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The Deregulation of RNA Polymerases I and III in Tumours

by
Nicole Louise Daly

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Division of Biochemistry and Molecular Biology

Institute of Biomedical and Life Sciences

University of Glasgow

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Declaration

I hereby declare that the thesis which follows is my own composition, that it is a record of the work done myself and that it has not been presented in any previous application for a Higher Degree.

Nicole L. Daly

RNA polymerases I and III are responsible for approximately 80% of all nuclear transcription. The activity of these enzymes is a major determinant of the biosynthetic capacity of cells, since they synthesise important products required for protein synthesis, such as tRNAs and rRNAs. Regulation of these polymerases is likely to be of fundamental importance, since their activity is controlled directly by two cardinal tumour suppressors, RB and p53.

Cervical, breast and colorectal malignancies were investigated to assess if activity of RNA polymerases I and III is deregulated in these cancers. Expression studies of genes transcribed by these polymerases demonstrated that most of the tumour biopsies examined displayed deregulated transcriptional activity in comparison with matched normal tissue.

In the cervical biopsy samples examined, the presence of human papillomavirus 16 correlated with dramatic and specific overexpression of genes transcribed by these polymerases. This may be explained by the fact that, upon integration into the host genome, this virus upregulates the levels of the oncoproteins E6 and E7, which are able to neutralise p53 and RB, respectively. Furthermore, Brf1, a subunit of TFIIIB, was also found to be overexpressed, and this correlated with the increased levels of RNA polymerase III transcripts in the human papillomavirus 16 infected samples. This subunit was also found to be elevated in some cases of the other tumour types tested.

When other factors were investigated that may contribute to upregulated rates of transcription, it was found that in the breast tumour biopsies, upregulation of c-Myc closely correlated with increased levels of RNA polymerase I transcripts as well as MRP and 7SK transcripts. In the colon tumour biopsies, a correlation was observed between the c-Myc target, cyclin D2, and increased levels of RNA polymerase III transcripts. It was also found through comparison of a transformed and untransformed cell line that RNA polymerase III transcription may be regulated through acetylation and deacetylation.

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List of Abbreviations

A	adenine
Arg	arginine
ARPP P0	acidic ribosomal phosphoprotein P0
Bdp1	B double prime 1
Brf1	TFIIB-related factor 1
Brf2	TFIIB-related factor 2
bp	base pair
C	cytosine
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
cdk	cyclin-dependent kinase
DNA	deoxyribonucleic acid
DSE	distal sequence element
EBV	Epstein-Barr virus
G	guanine
HAT	histone acetyltransferase
HBV	hepatitis B virus
HDAC	histone deacetylase
HMG	high mobility group
HPV	human papillomavirus
ICR	internal control region
IGS	intergenic spacer

Leu	leucine
mRNA	messenger RNA
ORF	open reading frame
PCR	polymerase chain reaction
pol	RNA polymerase
pre-rRNA	precursor rRNA
PSE	proximal sequence element
PTF	PSE-binding transcription factor
rDNA	ribosomal DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	reverse transcription
Sec	selenocysteine
SINE	short interspersed repeat
snRNA	small nuclear RNA
SV40	simian virus 40
T	thymine
TAF	TBP-associated factor
TBP	TATA-box binding protein
TFIIB	transcription factor IIB
TFIIH	transcription factor IIH
TFIIIA	transcription factor IIIA
TFIIIB	transcription factor IIIB
TFIIIC	transcription factor IIIC
tDNA	transfer DNA

TPA	12-O-tetradecanoylphorbol-13-acetate
tRNA	transfer RNA
TSA	trichostatin A
Tyr	tyrosine
UBF	upstream binding factor
UCE	upstream control element

Chapter 1

Introduction

1.1 Transcriptional Control of Cellular Activity.

The actions and properties of a cell are largely determined by the genes it expresses. The first step of this process, transcription, is subject to a wide variety of regulatory controls. This allows the cell to constantly adapt in response to environmental changes and metabolic requirements. Defects in transcription are responsible for a wide range of human diseases and play a crucial part in the development of cancer.

1.2 Eukaryotic RNA Polymerases.

Transcription of eukaryotic nuclear genes requires three different RNA polymerases, designated I, II, and III. Structurally, these multisubunit enzymes are quite similar to each other, the two largest subunits being closely related and some of the smaller ones being shared by more than one polymerase. Functionally, however, they are quite distinct, transcribing different sets of genes (White, 1998). RNA polymerase I (pol I) synthesises the precursor rRNA (pre-rRNA) which is processed into 5.8S, 18S, and 28S ribosomal RNAs (rRNAs), RNA polymerase II (pol II) synthesises messenger RNA (mRNA) and most small nuclear RNAs (snRNA), and RNA polymerase III (pol III) synthesises transfer RNA (tRNA), 5S rRNA, and an array of small cellular and viral RNAs (Chambon, 1975; Roeder & Rutter, 1969; Zieve, 1981).

1.3 RNA Polymerases I and III and Cancer.

The activities of pol I and pol III dominate cellular transcription, combining to exceed 80% of total RNA synthesis in growing cells. Therefore, tight regulation is of considerable importance to the balance of cellular metabolism, and ensures that the output of pols I and III is matched to a cell's biosynthetic requirement. The rapid growth of tumours requires elevated rates of biosynthesis. This would not be possible if rRNA or tRNA were limiting. Abnormally high rates of transcription by pols I and III are a general feature of transformed and tumour cells. Schwartz *et al.* (1974) demonstrated that the activities of pols I and III increase in mice myelomas, whereas the activity of pol II remains normal. Many different types of transformed cells have been found to overexpress the products of pols I and III, including cells transformed by DNA tumour viruses (e.g. SV40, hepatitis B virus), RNA tumour viruses (e.g. human T-cell leukaemia virus 1) and chemical carcinogens (e.g. 12-O-tetradecanoylphorbol-13-acetate) (Gottesfeld *et al.*, 1996; Larminie *et al.*, 1999; Scott *et al.*, 1983; Wang *et al.*, 1995, 1997; White *et al.*, 1990; Ying *et al.*, 1996; Zhai & Comai, 1999). Although many studies have examined transcriptional outputs by pols I and III in cultured cells, this has yet to be thoroughly addressed in human tumours. So far only two groups have carried out such experiments. Chen *et al.* (1997a) reported that a pol III transcript is abnormally abundant in 19 different types of tumour tissues relative to normal tissue from the same patient. Furthermore, Winter *et al.* (2000) showed that pol III transcripts important in protein synthesis are overexpressed in ovarian carcinomas.

1.4 Genes Transcribed by RNA polymerase III.

Pol III is responsible for approximately 10% of all nuclear transcription, producing a variety of transcripts, many of which have essential functions in cellular metabolism (White, 1998; Willis, 1993). These include tRNA and 5S rRNA, which are required for protein synthesis, 7SL RNA, which is involved in protein translocation, 7SK, which regulates a pol II elongation factor, and the U6, H1 and MRP RNAs, which are involved in post-transcriptional processing. Pol III also synthesises the VA RNAs encoded by adenovirus, which function in the selective translation of viral mRNA. Other class III genes encode transcripts with no known roles; these include the short interspersed repeat (SINE) gene families, such as Alu, which constitute the majority of pol III templates in mammals (reviewed in White, 1998). Generally, class III gene products are untranslated, short transcripts (usually less than 200bp), which are made and exported rapidly from the nucleoplasm. In a HeLa cell ~10 000 transcripts are made by pol III at any moment. These are concentrated in ~2000 sites which contain approximately five active polymerases (Pombo *et al.*, 1999).

1.4.1 5S rRNA Genes.

5S rRNA is approximately 120 nucleotides in length. It is found associated with the large ribosomal subunit providing binding sites for accessory molecules that are necessary for protein synthesis. Genes coding for the 5S rRNA are present in all eukaryotic organisms and are organised in clusters of tandem repeats, although there is evidence that some 5S genes are probably dispersed as single copies (Sørensen & Frederiksen, 1991). The total number of 5S related sequences in the haploid genome

is classically cited as 1700-2000, the majority of which were thought to be pseudogenes and gene variants, with 300-400 true 5S rRNA genes (Sørensen & Frederiksen, 1991). However, only four individual copies of 5S rDNA were identified in the draft human genome sequence, with 520 more distantly related sequences (International Human Genome Consortium, 2001).

1.4.2 tRNA Genes.

Eukaryotic cells contain 50-100 chromatographically distinct tRNA species (Sharp *et al.*, 1985). Each is designed to carry one of the 20 amino acids used for protein synthesis; a tRNA that carries tyrosine is designated $tRNA^{Tyr}$ and so on. The initial transcripts produced from tRNA genes are precursor molecules, which are processed into mature tRNAs, varying in length between 70-90 nucleotides. They have a cloverleaf secondary structure and an L-shaped tertiary structure. Their main role is translating mRNA into protein on the ribosome and their intracellular levels correspond strongly with the codon requirements of mRNA (Garel, 1976). They function in the initiation of peptide synthesis, the elongation of peptide chains, and the attachment of the growing polypeptide chain to the ribosome. $tRNA^{Sec}$ also functions as a carrier molecule upon which selenocysteine is synthesised (Low & Berry, 1996); it also differs from the other tRNAs in the fact that it is encoded by a single copy gene (O'Neill *et al.* 1985). The human haploid genome contains 821 tRNA related loci, 497 of which are tRNA genes, the other 324 are tRNA-derived putative pseudogenes (International Human Genome Consortium, 2001). These are scattered throughout the genome, often in clusters (International Human Genome Consortium, 2001). 28% of the tRNA genes are found in a 4 MB region on chromosome 6. Clustering also occurs

on chromosomes 1 and 7, whereas, chromosomes 22 and Y have no copies of tRNA genes (International Human Genome Consortium, 2001).

1.4.3 Virus Class III Genes.

Several viruses contain short class III transcriptional units within their genomes. The best-characterised example is that of adenovirus, which encodes two small pol III transcripts, called VAI and VAII, that are synthesised at high levels during the late stages of viral infection (Söderlund *et al.* 1976). These genes are approximately 160bp long and are separated from one another by 98bp. VAI is required for efficient expression of the adenovirus genome, whereas deletion of the VAII gene does not have a major effect upon the viral life-cycle (Thimmappaya *et al.* 1982). The VA RNAs act by subverting the host cells translational apparatus, in order to ensure the synthesis of viral proteins (Thimmappaya *et al.* 1982). The genome of Epstein-Barr virus (EBV) also contains two small adjacent genes that are transcribed by pol III.

1.4.4 7SL Genes.

7SL RNA is about 300 nucleotides long, and is a constitutive and indispensable part of the signal recognition particle. This aids in the secretion of newly formed polypeptides through the membranes of the endoplasmic reticulum (Walter & Blobel, 1982). 7SL is flanked on both ends by regions homologous to the highly repeated Alu family, and contains a central S-sequence region of 140 nucleotides of non-Alu DNA. In the human genome, there are over 770 sequences related to this S-sequence, the

majority of which are pseudogenes that are truncated at one or both ends. Only three or four represent functional 7SL genes (International Human Genome Consortium, 2001).

1.4.5 SINEs.

A variety of short interspersed elements (SINEs) constitute quantitatively important classes of pol III template in higher organisms (Jelinek & Schid, 1982; Singer, 1982). The functional role of these templates has not been unequivocally demonstrated, despite a broad range of speculations (reviewed by Howard & Sakamoto, 1990). These genes are commonly found in clusters, often in tandem arrays, and are spread out on all of the chromosomes (Bennett *et al.*, 1984). The major SINE in primates is the Alu family. Alu genes consist of two imperfect repeats separated by an 18bp spacer (Rubin *et al.*, 1980; Deininger *et al.*, 1981). The Alu consensus sequence is 282 nucleotides long, but transcription continues into a downstream A-rich region to produce poly(A)+ RNAs with an average length of ~400bp (Weiner *et al.*, 1986). There are over one million copies of the Alu gene in the haploid human genome (International Human Genome Consortium, 2001). In rodent species the most abundant SINEs are the B1 and B2 genes (Bennett *et al.*, 1984); the latter gene is specific to these mammals (Bennett *et al.*, 1984; Rogers, 1985). B1 genes are approximately 80% homologous to human Alu genes, but with only one of the two repeats. They are ~130bp long and are present in about 100,000 copies per haploid mouse genome (Bennett *et al.*, 1984; Krayev *et al.*, 1980). B2 genes are ~180bp long, with ~80,000 copies per haploid mouse genome (Bennett *et al.*, 1984; Rogers, 1985). Several SINE families, such as B2, appear to have evolved from tRNA genes, and so

can be regarded as amplified tRNA pseudogenes (Daniels & Deininger, 1985). It is thought that B1 and Alu families have evolved from the 7SL gene (Mighell *et al.*, 1997; Ullu & Tschudi, 1984).

1.4.6 RNase MRP Genes.

The site-specific endonuclease RNase MRP contains a 265 nucleotide RNA (Topper & Clayton, 1990). It has a major nuclear function in the late stage processing of rRNA (Clayton, 1994; Lygerou *et al.* 1996) and it also functions in mitochondria to process RNA primers for DNA synthesis. In humans, a single copy gene has been identified that encodes MRP RNA, which is located on chromosome 9 (Hsieh *et al.* 1990). However, several pseudogenes also exist.

1.4.7 U6 snRNA Genes.

U6 is an abundant, small nuclear RNA (snRNA) species, which is capped at its 5' end (Epstein *et al.* 1980). It forms a series of complexes with four other uridylic acid-rich RNAs and protein factors, which carry out splicing of pre-mRNA. These are present at approximately ten million copies per mammalian cell, and are involved in the splicing of pre-mRNA (Mattaj *et al.* 1993). It is the smallest of these snRNAs, 106 nucleotides in length (Epstein *et al.* 1980), and is the only one to be transcribed by pol III (Reddy *et al.* 1987); the others are pol II transcripts. There were 44 copies of the U6 sequence identified within the draft haploid human genome, and over 1100 related

pseudogenes (International Human Genome Consortium, 2001). These exist as dispersed middle repetitive DNA (Hayashi, 1981).

1.4.8 7SK Genes.

The 7SK gene encodes a snRNA transcript of 330 nucleotides in length. It functions as a negative regulator of the pol II elongation factor P-TEFb (Nguyen *et al.*, 2001; Yang *et al.*, 2001). Mammalian 7SK RNA has an abundance of approximately 200,000 copies per cell, and is evolutionarily conserved (Zieve *et al.*, 1977). The 50bp immediately flanking the start of transcription is homologous to 7SL and U6 RNA (Murphy *et al.*, 1986). The human genome contains a large family of truncated 7SK pseudogenes, but only one full-length 7SK gene (International Human Genome Consortium, 2001; Kruger, 1987; Murphy, 1984).

1.5 Class III Gene Promoters.

An unusual feature of promoters of most genes transcribed by pol III is that they include internal control regions (ICRs) downstream of the transcriptional start site, within the transcribed region. These structures are generally discontinuous, composed of essential blocks separated by non-essential regions. There are three general types of pol III promoters.

1.5.1 Type I Promoters.

Type I promoters, found in 5S rRNA genes, consist of three sequence elements; a 5' A-block which is well conserved, an intermediate element, and a 3' C-block (Pieler *et al.*, 1987). These elements span a region of approximately 50bp beginning at position +45.

1.5.2 Type II Promoters.

Type II promoters are the most common arrangement; examples include tRNA and the VA RNA genes. These promoters are identified by highly conserved A and B block elements. The A block is positioned close to the transcription start site (usually about 10-20bp) (Galli *et al.*, 1981). This reflects its role in start-site selection. The distance of the B block downstream of the A block is extremely variable, with an optimal spacing for transcription being 30-60bp (Baker *et al.*, 1987; Fabrizio *et al.*, 1987).

1.5.3 Type III Promoters.

Type III promoters, like the human U6, 7SK and RNase MRP genes, reside exclusively upstream of the coding sequence (Das *et al.*, 1988; Kunkel & Pederson, 1989; Lobo & Hernandez, 1989). These contain a TATA box located near position -30, and a proximal sequence element (PSE) at around position –60. These elements constitute a basal promoter, which is subject to activation by a variety of factors that bind to distal sequence elements (DSE).

1.5.4 Other Promoters.

Several genes have promoters that do not conform to the aforementioned groups. Among these is the 7SL gene. Transcription of this gene is dependent on a novel intragenic element, a DSE element and an ATF-binding site at position –50. The intragenic element is located proximal to the start site and overlaps, but is distinct from, a sequence that resembles the A block (Bredow *et al.* 1990).

1.6 The Transcription Machinery of Pol III.

Pol III alone has little specificity for particular DNA sequences. Its recruitment to specific genes requires the presence of transcription factors. The combination of these factors utilized during transcription initiation is dependent on promoter structure.

1.6.1 Transcription Complex Assembly at Type II Promoters.

It is transcription factor IIIC (TFIIIC) that initially recognises the promoter of type II genes. This is a large multisubunit and multifunctional complex, that in humans can be resolved into two components, called TFIIIC1 and TFIIIC2. TFIIIC1, which is not yet well characterised, contains at least four subunits with molecular masses of 70, 50, 45, and 40 kDa (Wang & Roeder, 1998). Purified TFIIIC2 has been shown to consist

of five polypeptides of 220, 110, 102, 90 and 63 kDa. These subunits form a stable complex.

During transcription complex assembly, TFIIIC2 binds preferentially to the B block region with high affinity (Yoshinaga *et al* 1987, 1989). This occurs through the largest subunit, TFIIIC220 (L'Etoile *et al.*, 1994; Lagna *et al.*, 1994). Weak interactions also occur with the A block through the smallest subunit, TFIIIC63 (Carrey *et al*, 1986; Hseih *et al.*, 1999a). The TFIIIC90 subunit provides key interactions that hold the transcription factor together (Hseih *et al.*, 1999a). This initial DNA-TFIIIC2 interaction then allows the recruitment of TFIIIC1 (Dean & Berk, 1988), which enhances and extends the footprint produced by TFIIIC2 (Yoshinaga *et al,* 1987; Wang & Roeder, 1996; Oettel *et al,* 1997).

TFIIIC2 can be isolated in both active (TFIIIC2a) and inactive (TFIIIC2b) forms. These promoter complexes were shown to be chromatographically distinct by Hoeffler *et al.* (1988). Silver staining revealed that the 110 kDa subunit was absent in TFIIIC2b, rendering it transcriptionally inactive; instead an additional band of 77 kDa was present (Kovelman & Roeder, 1992; Sinn *et al,* 1995). It has been shown that antibodies against TFIIIC110 do not recognise the 77 kDa component of TFIIIC2b. This suggests that the 77 kDa polypeptide is not a proteolytic product of TFIIIC110, although it is possible that the epitopes recognized by the antibody have been degraded.

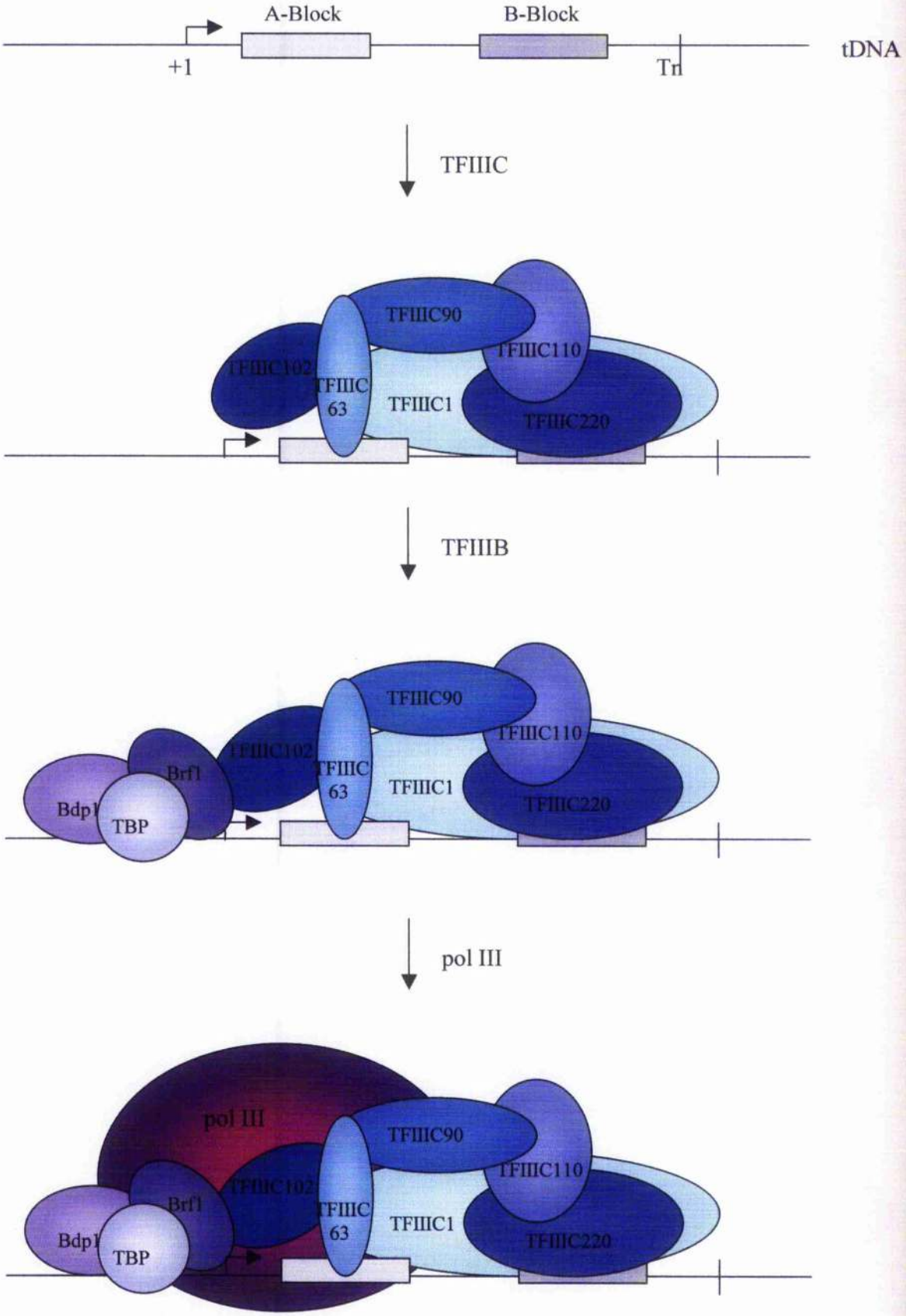
The primary function of TFIIIC is to recruit a second transcription factor TFIIIB, which is regarded as the pivotal initiation factor of the pol III system. TFIIIB is

Figure 1.1

A model for transcription initiation at a type II promoter.

Diagram indicating the order of interaction of transcription factors and pol III with a typical type II promoter such as that of a tRNA gene. The site of transcription initiation is depicted by +1 and the site of termination is indicated by Tn.

Type II Promoter



composed of three polypeptides (Kassavetis *et al.*, 1990), one of which is the TATA-box binding protein (TBP). The largest of the TBP-associated factors is the B double prime 1 (Bdp1) protein, which displays little homology to other proteins. The other component, because of its homology to TFIIB in its N-terminal half, is referred to as TFIIB-related factor 1 (Brf1). It has been shown that Brf1 is tightly associated with TBP, whereas Bdp1 is weakly associated with the TBP-Brf1 complex (Kassavetis *et al.*, 1991; Wang & Roeder, 1995).

