed, not only can the activation of pol III transcription in Py3T3 cells be partially blocked by the general kinase inhibitor DMAP *in vitro,* but *in vivo* studies utilising the specific

MEK1 and MEK2 inhibitor U0126 conferred a significant reduction in the overexpression of pol III transcripts observed in response to Polyomavirus transformation. This suggested that targets downstream, such as ERK, could be involved in the stimulation of pol III transcription. Analysis of the abundance of ERK in Polyomavirus-transformed cells relative to levels expressed in the untransformed parental cell line demonstrated a striking pattern of expression; despite total levels of ERK being more abundant in the 3T3 cells, the levels of the active forms of ERK were substantially higher following transformation by Polyomavirus. Moreover, specifically immunodepleting ERK from Py3T3 extracts revealed a reduction in transcriptional activation, an observation supported by the ability of a competitor ERK peptide to similarly diminish transcription levels in Py3T3 cells in proportion to increasing concentration. This study provided further insight to the potential mode of action of ERK on pol III transcription with the finding that endogenous ERK specifically co-immunoprecipitates with the BRF component of TFIIIB. Thus, it may be the case that the binding of ERK to BRF positions it in the transcription complex where it could potentially phosphorylate TFIIIC or pol III.

The region of the Polyomavirus genome that encodes the middle T antigen is poorly conserved with SV40 (Tooze, 1980). The equivalent SV40 sequence codes for a large T epitope that binds and inactivates p53, a function not performed by any Polyomavirus product (Lane and Crawford, 1979; Vousden, 1995; Zhu et al., 1992). Since p53 has been shown to bind and inactivate TFIIIB (Cairns and White, 1998; Chesnokov et al., 1996), release from p53 repression may provide yet another mechanism that facilitates the deregulation of pol III transcription in some types of SV40-transformed cells. A physical interaction between TFIIIB and the SV40 large T antigen has also been reported (Damania et al., 1998), although the contribution of

this towards pol III activation has yet to be established. Nevertheless, it seems clear now that papovavirus transformation can confer an impact on the pol III machinery through a diverse range of mechanisms. The targeting of both TFIIIB and TFIIIC2 may be important to maximise pol III transcription. Experiments with synchronised cell populations have revealed that alternative pol III transcription factors can be limiting during different phases of the cell cycle (White, 1995). Whereas TFIIIC activity limits the rate of VA_1 expression in extracts of S or G_2 phase cells, TFIIIB is the limiting factor in extracts of cells harvested during G_0 or early G_1 (Scott, 2001; White, 1995). Hence, stimulation of TFIIIB or TFIIIC alone may only influence the transcriptional output during a restricted interval of the cell cycle. Activating both TFIIIB and TFIIIC2 might therefore allow papovavirus-transformed cells to sustain elevated rates of pol III transcription throughout interphase, which may be a prerequisite for rapid growth.