There is a network of protein-protein interactions between TFIIIC and TFIIIB. Three such TFIIIC2 associations have been identified; TFIIIC102 interacts with Brf1 and TBP (Hseieh *et al.*, 1999b), TFIIIC90 interacts with Brf1 (Hsieh *et al.*, 1999a), and TFIIIC63 interacts with both Brf1 and TBP (Hsieh *et al.*, 1999b). Binding of TFIIIB just upstream of the transcription start site in turn allows the recruitment of pol III to the promoter, where it is positioned over the initiation region so that transcription can commence (Kassavetis *et al.*, 2001). This occurs mainly through protein-protein interactions with the Brf1 and TBP subunits of TFIIIB (Khoo *et al.*, 1994; Wang & Roeder, 1997; Werner *et al.*, 1993) (Figure 1.1).

1.6.2 Transcription Complex Assembly at Type I Promoters.

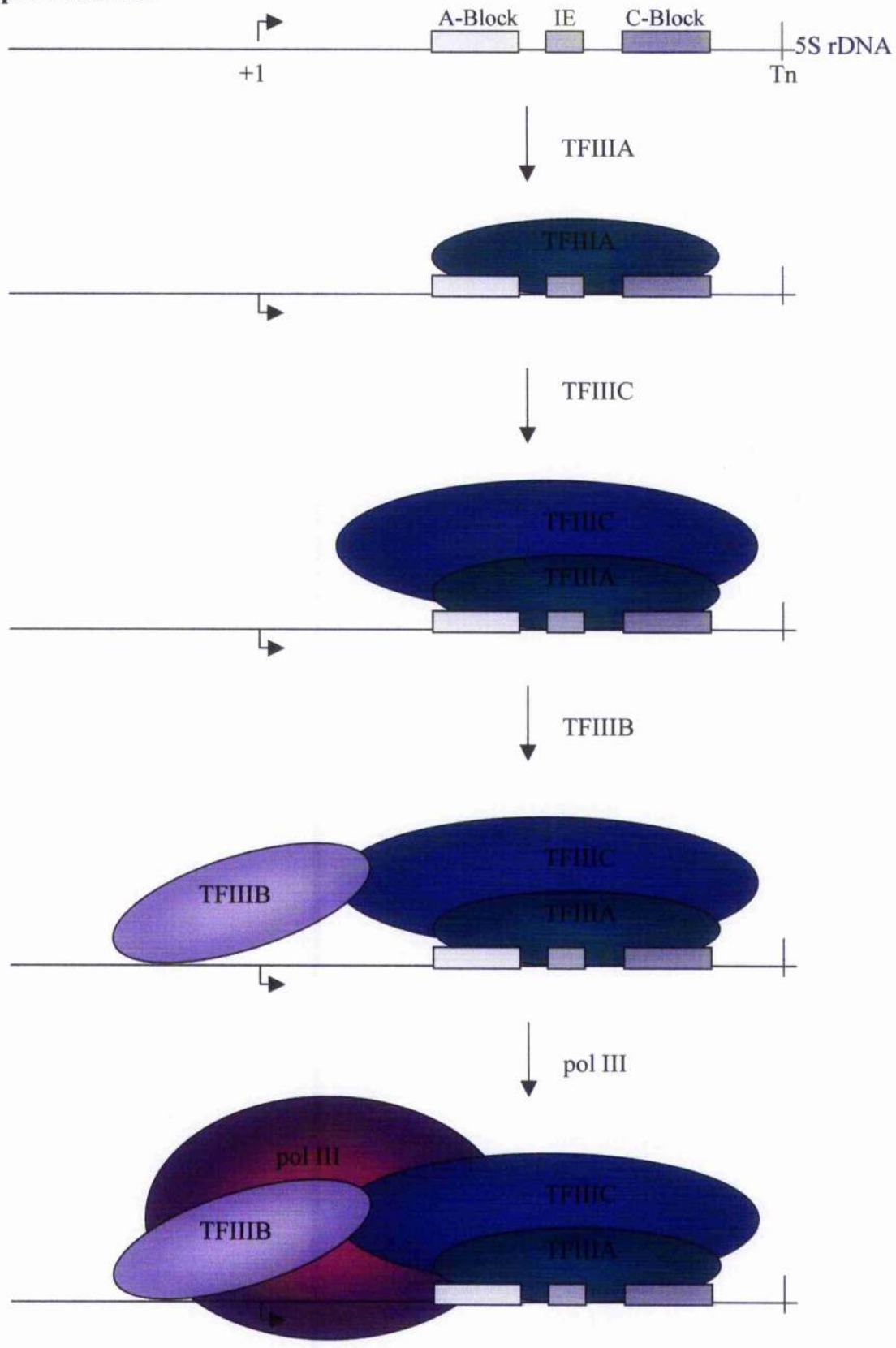
Although TFIIIC binds directly to the promoter sequences that are present within the coding regions of most pol III templates (Lassar *et al.*, 1983), an additional gene-specific factor TFIIIA is required by 5S rRNA genes to provide a platform for TFIIIC (Lassar *et al.*, 1983). TFIIIA is the founding member of the C_2H_2 zinc finger family of DNA-binding proteins, since most of its 344 residues are taken up by nine tandem,

Figure 1.2

A model for transcription initiation at a type I promoter.

Diagram indicating the order of interaction of transcription factors and pol III with the promoter of a 5S rRNA gene. The site of transcription initiation is depicted by +1 and the site of termination is indicated by Tn.

Type I Promoter



zinc-dependent DNA-binding domains (Miller *et al.*, 1985). These bind extensively to >50bp of 5S rDNA. The C block is recognised by the N-terminal three fingers (fingers 1-3), which contribute ~95% of the total binding energy of full-length TFIIIA; these fingers wrap smoothly around the major groove in a manner that is typical for this class of DNA-binding domain (Clemens *et al.*, 1992; Foster *et al.*, 1997; Nolte *et al.*, 1998). Fingers 7-9 are thought to contact the A block in a similar fashion, but with lower affinity (Clemens *et al.*, 1992). The middle three fingers adopt a completely different configuration in order to span the interblock DNA, which is twice as long as the regions bound by fingers 1-3 or 7-9 (Nolte *et al.*, 1998).

Surprisingly little is known about how TFIIIA recruits TFIIIC to the DNA. Only once TFIIIA and TFIIIC are both bound can TFIIIB be recruited onto the 5S rRNA gene (Bieker *et al.*, 1985; Carey *et al.*, 1986a; Setzer & Brown, 1985). It has been observed that TFIIIB recruitment to the promoter is the rate-limiting step (Bieker *et al.*, 1985). Pol III is then assembled onto the preformed TFIIIB/TFIIIC/TFIIIA/5S gene complex (Bieker *et al.*, 1985; Carey *et al.*, 1986a; Setzer & Brown, 1985) (Figure 1.2).

1.6.3 Transcription Complex Assembly at Type III Promoters.

The type III promoters associated with vertebrate 7SK and some U6 snRNA genes have distinct factor requirements from genes with type I or II promoters (Mital *et al*, 1996; Yoon *et al.*, 1995). The lack of an ICR obviates the need for TFIIIA or TFIIIC2 (Lagna *et al.*, 1994; Oettel *et al.*, 1997). Instead, other factors are recruited to the external upstream sequence elements in order to initiate transcription.

The DSE of these promoters is recognised by Oct-1. This factor can activate transcription of the 7SK gene both *in vitro* and *in vivo* (Murphy, 1997; Murphy *et al.*, 1989). Downstream from this, the PSE is bound by the five subunit PSE-binding transcription factor (PTF) (Murphy *et al.*, 1992; Sadowski *et al.*, 1993). Binding of PTF and Oct-1 occurs cooperatively (Mittal *et al.*, 1996; Murphy *et al.*, 1992). The relatively weak binding of Oct-1 is stabilized by interaction with DNA-bound PTF. In turn, PTF binding is strongly dependent on DNA-bound Oct-1 (Murphy, 1997). The poor DNA binding of PTF on its own is due to self-repression, as the PSE-binding domain is substantially occluded in the complete complex (Mittal *et al.*, 1996). However, through interaction with Oct-1 the occluding flap is removed enabling the partial complex to bind to the DNA more effectively (Ford *et al.*, 1998; Mittal *et al.*, 1996). Efficient binding of PTF, through interaction with Oct-1 and activation of transcription *in vitro*, occurs only when Oct-1 binding sites are close to the PSE (Murphy *et al.*, 1992; Stunkel *et al.*, 1997). DNase I digestion patterns have suggested that a nucleosome is positioned between the DSE and the PSE of class III genes *in vivo*, bringing these elements into juxtaposition, thereby enhancing physical interaction between factors binding to them (Boyd *et al.*, 2000).

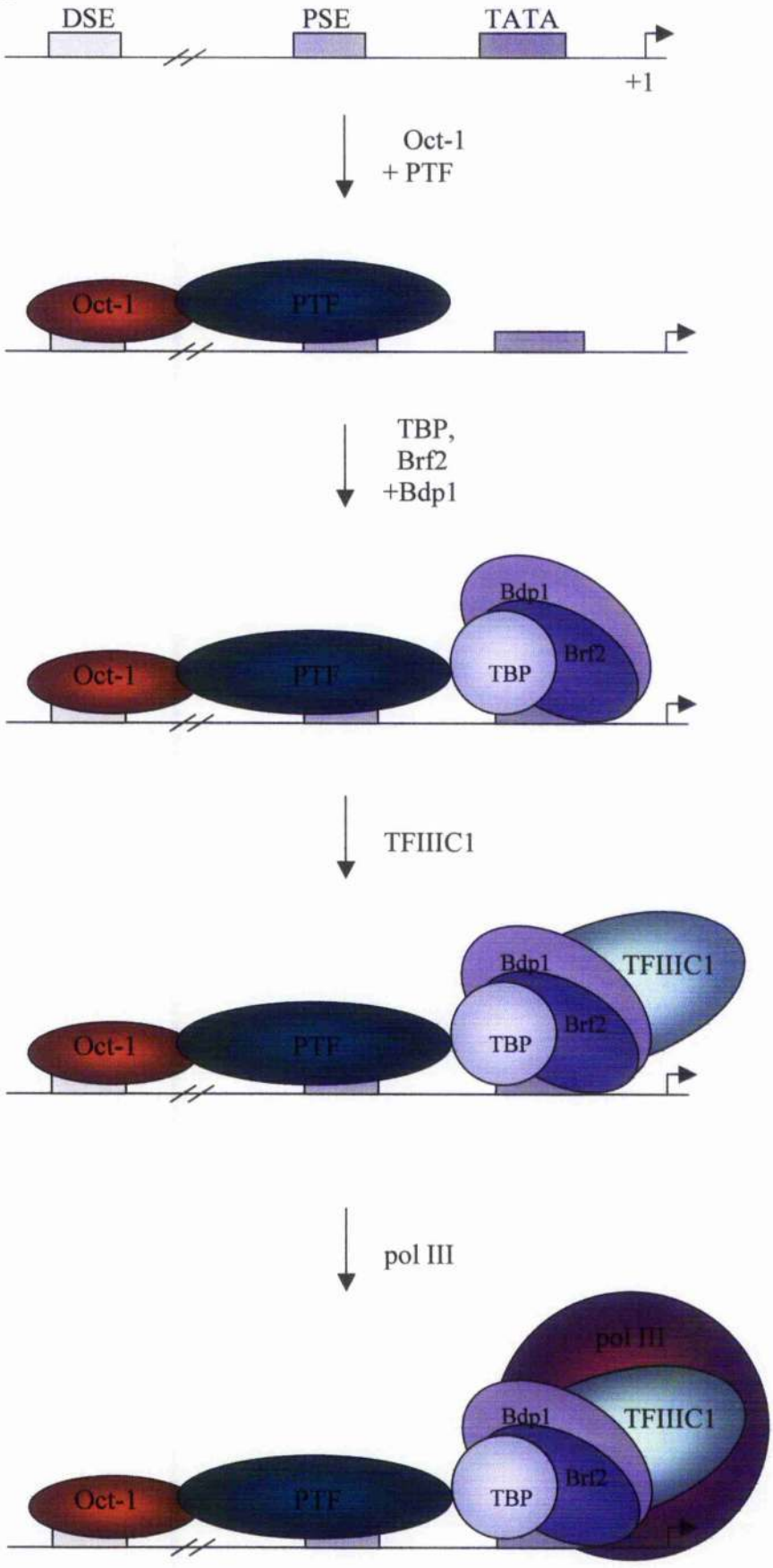
In addition to these interactions, TBP binds to the TATA box (Mittal & Hernandez, 1997). The N-terminal domain of TBP also potentiates binding of PTF to the PSE (Mittal & Hernandez, 1997). This binding occurs cooperatively with PTF, as TBP binds poorly to DNA on its own (Mittal & Hernandez, 1997). TBP also interacts with another factor, Brf2, which is related to both Brf1 and TFIIB in its N-terminal region (Schramm *et al.*, 2000; Teichmann *et al.*, 2000). Recruitment of Brf2 to the TATA box is TBP-dependent. Brf2 in turn stabilises TBP on TATA-containing templates,

Figure 1.3

A model for transcription initiation at a type III promoter.

Diagram indicating the order of interaction of transcription factors and pol III with a type III promoter such as that of a 7SK gene. The site of transcription initiation is depicted by +1.

Type III Promoter



and extends the TBP footprint both upstream and downstream of the TATA box (Čabart & Murphy, 2001). Once the Brf2-TBP-DNA subcomplex has assembled, the transcription factor Bdp1 is recruited (Čabart & Murphy, 2002). The N-terminal region of Brf2, containing a zinc ribbon, inhibits the direct association of Brf2 with Bdp1. This negative mechanism is deactivated by TBP through protein-protein contacts with both Bdp1 and Brf2 (Čabart & Murphy, 2002). In addition to the TATA box, a GC-rich sequence (the BURE) has been identified downstream from this promoter structure. This sequence, depending on the strength of the TATA box, can either enhance Brf2 binding to the TBP-DNA complex, or Bdp1 association with the Brf2-TBP-DNA complex, and substantially stimulate pol III transcription (Čabart & Murphy, 2002).

Although transcription at type I and II promoters requires both TFIIIC1 and TFIIIC2, TFIIIC1 alone is required at type III promoters (Yoon *et al.*, 1995). It remains to be determined how TFIIIC1 is recruited, but this may be a late step in complex assembly on type III promoters (Paule & White, 2000). The combination of binding by these transcription factors ultimately leads to the recruitment of pol III (Figure 1.3).

1.7 Genes Transcribed by RNA polymerase I.

Pol I transcribes a single RNA species, the large ribosomal RNA precursor molecule (pre-rRNA). Although it synthesises only one essential product, pol I may be responsible for as much as 70% of all nuclear transcription, and in an actively growing cell rRNA may constitute 80% of the total steady-state RNA (White, 2001). This high level of production from class I genes is necessary in order to make the

several million new ribosomes that are needed per generation to maintain protein biosynthetic capacity.

1.7.1 Pre-rRNA Genes.

During ribosome biogenesis, the large polycistronic transcript pre-rRNA is processed into 5.8S, 18S and 28S rRNAs by a series of specific endonucleolytic reactions that are mediated by U3 RNA, a small nucleolar RNA species (Sharma & Tollervey, 1999). 18S rRNA is approximately 2000 nucleotides long and is a component of the small ribosomal subunit, whereas 5.8S (~160 nucleotides) and 28S rRNA (~4000 nucleotides) are components of the large ribosomal subunit. During this processing, the mature rRNA sequences undergo extensive covalent nucleotide modification. In addition, the rRNAs must assemble with the 80 ribosomal proteins and the 5S rRNA. In human cells, there are about 200 highly conserved rRNA genes per haploid genome. These are found organised into five clusters of tandem head-to-tail repeats, and are located on five different chromosomes (Long & Dawid, 1980). A very high density of pol I is found at the active rRNA genes, with approximately one enzyme molecule every 100bp, and these sustain a rapid elongation rate (Miller & Beatty, 1969).

1.8 Class I Gene Promoters.

The DNA intervening between transcribed units, known as the intergenic spacer (IGS), contains sequence elements that mediate rRNA expression. In contrast to class

III gene promoters, the primary sequences of rRNA promoters are not well conserved between species. Despite this species specificity, the general layout of promoter elements is highly conserved from yeast to humans, containing two sequence elements (Moss & Stefanovsky, 2002). These are the upstream control element (UCE), which serves to modulate efficiency of transcription, and the core, which is essential for transcription initiation and contains the conserved AT-rich ribosomal initiator (Perna *et al.*, 1992; Radebaugh *et al.*, 1997). In humans, the 40bp core region is positioned overlapping the transcription start site, and the UCE is located between nucleotides –234 to –131 (Paule, 1998). The IGS of most species also contains other promoter elements, such as enhancers and the proximal terminator. This transcriptional terminator is located just upstream of the UCE, and is able to significantly stimulate transcription (Paule & White, 2000).

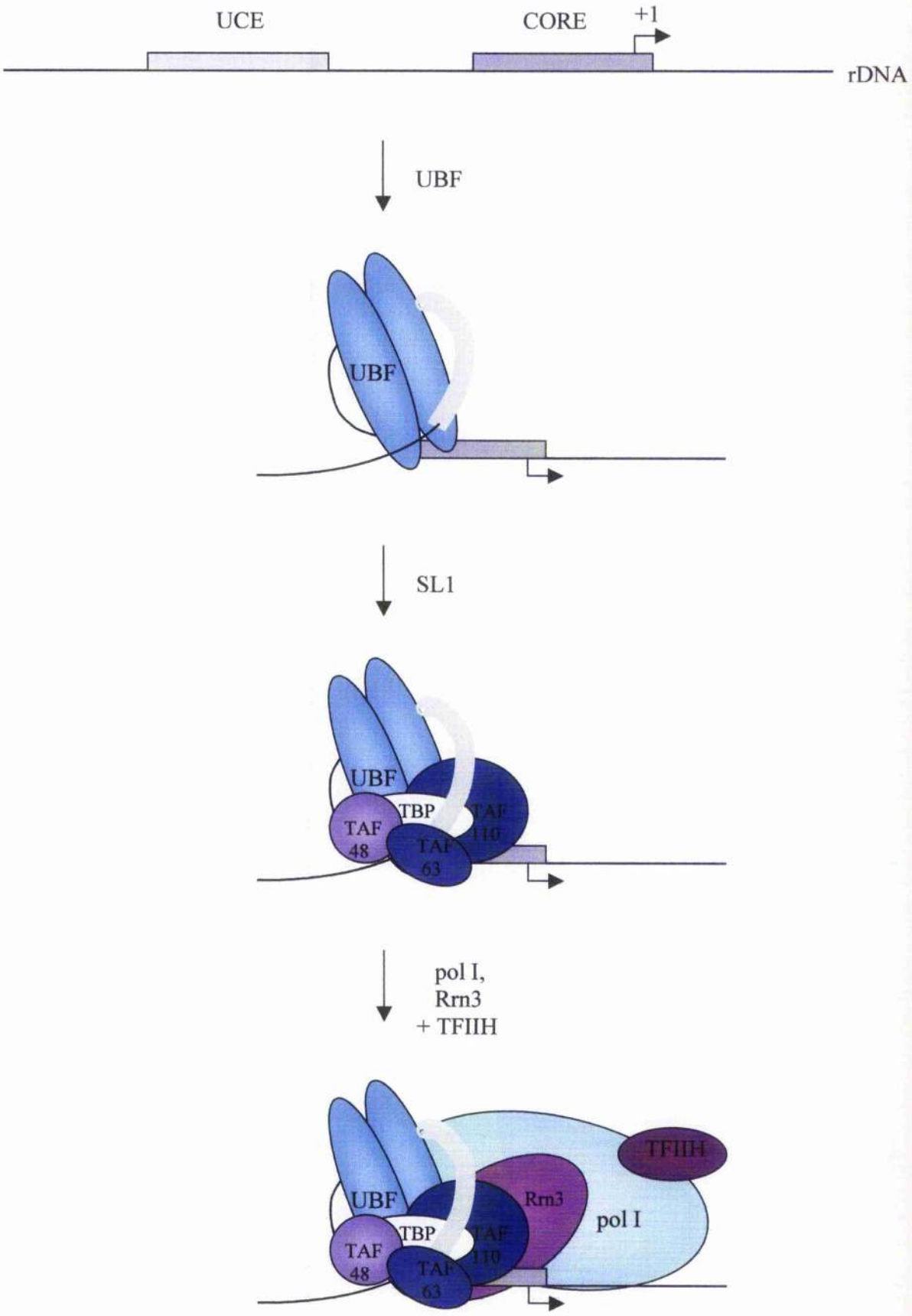
1.9 The Transcription Machinery of Pol I.

Template recognition in mammals is achieved by the upstream binding factor (UBF). This is a modular polypeptide of 97 or 95 kDa, the smaller splice variant being inactive (Grummt, 1999). It contains an N-terminal dimerization domain, multiple high mobility group (HMG) DNA-binding domains, and a C-terminal acidic tail (Reeder *et al.*, 1999). UBF functions as a dimer interacting with sequences in the UCE and core elements of the ribosomal promoter (Zomerdijk & Tijan, 1998). To accommodate this binding, the HMG boxes in this transcription factor wrap ~180bp of DNA around itself in a single turn of ~360° to provide a three-dimensional structure (Bazett-Jones *et al.*, 1994; Stefanovsky *et al.*, 1996). This higher-order nucleoprotein structure, called the enhancesome, necessitates major chromatin

Figure 1.4

A model for transcription initiation at a class I gene promoter.

Diagram indicating the order of interaction of transcription factors and pol I at a human ribosomal promoter. The site of transcription initiation is depicted by +1.


UCE
CORE
+1
rDNA
UBF
UBF
SL1
UBF
TBP
TAF 110
TAF 48
TAF 63
pol I,
Rrn3
+ TFIIH
TFIIH
UBF
Rrn3
pol I
TBP
TAF 110
TAF 48
TAF 63

remodelling, and also recruits a promoter selectivity factor. In humans this is referred to as SL1. These two transcription factors interact cooperatively (Bell *et al.*, 1988).

Isolated SL1 contains TBP, and three tightly bound associated factor (TAF) subunits, TAF_I48, TAF_I63, and TAF_I110 (Comai *et al.*, 1992), which are specific for pol I. The C-terminal of UBF contacts TBP and TAF_I48 in SL1, whereas TAF_I63 and TAF_I110 recognize and bind species-specific promoter sequences just upstream of the start site (reviewed by Moss & Stefanovsky, 2002; Paule & White, 2000). These latter two subunits carry binding sites for Rrn3. This is a polypeptide that has an essential function in linking pol I to SL1 at the rDNA promoter (Miller *et al.*, 2001). Additional polymerase-associated factors are also recruited along with pol I (Moss & Stefanovsky, 2002). One of these is TFIIH, a basal pol II transcription initiation factor, which is also required for a postinitiation step in pol I transcription (Iben *et al.*, 2002). Once pol I and its associated factors have been assembled at the promoter, transcription can commence (Figure 1.4).

1.10 Chromatin Structure.

The chromatin structure of the genes transcribed by both pols I and III can be a major determinant of their transcriptional activity (reviewed by Paule, 1998; White, 1998). The basic unit of chromatin consists of approximately 200bp of DNA and is termed a nucleosome. It comprises a disc-shape particle with a diameter of about 11nm and a variable length of linker DNA connecting adjacent subunits. In this particle, 146bp of DNA is wrapped twice around a histone octamer containing two copies each of the core histones H2A, H2B, H3 and H4 (Burlingame et al., 1985; Luger *et al.*, 1997;

Pruss *et al.*, 1995; Richmond *et al.*, 1984). The C-terminal domains of these core histones make substantial protein-DNA contacts (Hayes *et al.*, 1994; Luger *et al.*, 1997; Pruss *et al.*, 1995), while the N-terminal tails protrude outside the nucleosome (Luger *et al.*, 1997; Wolffe & Pruss, 1996). The two superhelical turns are fixed from the outside by histone H1 (Hayes & Wolffe, 1993; Pruss *et al.*, 1995). This linker histone binds at the point where DNA enters and exits the subunit (Crane-Robinson, 1997). The arrays of nucleosomes fold together, forming heavily compacted 30 nm fiber structures, which severely limit the accessibility of genes to transcription factors (Wolffe, 1990). Therefore, decondensation of such nucleoprotein complexes must occur to accommodate the transcription machinery.

The histone tails protruding from the nucleosome can be modified by histone acetyltransferases (HATs) and histone deacetylases (HDACs) during the cell cycle. Acetylation is associated with destabilization of nucleosomes (Grunstein, 1997), enabling the transcription apparatus to gain access to the DNA (Bannister & Miska 2000; Bronwell & Allis, 1996; Tse *et al.*, 1998; Ura *et al.*, 1997). While HDACs remove the acetyl groups, allowing condensation of the nucleosome structure (Kuo & Allis, 1998). The actions of these enzymes will be discussed in greater detail in chapter six.

Nucleosomes have been shown to block the DNA binding of a number of transcription factors by obscuring their recognition sites. For example, if a nucleosome is positioned over the C block of the 5S rRNA promoter, TFIIIA can no longer bind and 5S rRNA synthesis is repressed (Gottesfeld, 1987; Hayes *et al.*, 1991; Lee *et al.*, 1993). However, if the nucleosome is located further upstream, leaving the

C block open, TFIIIA can still gain access and transcription can proceed, but at a reduced rate (Almouzni *et al.*, 1991; Clark & Wolffe, 1991; Hansen & Wolffe, 1994; Tremethick *et al.*, 1990).

Deposition of histones onto naked DNA is generally accompanied by a substantial decrease in transcriptional activity. This ability of histones to inhibit both initiation and elongation is directly proportional to the density of the nucleosomes (Hansen & Wolffe, 1992). Class III genes that have been assembled into highly condensed structures are incapable of supporting significant levels of transcription (Hansen & Wolffe, 1992). In murine chromatin, 95% or more of transcriptionally competent B2 genes are subject to histone H1–dependent repression (Carey & Singh, 1988). Furthermore, it has been demonstrated that transcription of B2 genes increases by ~17 fold upon H1 depletion (Russanova *et al.*, 1995).

In the vast majority of eukaryotic cells, only a small subset of rRNA genes are selected for transcription. This results in the existence of two distinct types of rRNA gene organisation; a nucleosomal type corresponding to the transcriptionally inactive gene copies, and a non-nucleosomal type, representing the active copies (Paule, 1998). These transcriptionally active genes and their regulatory sequences are organised into more open chromatin conformations (Elgin, 1988; Mathias *et al.*, 1980; Weisbrod, 1982).

1.11 Coordination of Transcription by Pols I and III.

Protein constitutes most of a cell's dry mass; therefore, the rapid growth that occurs during tumour development requires high rates of protein synthesis. Indeed, Baxter & Stanners (1978) demonstrated that the rate of protein accumulation determines growth rate. Furthermore, it has been observed that cells withdraw from the cell cycle and quiesce when the rate of protein synthesis is reduced by 50% (Brooks, 1977; Ronning et al., 1981). Since rRNA and tRNA are essential for protein synthesis, elevated rates of biosynthesis would not be possible if the levels of transcription by pols I and III were limiting.

Actively proliferating eukaryotic cells use ~80 % of their energy in producing ribosomes and other components of the protein synthetic apparatus (Volarevic *et al.*, 2000). Therefore, transcription by pols I and III is often coregulated so that wastage is limited. This is especially important for the production of 5S rRNA, which is required in equimolar quantities with the other rRNA transcripts. For example, the outputs of mammalian pols I and III increase in parallel following serum stimulation, and fluctuate together during passage through the cell cycle (Klein & Grummt, 1999; Mauck & Green, 1974; White *et al.*, 1995a).

A combination of shared and distinct strategies may be involved in controlling pols I and III during the different stages of the cell cycle. For example, a number of phosphatases and kinases control the activity of specific transcription factors. As SL1 and TFIIIB pass through mitosis, both undergo specific inactivation through phosphorylation. This can be mediated by the mitotic kinase cdc2/cyclin B (Gottesfeld *et al.*, 1994; Heix *et al.*, 1998; Kuhn *et al.*, 1998). The inactivation of SL1

reflects the phosphorylation of TAF_I110, which compromises binding of UBF (Heix *et al.*, 1998). Similarly, TFIIIB repression is due to a specific loss of TAF activity (Lerosche *et al.*, 1996; White *et al.*, 1995b), which correlates with the hyperphosphorylation of Brf1. Since TFIID has also been shown to be phosphorylated and inhibited at mitosis (Segil *et al.*, 1996), it seems that the inactivation of TBP-containing complexes is a general mechanism for transcriptional control during M phase.

Protein kinases such as CK2 also regulate the output of pols I and III. This enzyme forms part of the Wnt signalling pathway (Song *et al.*, 2000: Willert *et al.*, 1997). Increases in the level, nuclear localisation and/or activity of CK2 are associated with cell growth and proliferation (Belenguer *et al.*, 1989; Bosc *et al.*, 1999; Orlandin *et al.*, 1998). Moreover, it has been demonstrated that CK2 induces the synthesis of rRNA and tRNA, a function that may be important for its growth promoting capacity (Belenguer *et al.*, 1989; Ghavidel & Schultz, 1997,2001; Hockman & Schultz, 1996; Johnston *et al.*, 2002). In the pol III system, CK2 regulates preinitiation complex assembly, as phosphorylation of TFIIIB by this enzyme appears to be necessary for efficient interaction with TFIIIC both *in vitro* and in fibroblasts (Johnston *et al.*, 2002). In rats, CK2 has been shown to stably interact with pol I itself, phosphorylating its largest subunit (Hannan *et al.*, 1998). In addition, the C-terminal tail of UBF is heavily phosphorylated by CK2 in mammals (Voit & Grummt, 2001; Voit *et al.*, 1992,1995). Removal of this region or phosphatase treatment of intact UBF blocks transcriptional activation (O'Mahony *et al.*, 1992; Tuan *et al.*, 1999; Voit *et al.*, 1992). Indeed, UBF phosphorylation regulates the ability of its C-terminal tail to bind and recruit SL1 (Kihm *et al.*, 1998; Tuan *et al.*, 1999). Thus, phosphorylation controls

promoter recruitment of the TBP-containing initiation factor (SL1 or TFIIIB) in both the pol I and pol III systems.

Other kinases are also involved in regulating assembly of the pol I and III preinitiation complexes. UBF can be phosphorylated at Ser484 by cdk4/cyclin D1 and cdk2/cyclin E, both *in vitro* and in NIH3T3 cells (Voit *et al.*, 1999). At the end of G1 phase, UBF also gets phosphorylated at Ser388 by cdk2/cyclin E and cdk2/cyclin A (Voit & Grummt, 2001). It has been demonstrated that the phosphorylation status of UBF at this residue regulates its interaction with pol I (Voit & Grummt, 2001).

1.12 Transcriptional Repression by Tumour Suppressor Genes.

In healthy cells, pols I and III are subject to repression by the tumour suppressors RB and p53 (Cairns & White, 1998; Cavanaugh *et al.*, 1995; Chesnokov *et al.*, 1996; White *et al.*, 1996; Zhai & Comai, 2000). The importance of controlling their output is clearly indicated by the fact that they are targeted by two major and unrelated tumour suppressors. Since the function of p53 and/or RB is compromised in most human cancers, it seems likely that pols I and III will be released from repression in the majority of tumours.

1.12.1 Repression of Pol III Transcription by RB.

The retinoblastoma protein (RB) is a 110 kDa nuclear phosphoprotein that is believed to be constitutively expressed by most normal cycling cells in mammals. It functions

as a tumour suppressor and growth inhibitor by regulating the activity of several key transcription factors (reviewed by Kouzarides, 1995; Weinberg, 1995a; Whyte, 1995).

Two additional proteins have been classified as RB-like proteins, p107 and p130. These share 30-35% identity at the amino acid level (reviewed in Grana *et al.*, 1998; Herwig & Strauss, 1997; Mulligan & Jacks, 1998). As most of their homology lies within a bipartite region called the pocket domain, these three proteins are referred to as the pocket proteins. This pocket is composed of an A domain (amino acids 379 – 572) and a B domain (amino acids 646 – 772), which are highly conserved between species and related proteins, and separated by a non-conserved spacer sequence (Herwig & Strauss, 1997; Knudson & Wang, 1997). A number of common target proteins have been found to interact with the pocket domains of all three proteins, including the members of the E2F family of cellular transcription factors and the oncoprotein products of several DNA tumour viruses (reviewed in Dyson, 1998; Herwig & Strauss, 1997; Mulligan & Jacks, 1998; Taya, 1997).

RB is able to inhibit transcription at all of the pol III templates (White *et al.*, 1996; Larminie *et al.*, 1997). Overexpression of RB represses transcription of pol III reporters in transfected cells, whereas control pol II promoters are unaffected (White *et al.*, 1996). Endogenous levels of RB have also been shown to control pol III *in vivo*. Analysis of the RB-deficient human osteosarcoma cell line SAOS2 and primary fibroblasts from RB-deficient mice demonstrates elevated levels of pol III activity in the absence of functional RB protein (White *et al.*, 1996).

RB appears to regulate pol III transcription by targeting TFIIIB (Chu *et al.*, 1997; Larminie *et al.*, 1997; Sutcliffe *et al.*, 1999,2000). Recombinant RB binds to TFIIIB *in vitro* and represses its activity (Chu *et al.*, 1997; Larminie *et al.*, 1997). Furthermore, immunoprecipitation and cofractionation experiments have indicated that a population of endogenous RB molecules stably associates with TFIIIB at physiological concentrations (Larminie *et al.*, 1997; Sutcliffe *et al.*, 2000). This interaction is diminished or abolished in SAOS2 cells, which contain only truncated RB (Larminie *et al.*, 1997). In addition, the activity of TFIIIB was shown to be specifically elevated in primary fibroblasts from RB-deficient mice (Larminie *et al.*, 1997). RB exerts this repression on pol III transcription by disrupting key interactions between TFIIIB and other components of the basal pol III transcription apparatus. Sutcliffe *et al.* (2000) showed that the binding of TFIIIB to TFIIIC2 can be blocked by RB. In addition, RB disrupts the interaction between TFIIIB and pol III (Figure 1.5).

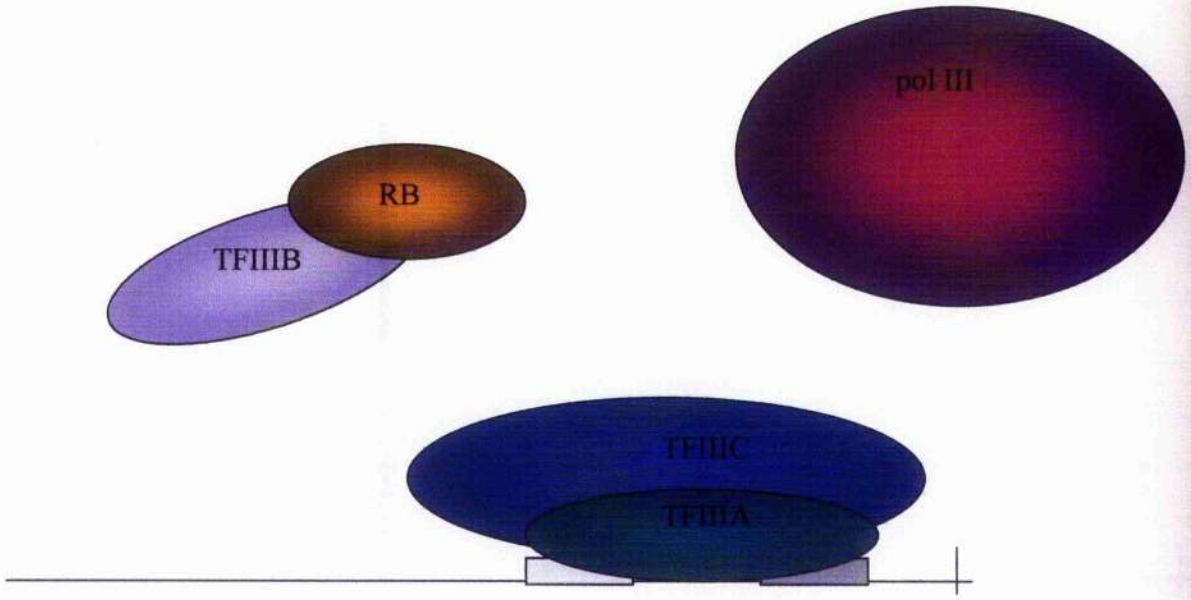
TFIIIB is targeted for repression not only by RB but also by its relatives p107 and p130 (Sutcliffe *et al.*, 1999). This has been demonstrated by a combination of *in vitro* and *in vivo* experiments. Recombinant p107 and p130 will both bind to TFIIIB and repress a variety of class III genes in cell extracts and in transfected cells. Furthermore, endogenous p107 and p130 were shown by cofractionation and coimmunoprecipitation analyses to associate stably with endogenous TFIIIB (Sutcliffe *et al.*, 1999). Disruption of this interaction *in vivo* results in a marked increase in pol III transcription (Sutcliffe *et al.*, 1999). Furthermore, fibroblasts derived from p107-/- p130-/- double-knockout mice display elevated levels of pol III transcripts (Sutcliffe *et al.*, 1999). The ability to bind and repress TFIIIB is therefore

Figure 1.5

Effects of RB on pol III transcription.

Schematic representation of the effects that RB can have on pol III initiation complexes. Cyclin-dependent kinases relieve RB mediated repression of TFIIIB at the G1/S phase transition.

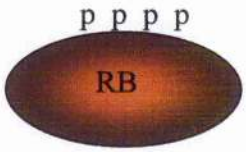
G1 Phase - (RB active)

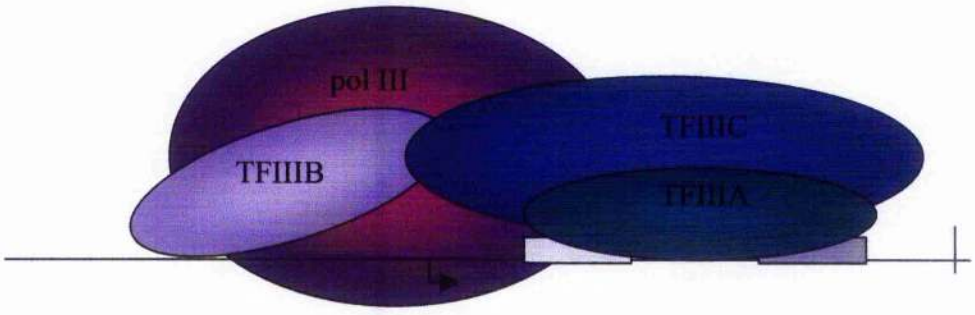


Cyclin D-dependent kinase

Cyclin E-dependent kinase

S Phase - (RB inactive)





a feature of each of the pocket proteins; this is consistent with deletion and substitution analyses which have shown that regulation of pol III transcription by RB is dependent on the pocket domain (Chu *et al.*, 1997; White *et al.*, 1996).

The functions of the pocket proteins are subject to cell cycle control (reviewed in Grana et al., 1998; Herwig & Strauss, 1997; Mittnacht, 1998; Mulligan & Jacks, 1998; Taya, 1997), with their phosphorylation status fluctuating through the different stages of the cell cycle. Hypophosphorylated proteins predominate during G0 and early G1 phases, but phosphorylation by cyclin D- and E-dependent kinases relieves the G1 restriction point and permits entry into S phase (Goodrich *et al.*, 1991; Hinds *et al.*, 1992). For much of the mammalian cell cycle, there is an inverse correlation between the activity of the pocket proteins and the level of pol III transcription. Thus, expression of class III genes is low during G0 and early G1 phases and then increases at the G1/S transition (Mauck & Green, 1974; White *et al.*, 1995a). It has been demonstrated that it is the hypophosphorylated form of RB that associates with TFIIIB during G0 and early G1 phases, and that this interaction substantially decreases as cells approach S phase (Scott *et al.*, 2001). p130 also interacts with RB in this cell cycle-dependent manner (Scott *et al.*, 2001). Consistent with these observations, pol III transcription is stimulated strongly when cells are transfected with vectors encoding cyclin D/cdk4 and cyclin E/cdk2 (Scott *et al.*, 2001). Once RB and its relatives have been silenced through phosphorylation, they remain in this inactive state until the start of the next G1 phase (Grana *et al.*, 1998; Herwig & Strauss, 1997; Mittnacht, 1998; Taya, 1997).

1.12.2 Repression of Pol I Transcription by RB.

In vitro experiments have demonstrated that RB can suppress the synthesis of rRNA by pol I (Cavanaugh *et al.*, 1995; Voit *et al.*, 1997). The physiological relevance of these findings was confirmed using immunofluorescence analyses to directly visualise the site of pol I transcription, the nucleolus (Rogalsky *et al.*, 1993; Cavanaugh *et al.*, 1995). This approach demonstrated that RB accumulates in the nucleolus when cells stop growing and begin to differentiate, in parallel with a decrease in pol I activity. This is also the case in confluent fibroblasts (Hannan *et al.*, 2000b), where the levels of rRNA transcription decreased ~five fold, which correlates with increasing amounts of the hypophosphorylated form of RB in the nucleolus. More direct evidence regarding the regulation of pol I by RB *in vivo* has been provided by transient transfections. These experiments showed that a RB expression vector can repress a cotransfected rDNA promoter (Hannan *et al.*, 2000b; Pelletier *et al.*, 2000). Inhibition of rRNA gene transcription is consistent with the RB-mediated negative regulation of cellular proliferation by preventing the progression of the cells from G1 to S phase.

p130 is also involved in repressing pol I transcription (Ciarmatori *et al.*, 2001; Hannan *et al.*, 2000a). Although pol I transcription is unaffected in RB-knockout fibroblasts, Rb-/- p130-/- double knockouts express ~50% more 18S and 28S rRNA than matched wild-type cells (Ciarmatori *et al.*, 2001). This implies that rRNA synthesis in living cells is subject to redundant control by endogenous RB and p130. Despite a high degree of homology and functional overlap with RB and p130 (Mulligan & Jacks, 1998), the third pocket protein p107 seems unable to regulate pol I transcription (Ciarmatori *et al.*, 2001; Hannan *et al.*, 2000a; Voit *et al.*, 1997).

The pol I transcription factor UBF has also been identified as an RB-binding protein by screening a cDNA expression library with purified RB as a probe (Shan *et al.*, 1992). Subsequent studies have confirmed the ability of recombinant RB to bind specifically to UBF (Cavanaugh *et al.*, 1995; Voit *et al.*, 1997). Furthermore, immunoprecipitation experiments have revealed that RB associates with UBF when present at physiological ratios, and that this interaction increases when cells down-regulate pol I transcription as their rate of growth decreases (Cavanaugh *et al.*, 1995).

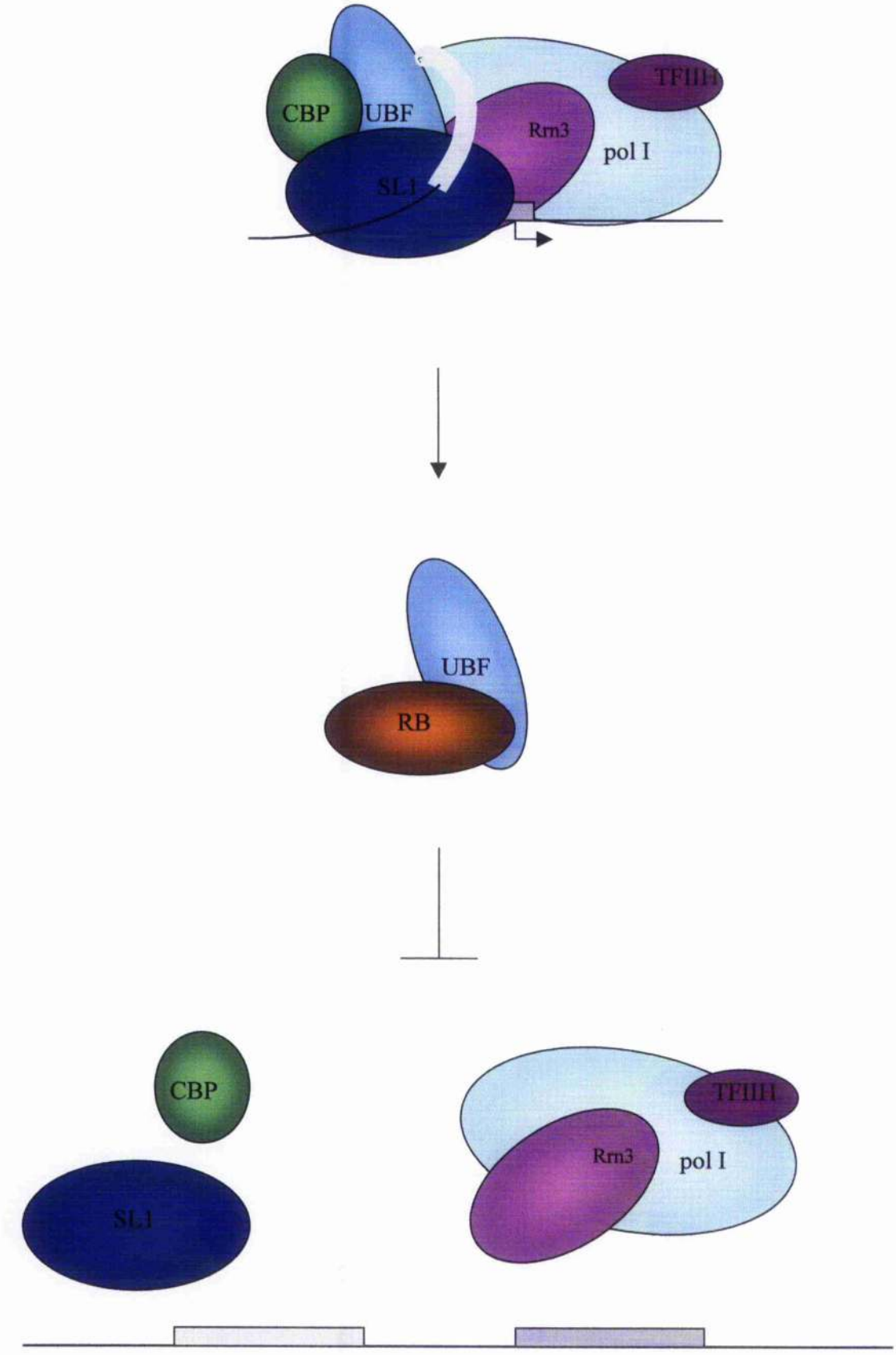
Again, p130, but not p107, also has the ability to bind and inactivate UBF (Ciarmatori *et al.*, 2001; Hannan *et al.*, 2000a). This pol I-sepcific transcription factor coimmunoprecipitates exclusively with the hypophosphorylated form of RB (Hannan *et al.*, 2000b), implying that the interaction between UBF and RB is regulated by cyclin-dependent kinase activity.