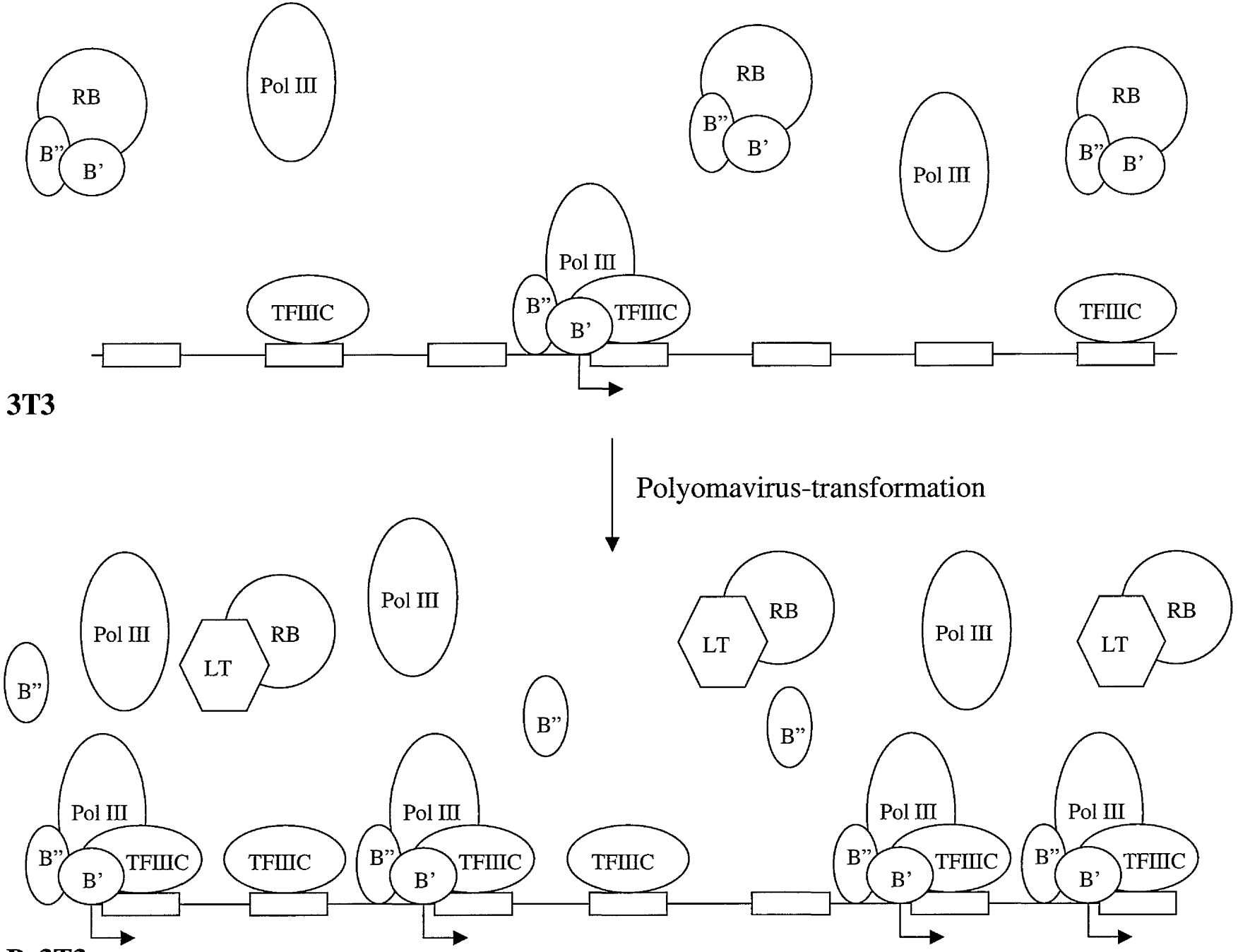
While the activation of two essential transcription factors, TFIIIB and TFIIIC, by papovaviruses clearly constitute vital mechanisms of deregulation, this study has demonstrated that Polyomavirus adopts an additional and apparently unique deregulatory approach. The polymerase itself is similarly targeted for activation, with pol III activity being substantially higher in Py3T3 cells relative to the untransformed 3T3 parental cell line. Furthermore, this corresponds to an increase in the abundance of pol III subunits that accompanies transformation by Polyomavirus. This appears to be the first instance documented of stimulation of pol III itself following viral transformation. The particular oncoprotein(s) responsible for the activation of pol III remains to be determined and it will be of interest to establish if this function is ascribed to an oncoprotein which is related to any expressed by similar tumour viruses.

Taken together, the results from this study clearly demonstrate the diversity of approaches utilised by both Polyomavirus (summarised in figure 7.2) and SV40 in their goal to deregulate pol III transcription. The employment of such a variety of mechanisms suggests that stimulating pol III transcription may be an important part of the viral transformation process.

Figure 7.2

Illustration summarising the mechanisms involved in the deregulation of pol III transcription following transformation by Polyomavirus

The situation reflecting pol III transcriptional output in untransformed 3T3 cells and the subsequent situation following Polyomavirus transformation. In 3T3 cells TFIIIB activity is limiting due to it being bound and repressed by RB. In Py3T3 cells, the large T antigen of Polyomavirus binds and neutralises RB, releasing TFIIIB from its repressive effects. Additionally, the B" subunit of TFIIIB is selectively increased and both pol III and TFIIIC2 activities increase in conjunction with raised levels of their subunit components. Consequently, the abundance of the essential transcription factors are elevated, allowing the increased formation of transcription complexes and an ensuing elevation in pol III transcriptional output.


RB
B"
B'
Pol III
TFIIIC
3T3
Polyomavirus-transformation
LT
Py3T3

Chapter 8

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