The precise mechanism that RB uses to inhibit pol I transcription is not yet clear. Several different groups have suggested alternative pathways that could lead to the down regulation of rRNA synthesis. Voit *et al.* (1997), found that RB can disrupt the DNA-binding activity of UBF, but it has also been demonstrated that it can interfere with the interaction between UBF and SL1 (Hannan *et al.*, 2000a). In addition, RB competes with the acetyltransferase CBP for UBF binding, and thereby suppresses the acetylation of UBF that activates pol I transcription (Pelletier *et al.*, 2000) (Figure 1.6).

Figure 1.6

Effects of RB on pol I transcription.

Schematic representation of the effects that RB can have on pol I transcription. Binding of RB to UBF disrupts its interactions with DNA, SL1 and CBP.


CBP
UBF
SL1
Rrn3
pol I
TFIIH
UBF
RB
CBP
SL1
Rrn3
pol I
TFIIH

1.12.3 Repression of Pol III Transcription by p53.

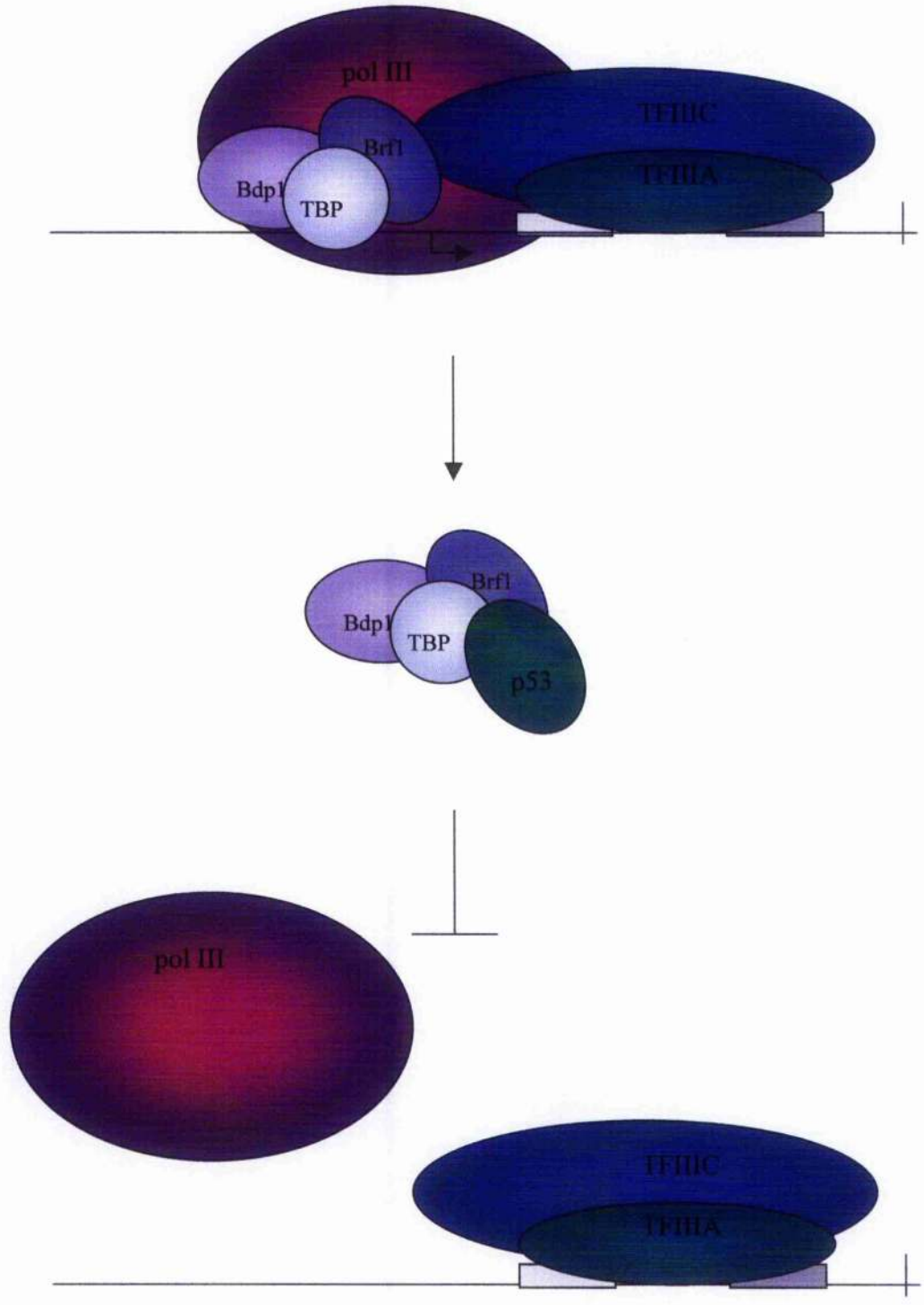
p53 protects cells in response to physiological stress conditions by either activating or repressing a variety of cellular promoters (Cox & Lane, 1995; Giaccia & Kastan, 1998; Ko & Prives, 1996; Levine, 1997; Sionov & Haupt, 1999; Vogelstein *et al.*, 2000). A well characterized example is the p21/WAF/CIP gene, which is activated by p53 and plays an important role in G1 arrest (El-Deiry *et al.*, 1993). Overexpression of p53 has a repressive effect on transcription of genes transcribed by pol III. This has been demonstrated both *in vitro* and in transfected cells (Cairns & White, 1998; Chesnokov *et al.*, 1996). Furthermore, the synthesis of tRNA and 5S rRNA *in vivo* is elevated four to six-fold following the targeted disruption of the *p53* gene in knockout mice (Cairns & White, 1998).

There are at least three distinct regions of p53 that contribute to its capacity to regulate transcription by pol III. These are located in both the N- and C-terminal domains, as well as the central core (Stein *et al.*, 2002b). Regulation of pol III transcription by p53 does not require sequence-specific DNA recognition. Instead, the pol III system is repressed through interactions of p53 with TFIIIB (Cairns & White, 1998; Chesnokov *et al.*, 1996). This is mediated through direct contacts with the TBP subunit (Crighton *et al.*, 2003). Furthermore, overexpressing TBP can reverse inhibition of tRNA gene transcription by p53. Coimmunoprecipitation assays have revealed that p53 does not disrupt the direct interaction between the TFIIIB subunits TBP and Brf1, but prevents the association of Brf1 complexes with TFIIIC2 and pol III, thereby impeding the formation of functional transcription complexes (Crighton *et al.*, 2003) (Figure 1.7).

Figure 1.7

Effects of p53 on pol III transcription.

Schematic representation of the effects that p53 can have on pol III transcription. Binding of p53 to TBP prevents initiation complex assembly.


pol III
TFIIIC
Brf1
TFIIIA
Bdp1
TBP
Brf1
Bdp1
TBP
p53
pol III
TFIIIC
TFIIIA

1.12.4 Repression of Pol I Transcription by p53.

Less research has been carried out to examine the molecular mechanism of pol I transcriptional inhibition by p53. Zhai & Comai (2000) have shown that the human rRNA promoter can be inhibited by overexpression of wild-type, but not mutant, p53, and, furthermore, that recombinant p53 inhibits rRNA transcription in a cell-free transcription system. In agreement with these results, p53-null epithelial cells display increased pol I transcriptional activity compared to that of epithelial cells expressing p53. Elevated levels of pre-rRNA were also observed by another group that examined fibroblasts from *p53* knockout mice (Budde & Grummt, 1999).

p53 represses pol I transcription by directly interfering with the assembly of productive transcriptional machinery on the rRNA promoter. Protein-protein interaction assays indicate that p53 binds to SL1. This interaction is mostly mediated by direct contacts with TBP and TAF_I110. Through this interaction, p53 prevents SL1 from binding UBF, and consequently represses rRNA synthesis (Zhai & Comai, 2000) (Figure 1.8).

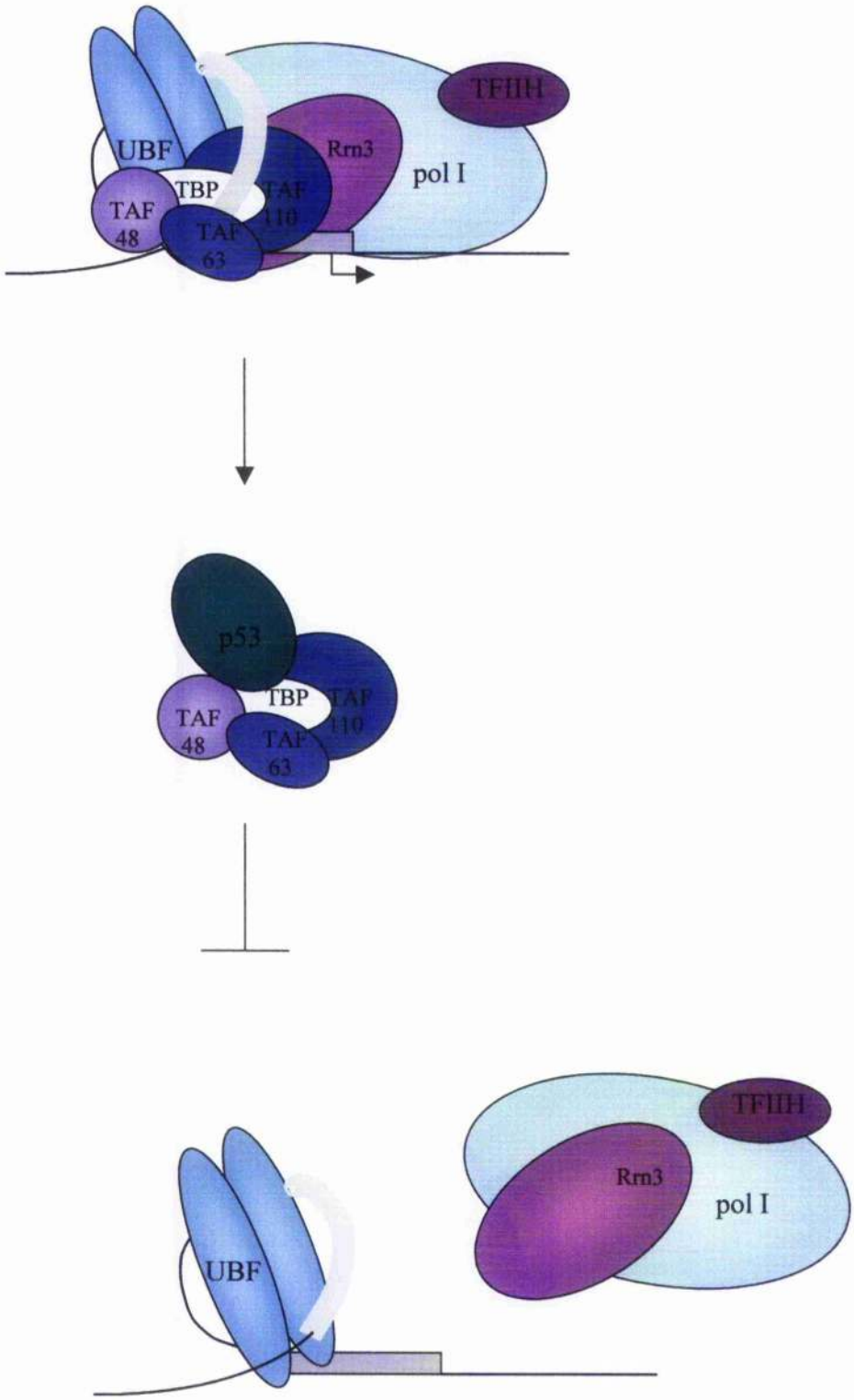
1.13 Deregulation of Pols I and III in Carcinogenesis.

Cancers arise as a result of cumulative genetic changes in somatic cells. The progression of normal cells to tumour cells involves alterations or mutations in tumour suppressor genes and oncogenes. These changes often result in the deregulation of an array of genes with different functions. Indeed, many studies have

Figure 1.8

Effects of p53 on pol I transcription.

Schematic representation of the effects that p53 can have on pol I transcription. Binding of p53 to SL1 prevents initiation complex assembly.


TFIIH
UBF
Rrn3
pol I
TBP
TAF
110
TAF
48
TAF
63
p53
TBP
TAF
110
TAF
48
TAF
63
TFIIH
Rrn3
pol I
UBF

observed that the abundance of pol I and III transcripts are abnormally elevated in transformed and tumour cell lines (Gottesfeld *et al.*, 1996; Larminie *et al.*, 1999; Scott *et al.*, 1983; Wang *et al.*, 1995; 1997; White *et al.*, 1990; Ying *et al.*, 1996; Zhai & Comai, 1999).

1.13.1 Inactivation of RB.

The RB pathway is frequently deregulated in tumours; in fact it has been suggested that this pathway may be disrupted in all human malignancies (Weinberg, 1995). Inactivating mutations in RB have been found in a variety of human tumours, including retinoblastomas, where the gene was first identified (Friend *et al.*, 1986), small-cell lung carcinomas, sarcomas, and bladder carcinomas (Weinberg 1995). Mutational events range from point mutation to chromosomal deletion, and invariably affect the pocket region (Hu *et al.*, 1990). Pocket domain mutations abolish the ability of RB to repress both the activities of pols I and III (Cavanaugh *et al.*, 1995; Chu *et al.*, 1997; Hannan *et al.*, 2000a; Larminie *et al.*, 1999; White *et al.*, 1996).

In many human malignancies, the function of RB, as well as p130 and p107, is lost due to the disruption of upstream control pathways (reviewed by Grana *et al.*, 1998; Mulligan & Jacks, 1998). The most common mechanism by which this occurs is through constitutive phosphorylation by cyclin-dependent kinases, which are hyperactive in many cancers (reviewed by Bates & Peters, 1995; Hunter & Pines, 1994). For example, in breast cancers the cyclin D1 gene is amplified in about 20% of cases, and its overexpression is detected in about half of all cases (Bartkova *et al.*, 1994; Buckley *et al.*, 1993; Gillet *et al.*, 1994, Lammie *et al.*, 1991; Schuuring *et al.*,

1992). Overexpression of cyclin E has also been associated with several types of cancers (Keyomarsi *et al.*, 1994). In addition, negative regulators of cyclin-dependent kinases, such as p16, are also found to be inactivated in a high percentage of human tumours. This occurs through point mutation, methylation, or deletion (Rocco & Sidransky, 2001).

1.13.2 Inactivation of p53.

The function of p53 is thought to be compromised in over 80% of human tumours (Lozano & Elledge, 2000). Mice that are homozygously null for the *p53* gene are strongly predisposed to cancer, such that 74% develop tumours by the age of six months (Donehower *et al.*, 1992). Furthermore, inherited mutations can cause Li-Fraumeni syndrome, a familial cancer predisposition in humans (Varley *et al.*, 1997). The primary fibroblasts from these patients often display abnormally elevated levels of pol III transcription (Stein *et al.*, 2002a).

About half of all sporadic tumours contain mutations of one *p53* allele coupled to deletion of the second allele (Hollstein *et al.*, 1991,1994). In most cases, the mutation lies in the central core of p53 (Bullock *et al.*, 2000). The most common substitution detected in human cancers converts the arginine 175 residue of the core domain of p53 to a histidine residue (Ryan & Vousden, 1998). This change abolishes the ability of p53 to repress pols I and III, both *in vitro* and *in vivo* (Stein *et al.*, 2002a; Zhai & Comai, 2000). Furthermore, this mutation is associated with high tumourigenicity and an extremely poor prognosis in patients (Goh *et al.*, 1995). Loss of p53 function can also occur by inactivation of the p53 protein through association with viral

proteins or with the MDM2 cellular oncoprotein (Hollstein *et al.*, 1991; Momand *et al.*, 1998). Inactivation of p53 through association with these proteins has been shown to release pol III from repression (Stein *et al.*, 2002a).

1.13.3 Deregulation by Viruses.

Transforming agents involved in carcinogenesis have also been shown to stimulate the activity of pols I and III. Among these agents are several viruses encoding oncoproteins that have the ability to bind and neutralize tumour suppressor genes. This stimulation of transcription in many cases seems to reflect a general requirement for increased biosynthetic capacity. However, transformation by adenovirus increases pol III transcription to enable high levels of expression of the viral VAI and VAII gene products during late infection (Söderlund *et al.*, 1976; Weinmann *et al.*, 1974). During the course of this infection, adenoviruses express the oncoprotein E1A. As well as binding and inactivating the pocket proteins, E1A also stimulates pol III transcription through increasing TFIIIC2 activity (Hoeffler & Roeder, 1985; Sinn *et al*, 1995; Yoshinaga *et al.*, 1986). This event is associated with a selective increase in the cellular concentration of the TFIIIC110 subunit and a concomitant rise in the ratio of the active-to-inactive forms of TFIIIC2 (Sinn *et al*, 1995). However, template commitment assays have also indicated that overall TFIIIC concentration is increased in extracts prepared after thirty hours of adenovirus infection (Yoshinaga *et al* 1986). Therefore, it may be that adenovirus acts initially by converting extant TFIIIC2b into TFIIIC2a, and then subsequently reinforces this effect by inducing an increase in TFIIIC2 abundance (White, 1998).

The oncoproteins of several other DNA tumour viruses have also been shown to increase pol III transcription. These include the large T-antigens of SV40 and polyomavirus (Carey *et al.*, 1986b; Felton-Edkins & White, 2002; Larminie *et al.*, 1999; Scott *et al.*, 1983; Singh *et al.*, 1985). RB mediated repression of pol III transcription has been shown to be reversed by E1A and the large T antigens of SV40 and polyomavirus, both *in vitro* and *in vivo* (Felton-Edkins & White, 2002; Larminie *et al.*, 1999; White et al., 1996). Furthermore, the interaction between RB and TFIIIB is diminished substantially in fibroblasts transformed by these viruses (Felton-Edkins & White, 2002; Larminie *et al.*, 1999). In addition, transformation by SV40 and polyomavirus also increases the levels of the TFIIIC2 subunits, in particular the TFIIIC110 subunit. This reflects an overproduction of the corresponding mRNAs (Larminie *et al.*, 1999; Felton-Edkins & White, 2002). In the pol I system, SV40 infection has a striking effect on rRNA half life, which increases from 72 hrs in primary human WI38 fibroblasts to 700 hrs in SV40-transformed derivatives (Liebhaber *et al.*, 1978). This is not a response to changes in growth rate, since rRNA stability is the same in serum-starved or rapidly growing WI38 cells (Liebhaber *et al.*, 1978). Immunoprecipitation experiments have revealed that the large T-antigen directly binds to SL1, *in vitro* as well as in SV40-infected cells (Zhai *et al.*, 1997). It has been suggested that this interaction occurs by direct association with three SL1 subunits, namely TBP, TAF_I48, and TAF_I110 (Zhai *et al.*, 1997).

Hepatitis B virus (HBV) is strongly linked to the development of hepatocellular carcinoma (Kim *et al.*, 1991). It encodes the oncoprotein X, which can induce liver cancer in transgenic mice (Kim *et al.*, 1991). X has been shown to stimulate transcription by both pol I and pol III (Aufiero & Schneider, 1990; Wang *et al.*,

1995,1997,1998). This coordinated activation can be explained by an increase in the level of TBP (Wang *et al.*, 1995,1997,1998). The effect is likely to be direct, since it can be obtained with recombinant TBP *in vitro* and also by transfecting cells with a TBP mutant that is specifically defective in supporting pol II transcription (Wang *et al.*, 1997,1998). Johnson *et al.* (2000) demonstrated that this occurs through promoter activation of TBP by the X oncoprotein, which occurs in a Ras-dependent manner that requires signalling through the Raf/MEK/Erk cascade (Johnson *et al.*, 2000).

One of the most clinically important viruses involved in carcinogenesis is the human papillomavirus. This virus encodes two major oncoproteins E6 and E7, which can bind to and inactivate p53 and RB, respectively (Dyson *et al.*, 1989; Münger *et al.*, 1989a). Cavanaugh *et al.* (1995) demonstrated that E7 is able to dissociate RB from UBF *in vitro*. Furthermore, transfections with the E7 protein stimulate pol III activity (Larminie *et al.*, 1999; Sutcliffe *et al.*, 1999). In addition to this, pol III transcriptional repression by p53 can be overcome by E6 (Stein *et al.*, 2002a). The transforming activities of this virus will be discussed in greater detail in chapter three.

1.13.4 Activation of Cellular Oncogenes.

As well as the exogenous viral oncogenes, several endogenous cellular genes have been demonstrated to cause cancer when deregulated. Among these are the intracellular messenger *ras*, and the nuclear-acting *myc* genes, both of which have been shown to affect the transcriptional activity of pols I and III.

Ras is commonly targeted for deregulation in carcinogenesis. The H-*ras*, K-*ras*, N-*ras*, and *rho* genes constitute a conserved mammalian family encoding structurally related proteins (Ishii *et al,* 1985; Stork *et al,* 1991). These are plasma membrane-associated guanine nucleotide-binding proteins that exhibit GTPase activity, and have a molecular mass of 21 kDa (Rozenberg & Howard, 1994). Mutant *ras* genes encoding altered proteins are found in many human and rodent tumour cells and are capable of the malignant transformation of NIH 3T3 cells, an established murine cell line (Ishii *et al*, 1986).

The oncogenic forms of Ras differ from their normal counterparts by mutations that result in amino acid substitutions at positions 12, 13, or 61 in the phosphate-binding domain of the protein (reviewed by Barbacid, 1987). Such mutations activate a number of different signal transduction cascades. One of these is the Ras/Raf/Mek/Erk cascade (Daum *et al.*, 1994). Recent studies have shown that Erk can stimulate both pol I and III transcription. With the pol I system, UBF is phosphorylated by Erk (Stefanovsky *et al.*, 2001), and this can result in a rapid induction of rRNA synthesis (Stefanovsky *et al.*, 2001). More recently, it has also been reported that Erk-dependent phosphorylation of the murine factor TIF-1A (known as Rrn3 in humans) is required for rRNA transcription (Zhao *et al.*, 2003). In the pol III system, Erk activates TFIIIB by binding and phosphorylating its Brf1 subunit (Felton-Edkins *et al.*, 2003). Coimmunoprecipitation assays have revealed that TFIIIB interactions with both TFIIIC and pol III are sensitive to Erk activity. Consequently, it has been demonstrated that promoter occupancy by Brf1 and pol III at endogenous tRNA genes decreases when Erk is inactivated (Felton-Edkins *et al.*, 2003). In addition to these effects, it has been demonstrated that constitutive

activation of Ras induces cyclin D1 overexpression (Filmus *et al.*, 1994; Kerkhoff & Rapp, 1997; Liu *et al.*, 1995; Sewing *et al.*, 1997; Winston *et al.*, 1996). This is likely to derepress pols I and III through hyperphoshorylation of RB.

Ras signalling is also involved in the induction of c-Myc expression (Kerkhoff & Rapp, 1998). The *myc* genes encode nuclear phosphoproteins that function as transcriptional regulators controlling cell proliferation, differentiation, and apoptosis (Evan *et al.*, 1992; Hermeking *et al.*, 1994; Marcu *et al.*, 1992). A strong causal link between c-Myc deregulation and malignancy has been established by the frequent deregulation of c-Myc in many different types of cancers, and by the ability of c-Myc expression vectors to function as potent transforming agents in primary cultures and in transgenic mice (DePinho *et al.*, 1991). A number of alterations affect c-Myc expression in various neoplasms, including gene amplification, chromosomal translocation, insertional mutations, altered transcriptional elongation rates, and prolonged mRNA half-life (Dang, 1999).

In c-Myc null cells, marked decreases in net RNA synthesis have been observed (Mateyak *et al.*, 1997). Since rRNA constitutes the bulk of cellular RNA, the c-Myc null phenotype could be explained by defects in pol I and III transcription. Indeed, anti-Myc antibodies and some c-Myc peptides from conserved regions of the c-Myc protein enhance the turnover of rRNA synthesized in homogenates of *Xenopus laevis* oocyte nuclei (Gibson *et al.*, 1992). It has also recently been demonstrated that TFIIIB is a target for c-Myc (Gomez-Roman *et al.*, 2003). Binding of c-Myc to TFIIIB directly activates pol III transcription at tRNA and 5S rRNA genes (Gomez-Roman *et al.*, 2003).

1.13.5 Cellular Transformation.

In addition to the cases already described, modulation of the relative levels of pols I and III activity has also been noted in response to several other transforming agents. These include transformation by several other viral regulatory proteins, such as the immediate early protein of pseudorabies virus and the Tax protein of human T-cell leukaemia virus type I, which stimulate pol III transcription, while infection by poliovirus has a repressive effect (Ahlers & Feldman, 1987; Fradkin *et al*, 1987; Gaynor *et al*, 1985; Gottesfeld *et al*., 1996; Piras *et al*., 1996). In addition, chemical carcinogens can also modify their transcription activities. An example of this is the tumour-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), which has been shown to rapidly increase rRNA and tRNA synthesis in dividing and nondividing *Drosphilia* cells (Chao & Pellegrini, 1993; Garber *et al*., 1991,1994; Vallet *et al*., 1993; Weber *et al*., 1991).

Although pols I and III have been shown to respond to a broad range of transforming agents in cultured cells, very little is known about the levels of transcription by these polymerases in human tumours. Two studies have addressed this, and have shown that certain pol III transcripts are abnormally abundant in tumours relative to normal tissue from the same patient. Chen *et al.* (1997a) showed that the pol III transcript 7SL is abnormally abundant in 19 different types of tumour tissues. Furthermore, Winter *et al.* (2000) showed that 7SL RNA, tRNA and 5S rRNA transcripts are overexpressed in ovarian carcinomas, along with the pol III specific factor TFIIIC2.

1.14 Aims.

As carcinomas develop and undergo changes in gene expression, the demands on the protein biosynthetic machinery are likely to be affected. The aim of this study was therefore to investigate transcription by pols I and III in cervical, breast and colorectal tumour biopsies in comparison to matched normal samples, to examine if deregulation occurs, and to identify transcription factors that may contribute to this. The effects of the histone deacetylase inhibitor TSA on the expression of pol III genes in matched transformed and untransformed cells was also assessed to determine whether elevated transcription after transformation results from abnormal acetylation.

Chapter 2

Materials & Methods

2.1 Cell Culture.

BALB/c 3T3 (A31), SV3T3 Cl38, SV3T3 Cl49, C33A and HeLa cells were cultured in DMEM (Dulbecco's Modified Eagle Medium). MCF-7 cells were cultured in RPMI 1640. Both types of media were supplemented with 10% fetal calf serum (FCS, Sigma), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were grown 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, by treatment with buffered trypsin-EDTA (0.05% trypsin, 0.02% EDTA). Cell lines were stored by cyro-freezing, in a solution of 72% DMEM or RPMI, 20% FCS and 8% dimethylsulphoxide (DMSO). For trichostatin A (TSA) (Sigma) treatment of A31, Cl38 and Cl49 cells, cells were grown to 40% confluency and then treated with 100, 200 or 300 nM TSA for 48 hours.

The W12 subclones 20863 and 20861, which contain episomal or integrated HPV16 DNA respectively (Jeon *et al.*, 1995), were cultured on mitomycin-C treated J2 3T3 fibroblast feeder layers in DMEM supplemented with 10% FCS, 2 mM glutamine, 8.4 ng/ml cholera toxin, 0.4 mg/ml hydrocortisone, 10 ng/ml EGF and 1.2 mM Ca^{2+}. Episomal maintenance or integration of viral DNA was confirmed by preparation of total genomic DNA and low molecular weight-enriched genomic DNA isolated by the Hirt method (Hirt, 1967), followed by Southern blotting using a whole HPV16 genome probe.

2.2 Preparation of Total Cellular RNA.

Total cellular RNA was extracted from cells grown in 10 cm tissue culture dishes using TRI reagent (Sigma), according to the manufacturer's instructions. Media was aspirated off the culture plates, and cells were harvested by scraping in 1 ml of TRI reagent per dish, and transferred to sterile eppendorf tubes. 0.2 ml of chloroform was then added to each tube, and the samples vortexed. Subsequently, they were centrifuged at 13000g for 15 minutes at 4°C. This resulted in separation of the samples into 3 phases: a lower organic phase containing protein, a middle interphase containing precipitated DNA, and an upper aqueous phase containing the RNA. The upper phase from each sample was carefully transferred to fresh eppendorf tubes, and 0.5 ml of isopropanol added. The tubes were then mixed thoroughly and were incubated for 5-10 minutes at room temperature. Following this, the samples were centrifuged at 13000g for 10 minutes at 4°C. The supernatant was then removed and the remaining RNA pellet was washed with 1 ml of 75% ethanol made up with diethypyrocarbonate (DEPC)-treated H_2O (0.1% DEPC). The samples were vortexed briefly, microcentrifuged at 7000g for 5 minutes at 4°C, and the supernatant aspirated off. RNA pellets were resuspended in appropriate volumes of DEPC H_2O, in the range of 10-30 µl. Samples were stored at -80°C. RNA concentration was determined by UV spectrophotometry using the calculation: RNA concentration (µg/ml) = absorbance at 260 nm x 40 x dilution factor.

Total cellular RNA was also extracted from human tissue samples. Cervical and breast biopsy material was obtained from the University Hospital of Heraklion, Crete, Greece, and colon biopsy material was obtained from the Institute for Cancer Studies, Birmingham, UK, all according to ethical guidelines. Tissue was removed surgically

and snap-frozen on dry ice. Frozen samples were crushed, and then ground in a homogeniser in 1 ml of TRI reagent. RNA was then extracted, as previously detailed.

2.3 Northern Blot Analysis.

20 µg total RNA was diluted to a total volume of 10 µl in DEPC H_20, and this was mixed with an equal volume of 2 X RNA sample buffer (1 X MOPS [20 mM MOPS pH 7.0, 8 mM sodium acetate, 1 mM EDTA pH 8.0], 4.4 M formaldehyde, 54% formamide). The samples were incubated at 65°C for 15 minutes to denature the secondary structure of RNA, and then immediately cooled on ice to prevent any renaturation. 2 µl of 10 X RNA loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol) and 2 µl of 1 mg/ml ethidium bromide was added to each sample. The samples were then loaded onto a denaturing formaldehyde 1% agrose gel (1% agrose, 2.2 M formaldehyde, 1 X MOPS) that had been pre-run in 1 X MOPS for 20 minutes at 40V. After electrophoresis for a further 5 hours at 40V, the fractionated RNA was visualised under a UV transilluminator and photographed.

The gel was then immersed in 20 X SSC (3 M NaCl, 0.3 M sodium citrate pH 7.0) and was washed by gentle shaking for 10-15 minutes. The gel was then placed inverted on a wick of Whatmann 3MM chromatography paper fed from a reservoir of 20 X SSC. A piece of pre-soaked Hybond N nitrocelluose filter (Amersham) which had been cut to size was then placed over the gel, followed by 2 pieces of pre-soaked Whatmann paper, cut to size, and a stack of paper towels. This was weighed down with a 500 g weight ensuring a tight connection between layers. Pieces of

autoradiography film were placed at the edges of the gel to block the towels from directly contacting the wick. The capillary transfer of RNA from the gel to the filter was allowed to proceed overnight. The nitrocellulose was then placed to dry and the RNA fixed to the membrane by UV-crosslinking (1200 μJ). It was then washed with DEPC H_20 and stored in Saran wrap until ready to hybridise.

To locate the RNA of interest, hybridisation was performed with a high specific activity radiolabelled DNA probe. The B2 gene probe was prepared from a 240bp *EcoR*I-PstI fragment from pTB14, and the ARPP P0 probe was prepared from a 1kb *EcoR*I-*Hind*III fragment from mouse cDNA. The probes were labelled by random oligonucleotide priming using a Megaprime DNA Labelling Kit (Amersham). 25 ng of purified DNA template was denatured by heating at 95°C for 5 minutes in the presence of random hexamer oligonucleotide sequences that can anneal to the DNA on the slow cooling of the mixture to room temperature. 10 μl of reaction buffer (dATP, dGTP, dTTP in Tris pH 7.5, β-mecaptoethanol and $MgCl_2$), 2 μl (2U) DNA polymerase I Klenow fragment, and 50 μCi of [α^{32}P] dCTP (Amersham) were added to initate labelling. This reaction was allowed to proceed for 1 hour at 37°C. The labelled DNA was then denatured by heating at 100°C for 5 minutes, and was then chilled on ice until ready for use.

While the probe was being prepared, the nitrocellulose was prehybridised by rotation in a hybridisation oven for 45 minutes at 45°C in 10 ml of hybridisation buffer (0.2M sodium phosphate buffer pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS, 45% formamide in DEPC H_20). The radiolabelled probe was then added to 10 ml of fresh hybridisation buffer and incubated with rotation at 45°C overnight. The filter was then washed

twice for 5 minutes at 65°C, and then twice for 15 minutes at 65°C in hybridisation wash solution (40 mM sodium phosphate buffer pH 7.2, 1 mM EDTA and 1% SDS). The filter was then exposed to autoradiography film overnight at -80°C. To reprobe the filter, it was added to boiling DEPC H_2O for 4 minutes and prehybridised and probed, as previously described.

2.4 RNA 6000 Nano Assay.

To prepare the gel-dye mix, 400 µl of RNA gel matrix (from RNA 6000 Nano Assay Kit, Agilent Technologies) was placed onto a spin filter. This was microcentrifuged at 1500g for 10 minutes. 130 µl of the filtered RNA gel matrix was then placed into an eppendorf tube and mixed with 2 µl of RNA dye concentrate by vortexing. To load the gel-dye mix, a RNA chip (RNA 6000 Nano LabChip, Agilent Technologies) was placed on the Chip Priming Station, and 9 µl of the gel-dye mix was dispensed into the appropriate well. The Chip Priming Station was then closed and the plunger pressed until it was held by the clip. The chip was then checked for air bubbles. 9 µl of the gel-dye mix was then pipetted into 2 further wells. 5 µl of RNA 6000 Nano Marker (Ambion) was then loaded into the ladder well, and then into each of the 12 sample wells. A further 1 µl of this marker was added to the ladder well, and 1 µl of each of the samples (approximately 200 ng/µl) was added to the 12 sample wells. The chip was then placed in an adapter and vortexed for 1 minute. The chip was then run in the Agilent 2100 Bioanalyzer. This piece of equipment assesses the purity and integrity of the RNA and allows concentration to be measured. When an electrical voltage is applied to the microchip, the sample migrates through micochannels etched

in the chip surface. As the sample moves, RNA of different sizes separate according to their mass. Intercalating dye within the sieving matrix allows the migrating RNA to be detected. The Agilent 2100 Bioanalyzer uses a laser for excitation of intercalating fluorescent dyes, and through connection to a computer depicts results as electropherograms.

2.5 RT-PCR.

cDNAs were prepared using 3 μg of RNA. Primer annealing was carried out at 80°C for 10 minutes with 200 ng of random hexamers (Roche), in a final volume of 24 μl. Reverse transcription (RT) was then performed for 1 hour at 42°C using 400 U of Superscript II reverse transcriptase (Life Technologies), 1 X First Strand buffer (Life Technologies), 10 mM dithiotreitol (DTT), and a 0.5 mM concentration of each deoxynucleoside triphosphate (dNTP) (Promega), in a total volume of 40 μl. This reaction was stopped by incubation at 70°C for 15 minutes.

PCRs were carried out using a Proteus II thermal controller (MJ Research, Helena Bioscience). Amplifications were performed using 2 μl cDNA with 20 pmol of relevant primers under various cycling parameters. Reaction mixtures also contained 0.5 U *Taq* DNA polymerase (Promega), 1 X *Taq* DNA polymerase buffer (Promega), 1.5 mM $MgCl_2$, 1 μCi of [α-^{32}P] dCTP (Amersham), and 0.2 mM of each dNTP, in a total volume of 20 μl.

ARPP P0 – 265bp

95°C	2 min	
95	1 min	
58	30 sec	20-25 cycles
72	1 min	
72	3 min	

Forward 5' – GAC CTG GAA GTC CAA CTA CTT C – 3'
Reverse 5' – TGA GGT CCT CCT TGG TGA ACA C – 3'

B2 – 90bp

95°C	3 min	
95	30 sec	
55	30 sec	15-20 cycles
72	30 sec	
72	5 min	

Forward 5' – GGG GCT GGA GAG ATG GCT – 3'
Reverse 5' – CCA TGT GGT TGC TGG GAT – 3'

Bdp1 – 293bp

95°C	2 min	
95	1 min	
56	1 min	25-30 cycles
72	1 min	
72	5 min	

Forward 5' – GCT GAT AGA GAT ACT CCT C – 3'
Reverse 5' – CCA GAG ACA AGA ATC TTC TC – 3'

Brf1 – 90bp

95°C	2 min	
95	1 min	
60	1 min	25-30 cycles
72	1 min	
72	5 min	

Forward 5' – CAG CCA GAA TGC ATG ACT TCA G – 3'
Reverse 5' – AAA TTC CGT GAG CCT CTT CCG CAG CG – 3'

c-Myc – 338bp

94°C	5 min	
94	30 sec	
65	1 min	25-30 cycles
72	1 min	
72	5 min	

Forward 5' – TCC AGC TTG TAC CTG CAG GAT CTG A – 3'
Reverse 5' – CCT CCA GCA GAA GGT GAT CCA GAC T – 3'

Cyclin D1 – 140bp

95°C	5 min	
95	1 min	
68	30 sec	25-30 cycles
72	1 min	
72	5 min	

Forward 5' – GGT GTC CTA CTT CAA ATG TGT GCA GAA GG – 3'
Reverse 5' – TCC AGC GAC AGG AAG CGG TCC AGG TAG –3'

Cyclin D2 – 112bp

94°C	2 min	
94	15 sec	
62	30 sec	25-30 cycles
72	30 sec	
73	5 min	

Forward 5' – CTG CCC CCA CCT AGA TCA TA – 3'
Reverse 5' – TCC CTT ATG CTG TAC TTC AAA TAG G –3'

MRP – 232bp

95°C	3 min	
95	30 sec	
58	30 sec	20-25 cycles
72	30 sec	
72	5 min	

Forward 5' – CGT GCT GAA GGC CGT TAT C – 3'
Reverse 5' – GGT GCG CGG ACA CGC AC – 3'

Rrn3 – 181bp

95°C	3 min	
95	30 sec	25-30 cycles
55	30 sec	
72	30 sec/min	
72	5 min	

Forward 5' – GGA AGA GAG AGA GGA TAT AG – 3'
Reverse 5' – GGA CAC TTG AGA GCC AAG – 3'

TBP – 154bp

95°C	3 min	
95	1 min	30-35 cycles
57	1 min	
72	1 min	
72	5 min	

Forward 5' – CCT GCC ACC TTA CGC TCA G – 3'
Reverse 5' – GCT GCT GCT GCC TTT GTT G – 3'

TFIIB – 262bp

95°C	3 min	
95	30 sec	25-30 cycles
57	30 sec	
72	30 sec	
72	5 min	

Forward 5' – GCA GAC AGA ATC AAT CTA C – 3'
Reverse 5' – CAG TTG TAA TCA AAT CCA CAC – 3'

TFIIIC63 – 300bp

95°C	3 min	
95	1 min	25-30 cycles
62	1 min	
72	1 min	
72	5 min	

Forward 5' – CGG CAG ATG TTC TAC CAG TTA TGC G – 3'
Reverse 5' – ATG GCT TGA AGT CCT CCT CC – 3'

TFIIIC 90 – 210bp

95°C	3 min	
95	1 min	
58	30 sec	25-30 cycles
72	1 min	
72	5 min	

Forward 5' – AAA CAG AAG TTG CTG AGT GC – 3'
Reverse 5' – ATG GTC AGG CGA TTG TCC – 3'

TFIIIC102 – 184bp

95°C	3 min	
95	1 min	
60	30 sec	25-30 cycles
72	1 min	
72	5 min	

Forward 5' – CCT ACT AAT GTC CGT TAT CTG TGG – 3'
Reverse 5' – GCA GAA GTA ACA TCA TTG GC – 3'

TFIIIC110 – 303bp

95°C	3 min	
95	1 min	
62	40 sec	20-25 cycles
72	40 sec	
72	5 min	

Forward 5' – CTT TCT TCA GAG ATG TCA AAG G – 3'
Reverse 5' – CCA GAA GGG GTC TCA AAG TCC – 3'

TFIIIC220 – 144bp

95°C	3 min	
95	20 sec	
62	30 sec	20-25 cycles
72	30 sec	
72	10 min	

Forward 5' – TCC AGC GAG ACC TTC ACA CC – 3'
Reverse 5' – GGA TTG AGT GTT GCT GGG CT – 3'

tRNA ^{Arg} – 74bp

95°C	2 min	
95	30 sec	25-30 cycles
65	30 sec	
72	15 sec	
72	5 min	

Forward 5' – GGC TCT GTG GCG CAA TGG ATA – 3'
Reverse 5' – TTC GAA CCC ACA ACC TTT GAA TTG CTC – 3'

tRNA ^{Leu} – 88bp

95°C	3 min	
95	30 sec	25-30 cycles
68	30 sec	
72	30 sec	
72	5 min	

Forward 5' – GTC AGG ATG GCC GAG TGG TCT AAG GCG CC – 3'
Reverse 5' – CCA CGC CTC CAT ACG GAG ACC AGA AGA CCC – 3'

tRNA ^{Ser} – 74bp

95°C	3 min	
95	30 sec	25-30 cycles
54	30 sec	
72	30 sec	
72	5 min	

Forward 5' – GGA TGA TCC TCA GTG GTC – 3'
Reverse 5' – GGT GGA ATT GAA CCA CTC – 3'

UBF – 205bp

95°C	3 min	
95	30 sec	25-30 cycles
57	30 sec	
72	30 sec	
72	5 min	

Forward 5' – GCA GTC CAA GTC GGA GTC – 3'
Reverse 5' – CCT CAT CGT CAT CCT CGT C – 3'

5S rRNA – 107bp

95°C	3 min	
95	30 sec	18-23 cycles
58	30 sec	
72	1 min	
72	5 min	

Forward 5' – GGC ATA CCA CCC TGA ACG C – 3'
Reverse 5' – CAG CAC CCG GTA TTC CCA GG – 3'

5.8S rRNA – 158bp

95°C	3 min	
95	30 sec	20-25 cycles
59	30 sec	
72	30 sec	
72	5 min	

Forward 5' – CGA CTC TTA GCG GTG GAT C – 3'
Reverse 5' – GAC GCT CAG ACA GGC GTA G – 3'

7SK – 247bp

95°C	3 min	
95	30 sec	25-30 cycles
57	30 sec	
72	30 sec	
72	5 min	

Forward 5' – CGA TCT GGT TGC GAC ATC TG – 3'
Reverse 5' – CGT TCT CCT ACA AAT GGA C – 3'

7SL – 150bp

94°C	2 min	
94	1 min	15-20 cycles
70	30 sec	
72	30 sec	
72	2 min	

Forward 5' – GTG TCC GCA CTA AGT TCG GCA TCA ATA TGG – 3'
Reverse 5' – TAT TCA CAG GCG CGA TCC CAC TAC TGA TC – 3'

18S rRNA – 302bp

95°C	3 min	
95	30 sec	25-30 cycles
54	30 sec	
72	30 sec	
72	5 min	

Forward 5' – GGT TGA TCC TGC CAG TAG – 3'
Reverse 5' – CGA GGT TAT CTA GAG TCA C – 3'

28S rRNA – 440bp

95°C	3 min	
95	30 sec	25-30 cycles
64	30 sec	
72	30 sec	
72	5 min	

Forward 5' – GCA CGA GAC CGA TAG TCA AC – 3'
Reverse 5' – GGA GGA CGG ACG GAC GGA C – 3'

Reaction products were resolved on 7% polyacrylamide sequencing gels containing 7 M urea and 0.5 X TBE (45 mM Tris, 45 mM boric acid, 0.625 mM EDTA pH 8.0). Gels were pre-run for 30 minutes at 40W in 0.5 X TBE. 2 µl of each sample after dilution 1:1 with formamide running buffer (98% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol, 5 mM EDTA) was loaded on the gel after being boiled at 100°C for 2 minutes and transferred to ice. Electrophoresis was carried out for 1 hour at 40W and the gel was vacuum-dried at 80°C for 1 hour. Radioactivity was then visualised by autoradiography.

2.6 Preparation of Genomic DNA.

DNA was also isolated from the cervical biopsy material. After the addition of chloroform to TRI reagent and isolation of RNA from the biopsy samples (as detailed in the preparation of RNA, section 2.2), DNA was precipitated by the addition of 0.3 ml of 100% ethanol. After mixing thoroughly and incubating for 2-3 minutes at room temperature, the samples were centrifuged at 2000g for 5 minutes at 4°C. The DNA pellet was then washed twice in 0.1 M sodium citrate, 10% ethanol solution. During each wash the pellet was allowed to stand for 30 minutes. Samples were then centrifuged at 2000g for 5 minutes at 4°C. Pellets were then resuspended in 1.5 ml 75% ethanol and allowed to stand for 20 minutes at room temperature. The samples were then microcentrifuged at 12000g for 5 minutes at 4°C, and the supernatant aspirated off. DNA pellets were allowed to air dry and were then resuspended in appropriate volumes of 8 mM NaOH, typically 50 µl. The samples were then centrifuged at 12000g for 10 minutes to remove insoluble material, the supernatant was then transferred to a fresh tube, and the pH adjusted to 8.4 using HEPES (66 µl of 0.1 M HEPES free acid/ml DNA solution). Samples were stored at -80°C. DNA concentration was determined by UV spectrophotometry using the calculation: DNA concentration (µg/ml) = absorbance at 260 nm x 50 x dilution factor.

2.7 PCR.

Genomic DNA from HPV11 infected samples was PCR amplified as described in section 2.5. Amplifications were performed using 2 µl DNA with 20 pmol ARPP P0 or Bdp1 primers.

ARPP P0 – 265bp

95°C	10 min	
95	1 min	⎫
58	30 sec	⎬ 21 cycles
72	1 min	⎭
72	3 min	

Forward 5' – GAC CTG GAA GTC CAA CTA CTT C – 3'
Reverse 5' – TGA GGT CCT CCT TGG TGA ACA C – 3'

Bdp1 – 308bp

95°C	10 min	
95	1 min	⎫
57	1 min	⎬ 30 cycles
72	1 min	⎭
72	5 min	

Forward 5' – CCA GTC TCA GTA GCT TG – 3'
Reverse 5' – CCT CAC GGC CGC TCA CAG – 3'

2.8 Preparation of Whole Cell Extracts.

Whole cell extracts were prepared from cells grown in 10 cm tissue culture dishes. Plates of cells were placed on ice and washed twice with 5 ml of chilled phosphate buffered saline (PBS) (170 mM NaCl, 3.4 mM KCl, 1 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2). They were then harvested by scraping in 5 ml PBS. Cells were collected in 50 ml Falcon tubes and pelleted by centrifugation at 12000g for 8 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 1 ml of PBS, and transferred to eppendorf tubes. They were then briefly microcentrifuged at 4°C, and the PBS was removed. Volumes of cell pellets were then estimated by comparison with pre-measured volumes of water (50-150 µl). The cells were resuspended in an equal volume of freshly prepared chilled microextraction buffer (450 mM NaCl, 50

mM NaF, 20 mM Hepes pH 7.8, 25% glycerol, 1 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, 40 µg/ml bestatin, 1 µg/ml trypsin inhibitor, 0.7 µg/ml pepstatin, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin). Upon addition of this buffer, the cells were immediately snap-frozen on dry ice. Cells were then placed in a 30°C water bath until they had just thawed, and then they were immediately returned to dry ice. This freeze-thaw procedure was performed a further 2 times to ensure optimal cell lysis. Following the final thaw, cells were microcentrifuged at 8000g for 8 minutes at 4°C. The supernatants were carefully aliquoted into fresh tubes, and were then stored at -80°C.

The protein concentration of the whole cell extracts was determined using Bradford's reagent (Biorad). A standard Bradford assay curve was constructed by measuring the absorbance at 595 nm of 0-20 µg of bovine serum albumin (BSA) in 1 ml of Bradford's reagent diluted 1:4 with H_2O. Absorbance readings of 1-3 µl of whole cell extract added to 1 ml of diluted Bradford's reagent were converted into protein concentrations accordingly.

2.9 Transformation of Competent Cells.

To store and propagate plasmids, *E.coli* XL-1 Blue supercompetent cells (Stratagene) were transformed. Cells stored at -80°C were thawed on ice to prevent loss of transformation ability. Typically, 10-20 ng of plasmid DNA was added to the thawed cells and mixed gently. This mixture was incubated on ice for 30 minutes; during this time the tube was occasionally gently tapped. Following this incubation, cells were

heat shocked at 42°C for exactly 45 seconds and then transferred to ice for a further 2 minutes. 450 µl of preheated (42°C) SOC medium (LB broth, 0.04% glucose, 10 mM $MgSO_4$, 10 mM $MgCl_2$) was then added and cells were incubated at 37 °C for 1 hour on an orbital shaker (225-250 rpm). Typically, 150 µl of the transformation mixture was then plated on LB agar (2% LB, 2% agar) plates containing 50 µg/ml ampicillin, and then incubated at 37 °C overnight to allow colony formation.

2.10 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 4 ml of LB medium containing 50 µg/ml ampicillin. This was incubated at 37°C on an orbital shaker (~300rpm) for approximately 6 hours to form a mini-culture, and was subsequently used to inoculate 250 ml of LB medium containing 50 µg/ml ampicillin. This was then incubated overnight under the same conditions. Cells were then harvested by centrifugation at 6000g for 15 minutes at 4°C, and plasmid DNA retrieved using the QIAGEN Plasmid Maxi Kit according to the manufacturer's instructions.

The harvested bacterial cells were resuspended in 10 ml of Buffer P1 (500 mM Tris pH 8.0, 10 mM EDTA, 100 µg/ml RNase A), and then were lysed by adding 10 ml of Buffer P2 (200 mM NaOH, 0.1% SDS). This reaction was allowed to proceed for 5 minutes at room temperature before neutralizing the lysate by the addition of 10 ml of chilled Buffer P3 (3 M potassium acetate, pH 5.5). This resulted in formation of a precipitate of potassium dodecyl sulphate, and the SDS-denatured proteins,

chromosomal DNA and cell debris were co-precipitated in insoluble salt-detergent complexes. Plasmid DNA, being circular and covalently closed, renatured correctly and remained in solution. Precipitation was enhanced by a 20 minute incubation on ice. Precipitated debris was then removed by centrifugation at 20000g for 30 minutes at 4°C. The supernatant was then promptly removed and applied to a QIAGEN-tip 500 pre-equilibrated with 10 ml of Buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100). This anion-exchange resin, to which plasmid DNA is able to bind tightly, was washed twice with 30 ml of Buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol). The purified plasmid DNA was subsequently eluted with 15 ml Buffer QF (1.25 M NaCl, 50 mM Tris pH 8.5, 15% isopropanol), and precipitated with 10.5 ml of isopropanol. The sample was then centrifuged at 15000g for 30 minutes at 4°C. Following this, the supernatant was carefully decanted out leaving the plasmid DNA pellet, which was then washed with 70% ethanol and recentrifuged. The pellet was finally dried at room temperature for 5-10 minutes and resuspended in an appropriate volume of sterile water.

2.11 Pol III *in vitro* Transcription Assay.

In vitro transcription of class III genes was reconstituted using 15-20 µg of cell extracts and fractionated factors to provide basal pol III transcription components and 250 ng of plasmid DNA containing the pol III templates tRNA ^{Leu} (a 240bp *EcoR*I-*Hind*III fragment of human genomic DNA carrying a tRNA ^{Leu} gene, subcloned into pAT153), or VA1 (a 221bp *Sal*I-*Bal*I fragment of adenovirus 2 DNA containing the VA1 gene subcloned into pUC18). Reactions were carried out in a 25 µl volume, with a final concentration of 12 mM HEPES pH 7.9, 60 mM KCl, 7.2 mM $MgCl_2$, 0.28

mM EDTA, 1.2 mM DTT, 10% glycerol, 1 mM creatine phosphate, 0.5 mM of each of rATP, rCTP and rGTP and 10 μCi [α-^{32}P] UTP (Amersham).

Cell extracts were preincubated with fractionated factors for 15 minutes at 30°C prior to the addition of template and nucleotides required to initiate transcription. Transcription was terminated by the addition of 250 μl of 1M ammonium acetate/0.1% SDS containing 20 μg of yeast tRNA, which stabilises the synthesised RNA. Phenol-chloroform extraction of samples was then performed to remove protein and DNA by adding 250 μl PhOH/$CHCl_3$/IAA of a 25:24:1 ratio. The samples were vortexed and microcentrifuged at 13000g for 5 minutes. 250 μl of the upper aqueous layer was then transferred to a fresh eppendorf tube containing 750 μl of 96% ethanol in order to precipitate the RNA. The samples were thoroughly mixed by inversion and left at –20°C overnight. The precipitated RNA was microcentrifuged at 13000g for 20 minutes, and the supernatant carefully removed to avoid dislodging the pellet. 750 μl of 70% ethanol was added to each sample to wash the pellet. This was carefully removed, and the pellets were placed at 47°C for 5-10 minutes to dry. 4 μl of formamide loading buffer (98% formamide, 10 mM EDTA pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol) was added to each sample, which was then vortexed for 20 minutes to ensure the RNA was fully resuspended. Samples were boiled at 95°C for 2 minutes and transferred to ice before 2 μl of each was resolved on a pre-run 7% polyacrylamide sequencing gel (described in section 2.5).

2.12 Separation of Proteins by Polyacrylamide Gel Electrophoresis.

Proteins were resolved on denaturing (SDS) polyacrylamide gels by electrophoresis. Typically samples were run on 7.8% polyacrylamide minigels (375 mM Tris pH 8.8, 0.1% SDS) with a 4% polyacrylamide stacking gel (125 mM Tris pH 6.8, 0.1% SDS). Before loading, samples were boiled for 2 minutes in 4 X protein sample buffer (250 mM Tris pH 6.8, 2% SDS, 20% β-mercaptoethanol, 40% glycerol, 0.5% bromophenol blue) and then transferred to ice. After the samples had been loaded, electrophoresis was performed in 1 X SDS running buffer (0.1% SDS, 76.8 mM glycine, 10 mM Tris pH 8.3), at an initial voltage of 70V while the bromophenol blue dye moved through the stacking gel, and a subsequent voltage of 140V after reaching the resolving gel. This was carried out for approximately 1 – 1.5 hours.

2.13 Western Blot Analysis.

Proteins resolved by SDS-PAGE were transferred to 0.2 μM pore nitrocellulose membrane (Biorad) using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system. Transfer was carried out overnight at 4°C in 1 X transfer buffer (76.8 mM glycine, 10 mM Tris pH 8.3, 16.5% methanol) at 40V. Membranes were then blocked in milk buffer (32.5 mM Tris, 150 mM NaCl, 0.2% Tween-20, 4% skimmed milk powder (Marvel)) for 1 hour at room temperature. Membranes were incubated for 2 hours with primary antibodies diluted 1:1000 in milk buffer.

06-866	anti-acetylated histone H4	UpState
1900-4	anti-pol III (RPC 155)	In house
128-4	anti-Brf1	In house
4289-4	anti-TFIIIC110	In house
C18	anti-TFIIB	Santa Cruz

The membranes were then washed 3 times for 3 minutes in fresh milk buffer to remove excess primary antibody, before incubating for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000 dilution in milk buffer). The blot was then washed 3 times for 3 minutes in milk buffer, followed by a further 2 milk buffer washes lasting for 15 minutes each. After 1 further 5 minute wash using 1 X TBS (2.5 mM Tris pH 7.6, 15 mM NaCl), the bound antibody was detected using the enhanced chemiluminescence method (ECL, Amersham) according to the manufacturer's instructions.

2.14 Chromatin Immunopreciptiation Assay.

Formaldehyde was added directly to plates of cells containing media to a final concentration of 1%. They were then incubated at 37°C for 10 minutes. Glycine was then added to a final concentration of 0.125 M to stop the crosslinking. The plates were then transferred to ice, and 7 ml of the media removed. Cells were then scraped in the remaining media, transferred to Falcon tubes, and centrifuged at 500g for 5

minutes at 4°C. The cells were washed twice by resuspension in 40 ml chilled PBS and pelleted by centrifugation at 500g. The supernatant was then decanted off and the cells were incubated on ice for 30 minutes in 40 ml of high salt buffer (0.5% NP-40/ PBS, 1 M NaCl). Cells were then recentrifuged and washed with 0.5% NP-40/PBS. This was followed by hypotonic disruption for 30 minutes on ice with 40 ml of low salt buffer (0.1% NP-40, 10 mM Tris pH 8.0, 1 mM EDTA, 0.1 M NaCl). The samples were then centrifuged at 500g at 4°C, the supernatant decanted off, and the pellets resupended in 1 ml of low salt buffer. Nuclei were then obtained by passing the sample through a 26 gauge needle 3 times. The samples were recentrifuged and the nuclei resuspended in 2.7 ml of low salt buffer and lysed with 300 µl sarkosyl. The samples were then transferred to a sucrose solution (40 ml low salt buffer/100 mM sucrose) and centrifuged at 4000g for 10 minutes at 4°C. The supernatant was then discarded and the pellet was resuspended in 3 ml TE. The process was then repeated. The pellet containing the genomic DNA was then resuspended in 2 ml TE, and sheared by sonication (Branson sonifier 250, 10 X for 10 second intervals, 30% duty cycle).

Sonicated material was adjusted with 1/10 volume of 11 X NET (1.65 M NaCl, 5.5 mM EDTA, 5.5% NP-40, 550 mM Tris pH 7.4). 10% of each sample was stored at 4°C to use as input controls. 4 µg of antibody was added to the samples and they were incubated overnight at 4°C. Protein-A-Sepharose beads were added for a further 2 hours and then recovered on Polyprep columns (Biorad). The negative control was incubated with beads alone. After washing twice in 10 ml RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40), twice in 10 ml of LiCl buffer (10 mM Tris, 250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1mM

EDTA pH 8.0) and twice in 10 ml TE, beads were transferred to 1.5 ml tubes. Immunoprecipitated material was then eluted twice with 200 µl of 1% SDS in TE; this was carried out by rotating the tubes for 10 minutes at room temperature. 5 µl of proteinase K was then added to the supernatants and the samples were incubated at 42°C overnight to degrade proteins and antibodies. DNA was extracted twice using 400 µl PhOH/$CHCl_3$/IAA, and ethanol precipitated. The immunoprecipitated DNA was then resuspended in 40 µl TE (10 mM Tris pH 8.0, 1 mM EDTA), and quantified by PCR.

Chapter 3

Cervical Cancer

3.1 Introduction.

Cervical cancer is among the most common female cancers in many developed countries. Out of around 200 different types of cancer, it is ranked the eleventh most common cancer in women in the UK, and accounts for around 2% of all female cancers. In 1999, there were over 3000 new cases and 1000 deaths from this cancer in the UK (Cancer Research UK, 2003a, b). Although there is a higher chance to develop cervical cancer later in life, it is the second most common cause of cancer in women under the age of 35 (Cancer Research UK, 2003c).

Normally, proliferation occurs only at the basal layer of the cervix generating cells that then move outward toward the surface, differentiating into flattened, keratin-rich, nondividing cells, and finally being sloughed off from the surface. During cervical cancer, the usual pattern of cell division is disrupted and all the layers of the epithelium consist of undifferentiated proliferating cells. In the advanced stages of the disease cells break out of the epithelium by crossing the basal lamina and begin to invade the underlying connective tissue (reviewed in Alberts *et al.*, 1994). Around two thirds of cervical cancers are squamous cell carcinomas (Cancer Research UK, 2003c), arising within a specific region of the cervix referred to as the transformation zone. In this area the columnar epithelium located at the junction between the ectocervix and endocervix is replaced by squamous epithelium (reviewed in Cho, 2002). Adenocarcinoma, arising from the endocervical columnar/glandular epithelium is the next most common histology (around 15%); a further 15% are poorly specified carcinomas (Cancer Research UK, 2003c).

Risk factors for cervical cancer include sexual behaviour, smoking, human immunodeficiency virus, and most importantly human papillomavirus (HPV) infection. The etiological association of HPV with cervical cancer is well established (Baldwin *et al.*, 2003; zur Hausen, 2000). Over 90% of invasive cervical carcinomas have been shown to contain HPV DNA sequences, and precursor lesions have also been associated with the presence of HPV DNA (Bosch *et al.*, 1995).

HPVs belong to the Papovaviridae family of viruses. These are small nonenveloped viruses with circular genomes, composed of 8 kb of double stranded DNA (zur Hausen, 1996). They have a slow growth cycle, stimulate cell DNA synthesis, and replicate within the nucleus. HPVs comprise a heterogenous group of more than 100 different genotypes that are highly trophic for epithelial cells of the skin and mucous membranes. Of those, more than 35 distinct types are known to infect the anogenital mucosa (Bernard *et al.*, 1994). These are broadly divided into different categories. Low risk HPV types such as 6 and 11 induce benign proliferative lesions. Types 31 and 33 are classified as intermediate risk, whereas types 16 and 18 are considered high risk and can potentially induce neoplastic transformation (Lorincz *et al.*, 1992).

During the initial stages of HPV infection, viral DNA is maintained as episomes in the basal layer of stratified epithelia, with a copy number of 50 to 100 copies per cell (del Mar Peña & Laimins, 2001). As a pre-malignant HPV infected lesion progresses to cervical cancer, the viral DNA often becomes integrated into host cell DNA (Cullen *et al.*, 1991; Matsukura *et al.*, 1989). Indeed, the majority of invasive carcinomas contain high risk HPV DNA integrated into human chromosomal DNA as one or more tandem copies (Cullen *et al.*, 1991; Dürst *et al.*, 1985; Matsukura *et al.*, 1989).

Upon integration, most of the open reading frames (ORFs) of the integrated viral DNA are lost or interrupted (Schwarz *et al.*, 1985). The HPV oncoproteins E6 and E7, however, are still expressed. Furthermore, their expression is deregulated, as the ORF of the viral transcriptional regulator protein E2 is generally lost during this process, and the mRNA encoding E6 and E7 is stabilised (Cripe *et al.*, 1987; Jeon *et al.*, 1995; Jeon and Lambert 1995). E6 and E7 are constantly expressed in malignant tissue, and inhibiting their expression blocks the malignant phenotype of cervical cancer cells. They are independently able to immortalize various human cell types in culture, but efficiency is increased when they are expressed together (de Villiers *et al.*, 1989; Münger *et al.*, 1989a). High risk HPVs may themselves contribute to integration of HPV DNA into the host genome. Indeed, studies have shown that the frequency of foreign DNA integration is enhanced in cells expressing high risk, but not low risk, HPV E6 and E7 (Kessis *et al.*, 1996).

The E6 oncoprotein of high risk HPV types binds to p53 with much higher affinity than E6 of low risk HPV types, and this binding appears to promote p53 degradation (Scheffner *et al.*, 1990; Werness *et al.*, 1990). The interaction between high risk HPV E6 and p53 is mediated by another protein, E6-AP, that functions as a ubiquitin ligase in the ubiquitination of p53 (Huibregste *et al.*, 1991, 1993; Scheffner *et al.*, 1993). p53 is polymorphic at amino acid 72, and may contain either a proline or an arginine residue at this position. Storey *et al.* (1998) have revealed that the arginine form is more susceptible to degradation by the E6 protein than the proline form. Moreover, patients with HPV-associated cervical cancer are much more likely to contain the arginine form of p53 compared with the rest of the population. This study suggested that patients homozygous for arginine-72 p53 are seven times more susceptible to

developing cervical cancer than people with the proline form. Similarly, the E7 protein of high risk HPV has been shown to bind RB and the related pocket proteins, with much greater affinity than its low risk counterpart (Davies *et al.*, 1993; Dyson *et al.*, 1989, 1992; Münger *et al.*, 1989b). This binding results in enhanced degradation of RB via the ubiquitin-proteosome pathway (Boyer *et al.*, 1996). Therefore, by interfering with the two master regulators of the cell cycle, differentiation and apoptosis, HPV infection leads to uncontrolled cellular proliferation.

It has been found that most HPV-positive cervical carcinomas and carcinoma-derived cell lines lack *p53* and *Rb* mutations; this is unsurprising since high risk infection provides a means with which to inactivate these tumour suppressors (Crook *et al.*, 1991; Scheffner *et al.*, 1991). However, it has been demonstrated that mutation of *p53* and HPV infection are not mutually exclusive, suggesting that in at least some cases the gene mutation confers an additional growth advantage to the HPV infected cell (Fujita *et al.*, 1992; Hellend *et al.*, 1993; Kessis *et al.*, 1993). It has also been suggested that the acquisition of *p53* mutations may play a role in the progression of some HPV associated carcinomas, since *p53* point mutations have been identified more frequently in metastases arising from HPV-positive cervical carcinomas than in primary tumours (Crook & Vousden , 1992).

Previous work has shown that HPV16 E6 and E7 can both upregulate pol III transcription. For example, when a vector encoding wild-type p53 is transfected into p53-negative osteosarcoma cells, transcription of a pol III reporter gene is repressed. This effect can be overcome by expression of the E6 oncoprotein of HPV16 (Stein *et al.*, 2002a). It has also been shown that acute expression of HPV16 E7 in

untransformed RB-positive murine fibroblasts activates pol III transcription (Sutcliffe *et al.*, 1999). This is not an indirect response to cell transformation, because pol III is also activated by a nontransforming mutant version of E7 that has retained its ability to inactivate RB. In contrast, no stimulation is observed with deletions or substitutions in the pocket-binding domain of E7. It has also been demonstrated by affinity chromatography experiments that an E7 peptide can prevent UBF from binding to RB, whereas a mutant E7 peptide lacking the RB binding motif has no effect. Moreover, the inhibitory effects of RB on rDNA transcription are overcome by E7 (Cavanaugh *et al.*, 1995). This suggests that E7 can activate transcription by pols I and III by overcoming the repressive effects of the pocket proteins.

Although these observations suggest that HPV16 infection will increase pol I and III output, this remains to be tested in cervical cells, the principle context in which this virus impacts on human health. Therefore, the aim of this chapter was to investigate the effect of HPV infection in tissue samples removed during cervical biopsies.

3.2 Results.

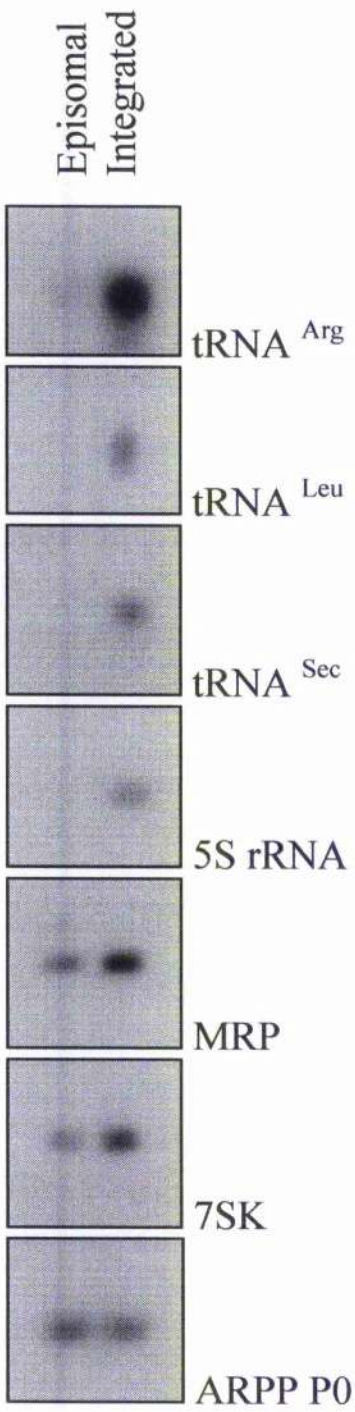
3.2.1 Integration of HPV16 Raises Expression of Pol III Transcripts.

During the development of cervical carcinomas, HPV DNA frequently becomes integrated into the host genome (Alazawi *et al.*, 2002; Boshart *et al.*, 1984; Scharz *et al.*, 1985). This results in elevated expression of both E6 and E7 (Alazawi *et al.*, 2002; Jeon *et al.*, 1995; Jeon & Lambert, 1995). To test whether integration of HPV16 results in changes in the expression of pol III transcripts, a cervical keratinocyte cell line (W12), generated from a squamous epithelial lesion, was investigated. The matched subclones 20863 and 20861, which carry either episomal or integrated copies of the viral DNA (Jeon *et al.*, 1995) were compared by RT-PCR. It was found that a range of pol III products were upregulated in the cells where HPV16 was integrated in comparison with cells where the viral DNA was episomal (Figure 3.1). tRNA ^{Arg}, ^{Leu}, ^{Sec} as well as 5S rRNA were strongly induced following HPV16 integration, whereas the increase in transcription observed with MRP and 7SK was less striking. This effect was specific, as no increase in transcription was observed in the mRNA encoding acid ribosomal phosphoprotein P0 (ARPP P0), which is synthesised by pol II. For tRNA ^{Leu} and tRNA ^{Arg}, which contain introns, the RT-PCR primers detect the short-lived primary transcript that is considered to provide a strong indicator of ongoing transcription, because of its rapid processing into mature tRNA (Winter *et al.*, 2000). In all the other cases, steady-state RNA levels were measured. These data suggest a marked and specific elevation of pol III-dependent gene expression when HPV16 integrates into the genome of cervical cells.

Figure 3.1

Pol III induction accompanies viral integration.

cDNAs were generated by reverse transcription of total RNA from W12 cells, which maintain the HPV16 genome episomally (subclone 20863; lane 1), and W12G cells (subclone 20861; lane 2), which have the HPV16 genome integrated. These cDNAs were PCR amplified using primers specific for tRNA ^{Arg}, tRNA ^{Leu}, tRNA ^{Sec}, 5S rRNA, MRP, 7SK and ARPP P0.


Episomal
Integrated
tRNA Arg
tRNA Leu
tRNA Sec
5S rRNA
MRP
7SK
ARPP P0

3.2.2 Cervical Biopsies Infected with HPV16 Express Elevated Levels of tRNA and 5S rRNA.

RT-PCR was also used to examine cervical biopsy samples. Initially ten samples were examined, seven of which were HPV16-positive, and three in which HPV was not detected. There was a clear correlation between the presence of HPV16 and an increase in transcription of the pol III templates tRNA ^{Arg}, ^{Leu}, ^{Sec}, and 5S rRNA (Figure 3.2). Since samples #2, 4, 6, 8 and 10 were normal tissue taken from areas adjacent to tumours, it is clear that this increase was independent of the disease status of the sample, as the HPV-16 infected normal samples displayed the same increase in pol III transcription as the tumours themselves. RT-PCRs show that levels of the primary transcript tRNA ^{Leu} in one HPV-negative tumour reached those of the least active HPV16-positive biopsy, although this was not the case for the other tRNAs. The significant elevation in pol III transcription observed in samples infected with HPV16 when compared with the HPV-negative biopsies was not seen in the pol II transcript ARPP P0.

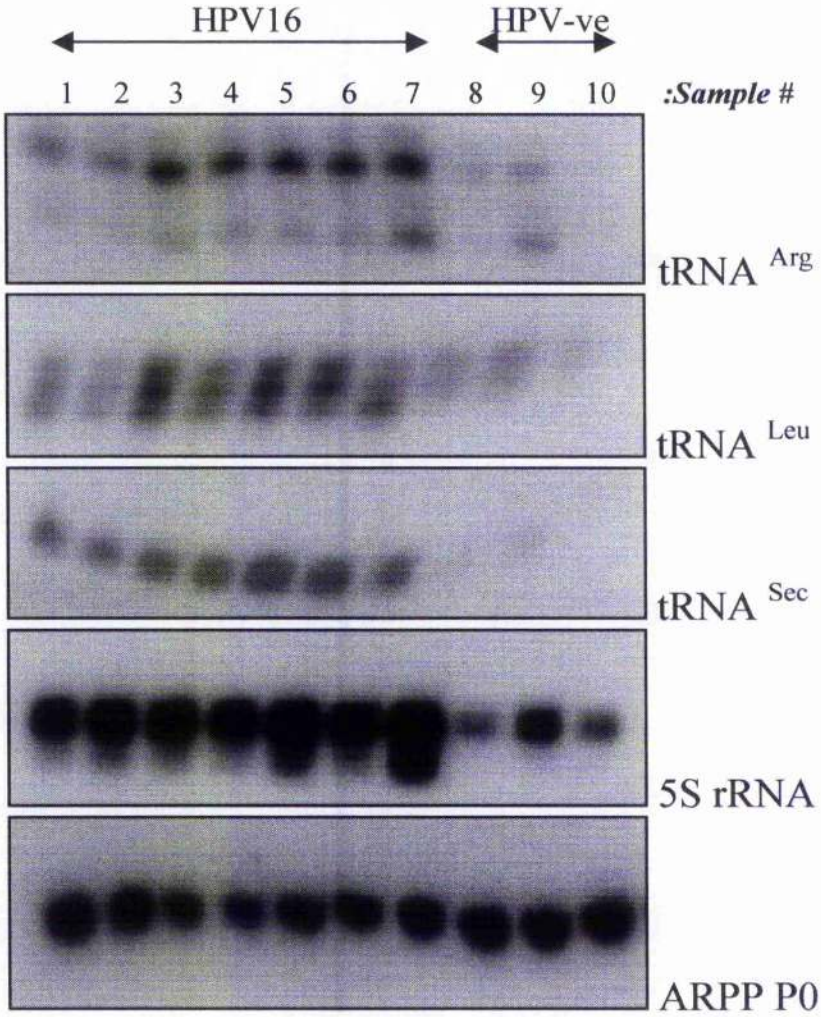
3.2.3 Genes with a Type III Promoter Structure are not Overexpressed in Cervical Biopsies Infected with HPV16.

When the pol III transcripts MRP and 7SK were examined, quite different expression patterns were observed (Figure 3.3). The presence of HPV16 in biopsy samples did not result in overexpression of these transcripts. While 7SK remained relatively constant, MRP varied markedly between samples, but not in a manner that correlated with HPV status or tRNA expression. These variations do not reflect differential RNA

Figure 3.2

The presence of integrated HPV16 DNA in human biopsies correlates with elevated expression of tRNA and 5S rRNA.

cDNAs were generated by reverse transcription of total RNA extracted from normal and tumour tissues. These cDNAs were PCR amplified using primers specific for $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Sec}$, 5S rRNA and ARPP P0.


HPV16
HPV-ve
1 2 3 4 5 6 7 8 9 10
:Sample #
tRNA Arg
tRNA Leu
tRNA Sec
5S rRNA
ARPP P0

extraction or integrity, as they were not found in the pol II derived ARPP P0 mRNA control.

3.2.4 The Presence of Integrated HPV16 DNA in Cervical Biopsies does not Correlate with any Change in Expression of mRNAs Encoding TFIIIC2.

As a variety of different genes transcribed by pol III are upregulated in samples infected with HPV16, the different transcription factors utilized at these templates were investigated. The five subunits of TFIIIC2, which are necessary for expression of tRNA and 5S rRNA, but are not used at type III promoters such as MRP and 7SK (Geiduschek & Kassavetis, 2001, Schramm & Hernandez, 2002; White, 1998), were examined by RT-PCR. Previous studies have shown that all five mRNAs encoding the subunits of TFIIIC2 are induced when fibroblasts are transformed by SV40 or polyomavirus (Felton-Edkins & White, 2002; Larminie *et al.*, 1999). Furthermore, adenoviral infection can selectively increase the level of TFIIIC110 (Sinn *et al.*, 1995). However, it was found that HPV16 infection did not deregulate transcription of the TFIIIC2 subunits. It was observed that the subunit's expression is not upregulated upon HPV16 infection (Figure 3.4). In fact, the expression of mRNAs encoding these subunits varied substantially between the different samples examined, but not in a way that reflected either HPV status or tumour status. Furthermore, there was no correlation between the relative levels of these mRNAs and pol III transcript expression. These data suggest that TFIIIC2 may not be limiting in cervical epithelium and that HPV16 does not induce expression of TFIIIC2 genes.

Figure 3.3

Genes transcribed by pol III that have a type III promoter structure are not overexpressed in cervical biopsy samples infected with HPV16.

cDNAs were generated by reverse transcription of total RNA extracted from normal and tumour tissues. These cDNAs were PCR amplified using primers specific for MRP, 7SK and ARPP P0.

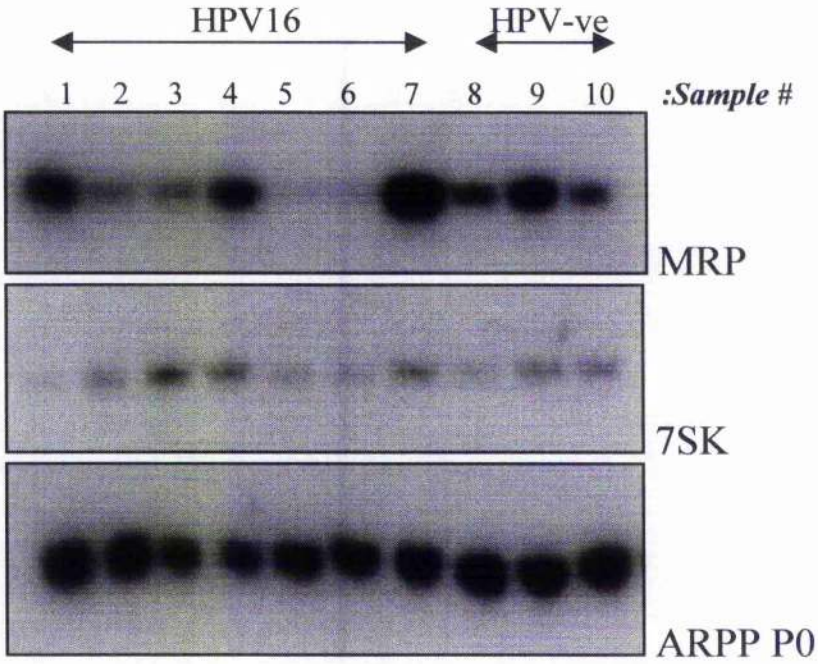
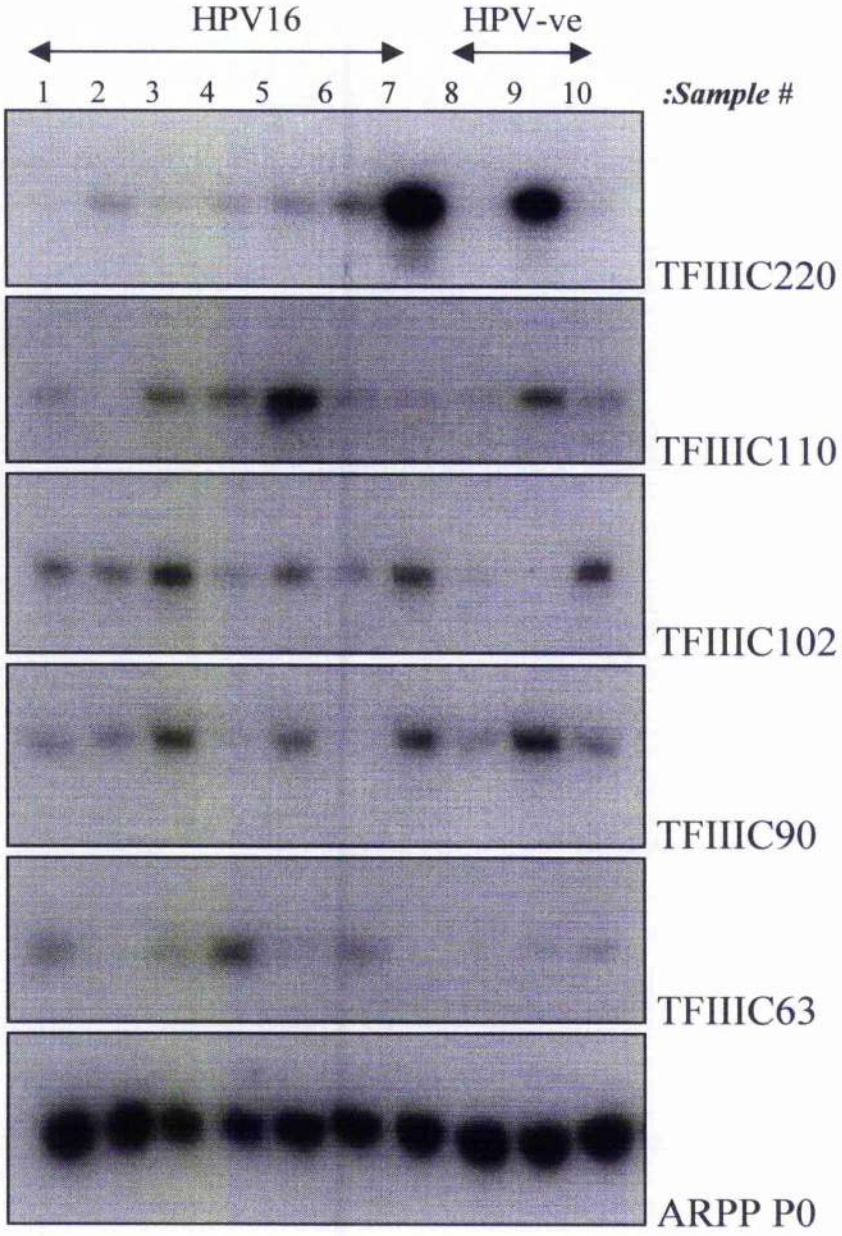

HPV16
HPV-ve
1 2 3 4 5 6 7 8 9 10
:Sample #
MRP
7SK
ARPP P0

Figure 3.4

The presence of integrated HPV16 DNA in human biopsies does not correlate with any change in expression of mRNAs encoding TFIIIC2.

cDNAs were generated by reverse transcription of total RNA extracted from normal and tumour tissues. These cDNAs were PCR amplified using primers specific for TFIIIC220, TFIIIC110, TFIIIC102, TFIIIC90, TFIIIC63 and ARPP P0.


HPV16
HPV-ve
1 2 3 4 5 6 7 8 9 10
:Sample #
TFIIIC220
TFIIIC110
TFIIIC102
TFIIIC90
TFIIIC63
ARPP P0

3.2.5 Brf1 is Upregulated in Cervical Biopsy Samples Infected with HPV16.

One of the important transcription factors that is utilized at all pol III templates is TFIIIB. RT-PCR was therefore used to assay the relative levels of the three subunits composing this transcription factor. It has been demonstrated that Bdp1 is strongly induced in fibroblasts by SV40 and polyomavirus, whereas TBP and Brf1 do not respond to these viruses (Felton-Edkins & White, 2002). HPV16 infection in these cervical samples did not have this effect. The Bdp1 and TBP subunits displayed variable levels of mRNA in the different samples examined, which did not reflect HPV status or expression of particular pol III products. However, the Brf1 subunit of TFIIIB was upregulated in the samples in which HPV16 was present (Figure 3.5). This correlated with the samples which displayed elevated levels of tRNA and 5S rRNA.

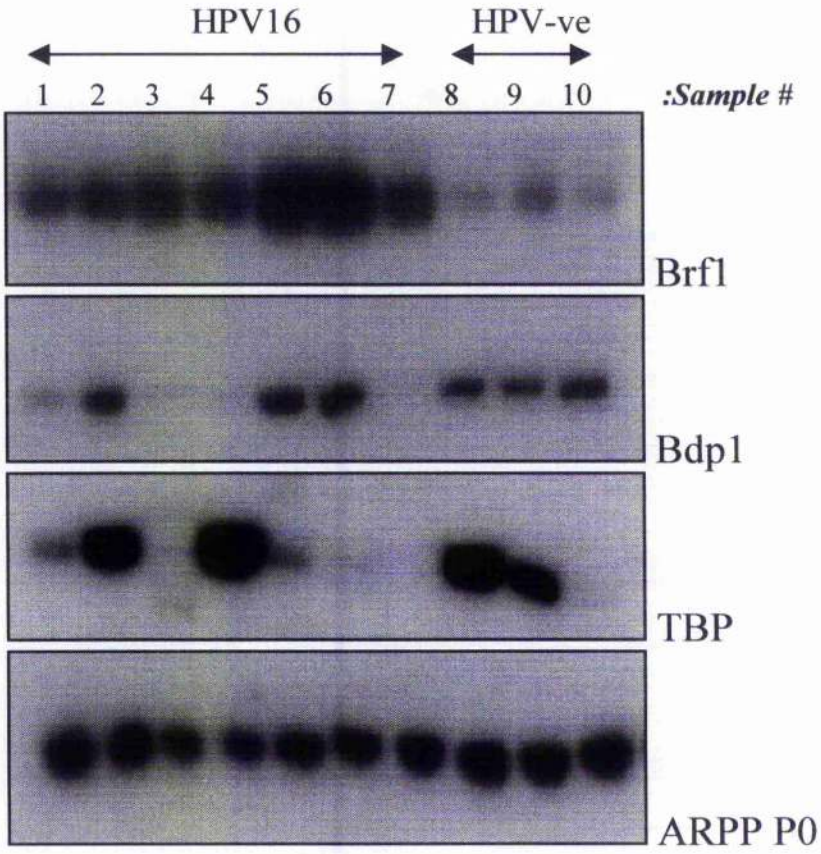
3.2.6 tRNAs and 5S rRNA are not Overexpressed in Cervical Biopsy Samples Infected with Medium or Low Risk HPVs.

Further cervical biopsies were investigated to examine whether presence of other HPV types also induced overexpression of pol III templates. Samples containing medium risk HPV33 and low risk HPV11 (Lorincz *et al.*, 1992) were compared to samples infected with HPV16 by RT-PCR. It was found that presence of HPV33 and

Figure 3.5

The Brf1 subunit of TFIIIB is upregulated in cervical biopsy samples infected with HPV16.

cDNAs were generated by reverse transcription of total RNA extracted from normal and tumour tissues. These cDNAs were PCR amplified using primers specific for Brf1, Bdp1, TBP and ARPP P0.


HPV16
HPV-ve
1 2 3 4 5 6 7 8 9 10
:Sample #
Brf1
Bdp1
TBP
ARPP P0

HPV11 did not increase transcription of tRNA ^{Arg}, ^{Leu}, ^{Sec} or 5S rRNA in comparison with the cervical biopsy samples that are HPV16-positive (Figure 3.6). As seen with the HPV16 infected samples, when RT-PCRs were carried out with primers for 7SK and MRP no increase in transcription was correlated with the presence of HPV33 or HPV11. However, a dramatic increase in transcription was observed for one of the HPV11-positive cervical samples (Figure 3.7). The elevated transcription in this particular sample was not seen with tRNA or 5S rRNA templates.

3.2.7 Brf1 is not Upregulated in Cervical Biopsy Samples Infected with Medium or Low Risk HPVs.

When the three subunits of TFIIIB were examined in these biopsy samples, it was found that Bdp1 showed a massive increase in transcription in the same HPV11-positive cervical sample that displayed overexpression of the 7SK and MRP transcripts. Although this suggests that very high levels of Bdp1 might activate 7SK and MRP gene transcription, there is no clear correlation between Bdp1, 7SK and MRP RNAs in the other biopsy samples (Figure 3.8). To investigate these high levels of expression in the HPV11 infected sample, PCRs were carried out on genomic DNA to examine if the Bdp1 gene had been amplified. However, no evidence of this event was discovered (Figure 3.9).

As with the samples infected with HPV16, there were variations in the levels of TBP in the different biopsy samples that were infected with HPV33 or HPV11. Brf1 levels also varied in HPV33, and HPV11-positive samples, but did not show the substantial

Figure 3.6

Pol III transcripts are only overexpressed in HPV16 infected cervical biopsies, and not in samples infected with medium or low risk HPVs.

cDNAs were generated by reverse transcription of total RNA extracted from normal and tumour tissues. These cDNAs were PCR amplified using primers specific for $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Sec}$, 5S rRNA and ARPP P0.

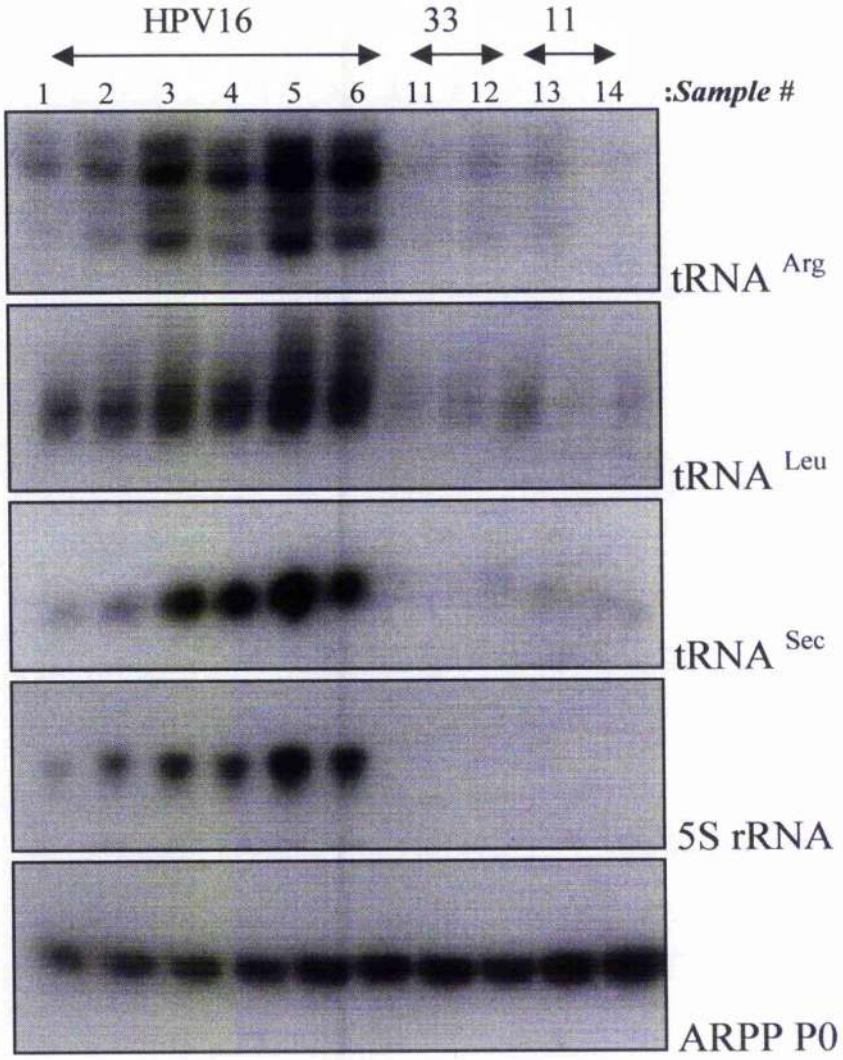
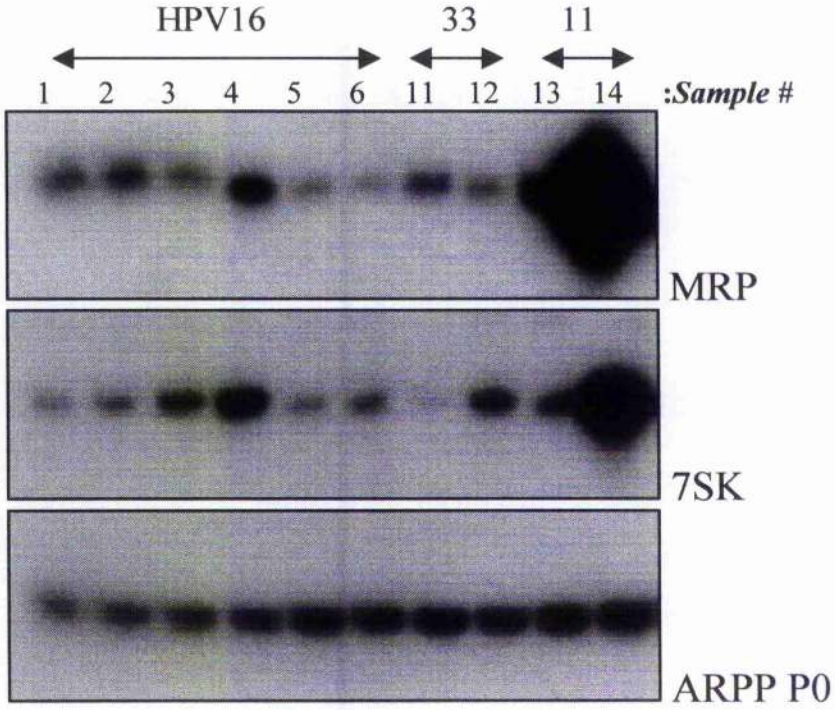

HPV16
33
11
1 2 3 4 5 6 11 12 13 14
:Sample #
tRNA Arg
tRNA Leu
tRNA Sec
5S rRNA
ARPP P0

Figure 3.7

Genes transcribed by pol III that have a type III promoter structure are not overexpressed in cervical biopsy samples infected with high, medium or low risk HPVs.

cDNAs were generated by reverse transcription of total RNA extracted from normal and tumour tissues. These cDNAs were PCR amplified using primers specific for MRP, 7SK and ARPP P0.


HPV16
33
11
1 2 3 4 5 6 11 12 13 14
:Sample #
MRP
7SK
ARPP P0

increase seen in the HPV16-positive samples. Although the biopsy samples that were HPV16-positive showed a correlation between presence of this high risk virus and upregulation of Brf1, the first sample examined (Figure 3.8) did not show elevated expression of this mRNA in comparison with the biopsy samples infected with medium or low risk HPVs. This sample also showed the lowest increase in tRNA and 5S rRNA expression. In contrast to the other biopsy samples examined, this sample maintained the viral genome episomally. This is consistent with the results from the W12 model, which suggest that pol III activity is enhanced by HPV16 integration. It also raised the possibility that viral integration may also stimulate Brf1 expression. To test this, the W12 model was investigated to examine whether the cells with integrated HPV16 contain higher levels of Brf1 mRNA than cells in which the viral genome remains episomal. Indeed, this was found to be true (Figure 3.10).

3.2.8 Raising the Concentration of TFIIIB Stimulates Pol III Transcription in Cervical Cells.

The correlation observed in biopsy samples between levels of tRNA, 5S rRNA and Brf1 mRNA suggests that availability of TFIIIB subunits may limit the rate of transcription of many pol III templates in cervical epithelium. It was therefore investigated whether raising the level of TFIIIB is sufficient to stimulate pol III transcription in extracts of cervical cells. These experiments used a crude fraction prepared by phosphocellulose chromatography. Upon incubation of cervical cell extracts with a PC-B fraction that is enriched for TFIIIB, transcription of the pol III

Figure 3.8

The Brf1 subunit of TFIIIB is only upregulated in cervical biopsy samples infected with high risk HPV16, and not in samples infected with medium or low risk HPVs.

cDNAs were generated by reverse transcription of total RNA extracted from normal and tumour tissues. These cDNAs were PCR amplified using primers specific for Brf1, Bdp1, TBP and ARPP P0.

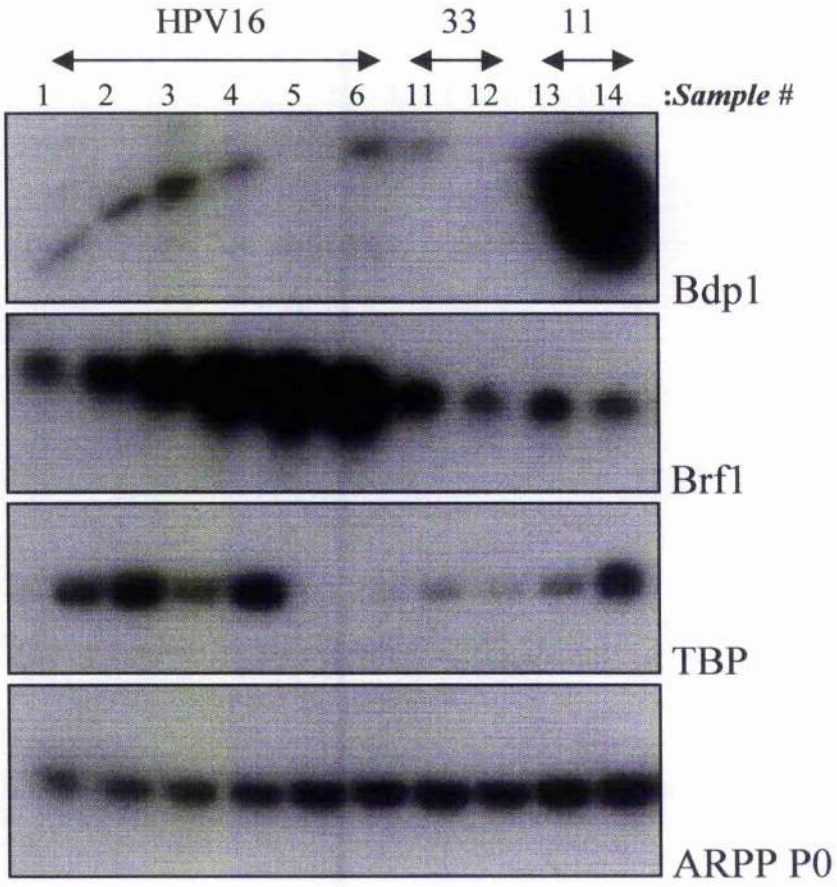

HPV16
33
11
1 2 3 4 5 6 11 12 13 14
:Sample #
Bdp1
Brf1
TBP
ARPP P0

Figure 3.9

Gene amplification was not detected in the HPV11 cervical biopsy sample that displayed massive upregulation of Bdp1 mRNA.

Genomic DNA from HPV11 infected cervical biopsy samples was PCR amplified using primers specific for Bdp1 and ARPP P0.

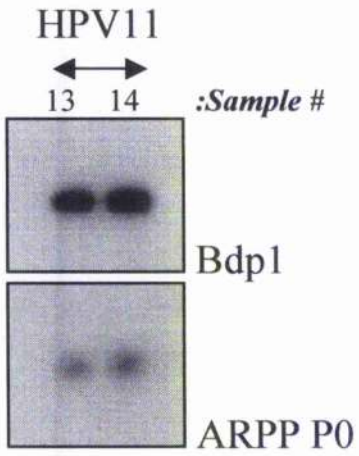

HPV11
13 14
:Sample #
Bdp1
ARPP P0

Figure 3.10

Viral integration upregulates expression of Brf1.

cDNAs were generated by reverse transcription of total RNA from W12 cells, which maintain the HPV16 genome episomally (subclone 20863; lane 1), and W12G cells (subclone 20861; lane 2), which have the HPV16 genome integrated. These cDNAs were PCR amplified using primers specific for Brf1 and ARPP P0.

Episomal
Integrated
Brf1
ARPP P0

templates VA1 and tRNA ^{Leu} was stimulated in a dose dependent manner (Figure 3.11).

3.2.9 Brf1 is a Limiting Factor for Pol III Transcription in Cervical Cells.

Previous work showed that pol III transcription increases markedly at the G1/S phase transition due to a substantial increase in TFIIIB activity as it dissociates from RB and p130 (Scott *et al.*, 2001; White *et al.*, 1995a). The response to Brf1 in both G1 and S phases was therefore examined. In experiments carried out by J. Fairley (University of Glasgow), cervical cells were transfected with an expression vector encoding Brf1 and were then synchronized using either thymidine or nocodozole blocks to arrest cell cycle progression; after release from these synchronizing blocks, the cells were harvested in either early G1 or S phase. As previously demonstrated with nuclear run-on assays (White *et al.*, 1995a), pol III transcription is substantially less active before the G1/S transition. Transfection of Brf1 only marginally increased transcription of a 5S rRNA gene in S phase, but it strongly stimulated expression in G1 phase cells (Figure 3.12). However, Brf1 markedly increased tRNA ^{Leu} gene transcription in both G1 and S phase cells. In contrast, Brf1 had no effect on transcription of 7SK. These data provide clear evidence that an increase in Brf1 levels can selectively stimulate synthesis of tRNA and 5S rRNA in cervical cells. This may explain the observed correlation in cervical biopsies between levels of these pol III products and the Brf1 mRNA.

Figure 3.11

Raising the concentration of TFIIIB in cervical cells stimulates pol III transcription of genes encoding VA1 and tRNA ^{Leu}.

C-33A whole cell extracts were prepared and incubated with a phosphocellulose B fraction enriched with TFIIIB. *In vitro* transcription assays were then carried out with the pol III templates VA1 or tRNA^{Leu}.

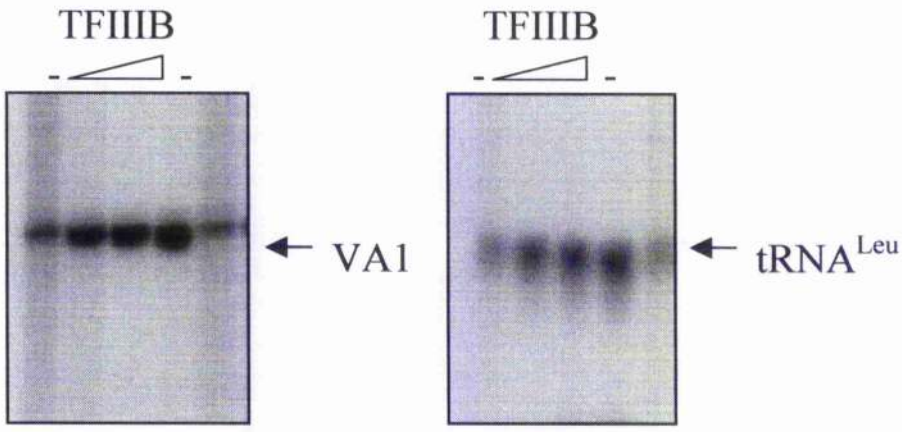
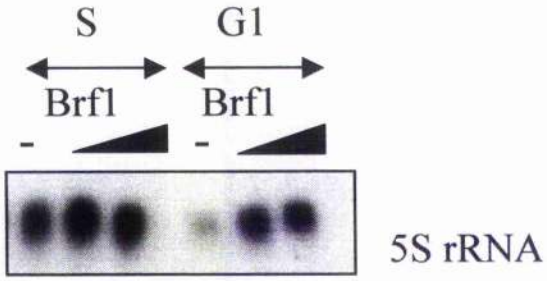

TFIIIB
-
-
VA1
TFIIIB
-
-
tRNA^{Leu}

Figure 3.12

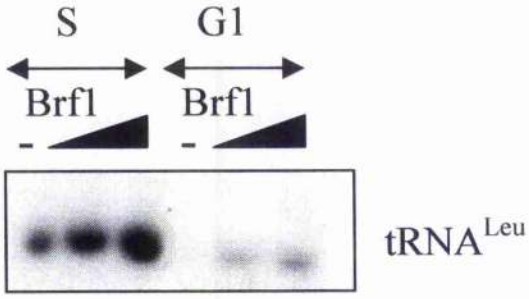
Raising the concentration of Brf1 in cervical cells stimulates pol III transcription of genes encoding tRNA and 5S rRNA, but not 7SK RNA.

(*A*) Transcription of a 5S rRNA gene using extracts made from HeLa cells transfected with empty vector (10μg lanes 1 and 4; 5μg lanes 2 and 5) and pcDNA3HA.BRF (5μg lanes 2 and 5; 10μg lanes 3 and 6). After transfection, the cells were synchronized in S (lanes 1-3) or G1 (lanes 4-6) phases prior to harvesting. (*B*) As above, except that a tRNA ^{Leu} gene was used as transcription template. (*C*) Transcription of a 7SK gene using extracts made from HeLa cells transfected with empty vector (lanes 1 and 3) or 10 μg of pcDNA3HA.BRF (lanes 2 and 4). After transfection, the cells were synchronized in G1 (lanes 1 and 2) or S (lanes 3 and 4) phases prior to harvesting. Experiments carried out by J. Fairley, University of Glasgow.

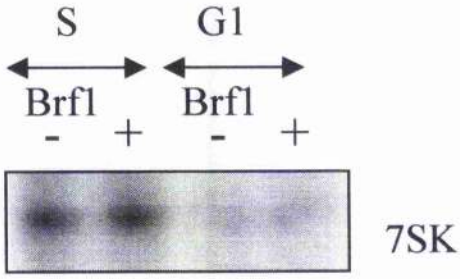
(A)



(B)



(C)



3.2.10 Pol I Transcripts are Overexpressed in HPV16 Infected Cervical Biopsy Samples.

RT-PCRs were also carried out to investigate if the presence of HPV in cervical biopsy samples would have an effect on pol I transcription. It was found that the three processed transcripts of pol I, 5.8S rRNA, 18S rRNA and 28S rRNA, each showed overexpression in the samples infected with HPV16 (Figure 3.13). These are the same samples which displayed elevated levels of pol III transcription. As seen with the pol III transcripts tRNAs and 5S rRNA, this overexpression only occurred in cervical biopsy samples infected with high risk HPV16 and not in the medium risk HPV33, or low risk HPV11 infected samples (Figure 3.14).

Figure 3.13

Pol I transcripts are overexpressed in HPV16 infected cervical biopsy samples.

cDNAs were generated by reverse transcription of total RNA extracted from normal and tumour tissues. These cDNAs were PCR amplified using primers specific for 28S rRNA, 18S rRNA, 5.8S rRNA and ARPP P0.

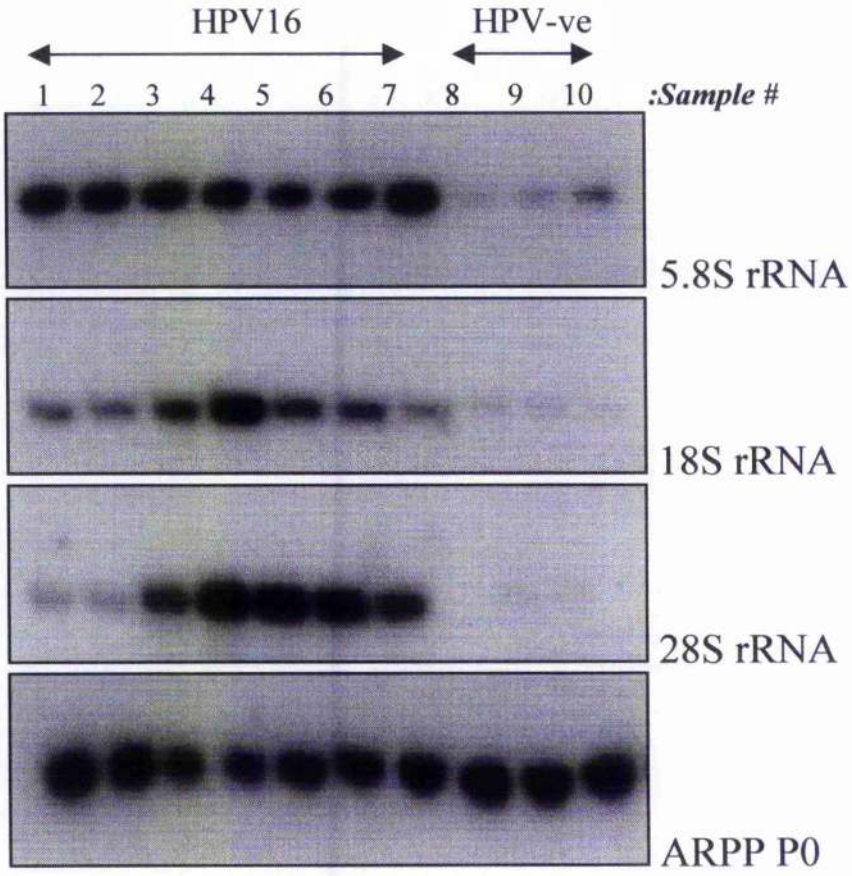
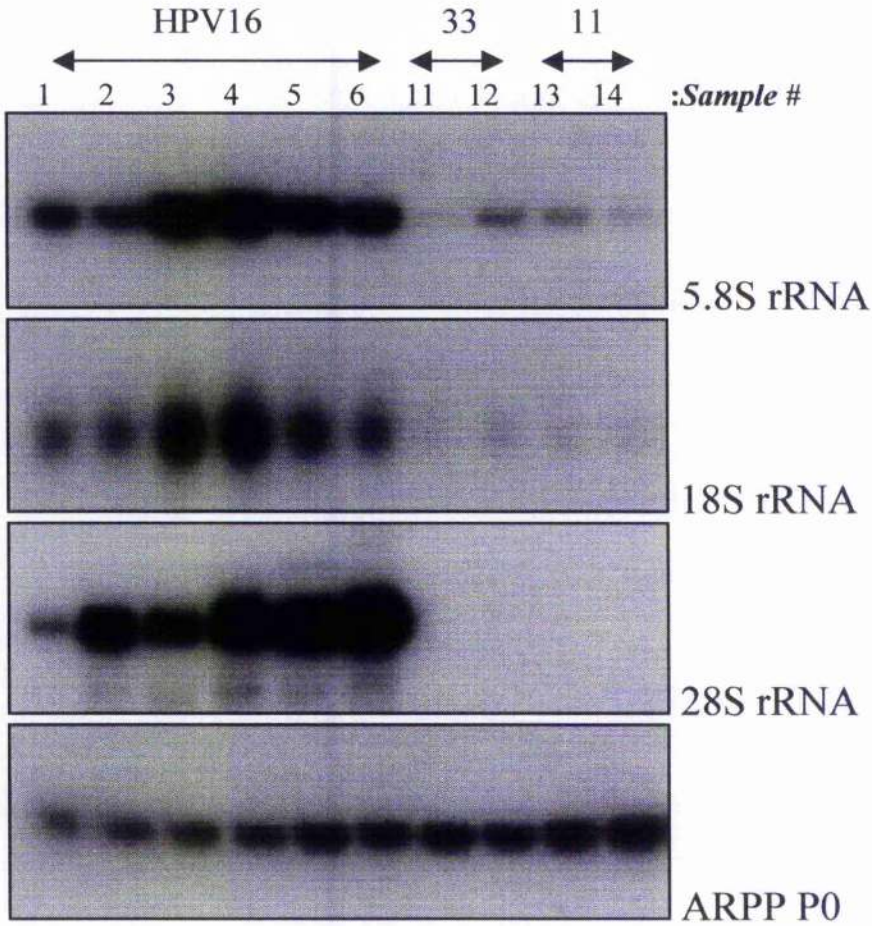

HPV16
HPV-ve
1 2 3 4 5 6 7 8 9 10
:Sample #
5.8S rRNA
18S rRNA
28S rRNA
ARPP P0

Figure 3.14

Pol I transcripts are only overexpressed in HPV16 infected cervical biopsies and not in samples infected with medium or low risk HPVs.

cDNAs were generated by reverse transcription of total RNA extracted from normal and tumour tissues. These cDNAs were PCR amplified using primers specific for 28S rRNA, 18S rRNA, 5.8SrRNA and ARPP P0.


HPV16
33
11
1 2 3 4 5 6 11 12 13 14
:Sample #
5.8S rRNA
18S rRNA
28S rRNA
ARPP P0

3.3 Discussion.

The presence of the high risk HPV16 in human cervical biopsy samples, but not lower risk HPVs, correlates with a specific increase in the expression of genes encoding tRNA ^{Arg}, ^{Leu}, ^{Sec} and 5S rRNA. This was shown by RT-PCR to correlate with the expression of Brf1. It was also found that raising the concentration of Brf1 in cervical epithelial cells increases transcription of tRNA ^{Leu} and 5S rRNA genes.

It was also demonstrated that the integration status of the virus correlates with pol III transcription. In a sample in which the HPV16 genome is maintained episomally, levels of Brf1 as well as the aforementioned pol III transcripts were overexpressed to a lesser extent than in the samples in which the HPV16 genome was integrated. Thus, Brf1 appears to have a major role in regulating the expression of the pol III transcripts tRNA and 5S rRNA. Due to the very limited amounts of biopsy material available, it was not possible to analyse this transcription factor at the protein level. Therefore, in interpreting these results, the assumption is made that transcription factor levels in these tissue samples reflect the expression of their corresponding mRNAs. Thus, it is assumed that tumours with high Brf1 mRNA are likely to have elevated Brf1 protein content. However, this might not be the case, as posttranscriptional controls may have a significant influence.

Unlike SV40, polyomavirus, and adenovirus (Felton-Edkins & White, 2002; Larminie *et al.*, 1999; Sinn *et al.*, 1995), the presence of HPV did not deregulate expression of the mRNAs encoding the TFIIIC2 subunits. Evidently, TFIIIC2 expression does not respond to all types of DNA tumour virus. Another group has shown that the oncoprotein from a further DNA virus, HBV, stimulates pol III transcription by

increasing TBP expression (Wang *et al.*, 1997). Although the levels of TBP mRNA fluctuated dramatically in the cervical biopsies analysed, they did not correlate with any of the pol III transcripts examined. It has also been demonstrated that SV40 or polyomavirus transformed fibroblasts display increased levels of Bdp1 (Felton-Edkins & White, 2002). In the cervical biopsy samples, like TBP, the levels of Bdp1 mRNA varied markedly, showing little correlation with expression of pol III templates, suggesting that both factors may be in relative excess for pol III transcription in the cervix.

7SK and MRP RNAs respond very differently from tRNA and 5S rRNA. In the W12 model system, cells which have the HPV16 integrated into the host genome show slightly higher expression of these transcripts than the cells that maintain HPV16 episomally. However, 7SK and MRP levels in human tissue do not correlate with viral status. It was also observed that raising the concentration of Brf1 in cervical epithelial cells had no effect on transcription of 7SK. The distinct response of 7SK and MRP might reflect their very different promoter arrangements from those of 5S rRNA and tRNA genes. Unlike tRNA and 5S rRNA genes, which have internal promoters, 7SK and MRP genes do not utilize TFIIIC2 or Brf1 (Schramm & Hernandez, 2002). However, they do require TBP and Bdp1 (Schramm & Hernandez, 2002), and it is striking that 7SK and MRP are both expressed at high levels in a tumour that selectively overproduces Bdp1. However, neither TBP nor Bdp1 mRNA expression correlates with 7SK or MRP RNA in the other biopsy samples. Availability of some other factor (e.g. PTF) may therefore limit transcription from external pol III promoters in cervical epithelium. These results suggest that transcription of MRP and 7SK genes might be activated by very high levels of Bdp1, even if it is not normally

limiting. It is not clear what caused the dramatic overexpression of Bdp1 mRNA in the tumour in biopsy #14, but no evidence for gene amplification was found.

As well as correlating with an increase in pol III transcription, infection of cervical epithelium with HPV16 also correlated with elevated levels of pol I products. The three processed transcripts, 5.8S rRNA, 18S rRNA and 28S rRNA, were found to be overexpressed in samples that were HPV16-positive, but not in samples infected with HPV33 or HPV11.

Alterations in the levels of pol I and pol III transcription in HPV16 infected cervical samples may be explained by the fact that this high risk virus expresses the oncoproteins E6 and E7 which can inactivate tumour suppressor genes (Larminie *et al.*, 1999; Stein *et al.*, 2002a; Sutcliffe *et al.*, 1999). E6 is able to inactivate p53, which causes the pol III transcription factor TFIIIB and the pol I transcription factor SL1 to be released from repression. E7 inactivates RB, which also causes the release of TFIIIB, and the pol I transcription factor UBF (Figure 3.15). Both these oncoproteins from high risk HPV16 bind to these tumour suppressors with a higher affinity than the proteins from lower risk HPVs. This might explain why HPV33 and HPV11 do not elevate pol III transcription. Viral integration stimulates production of E6 and E7 and also correlates in cultured W12 cells with increased levels of pol III transcripts and Brf1 mRNA, although no increase was observed when the processed transcripts of pol I were examined in this system (data not shown). It is striking that HPV16 seems to have evolved an additional mechanism for activating the pol III system. The benefit for HPV16 of targeting Brf1 can be readily explained by the fact

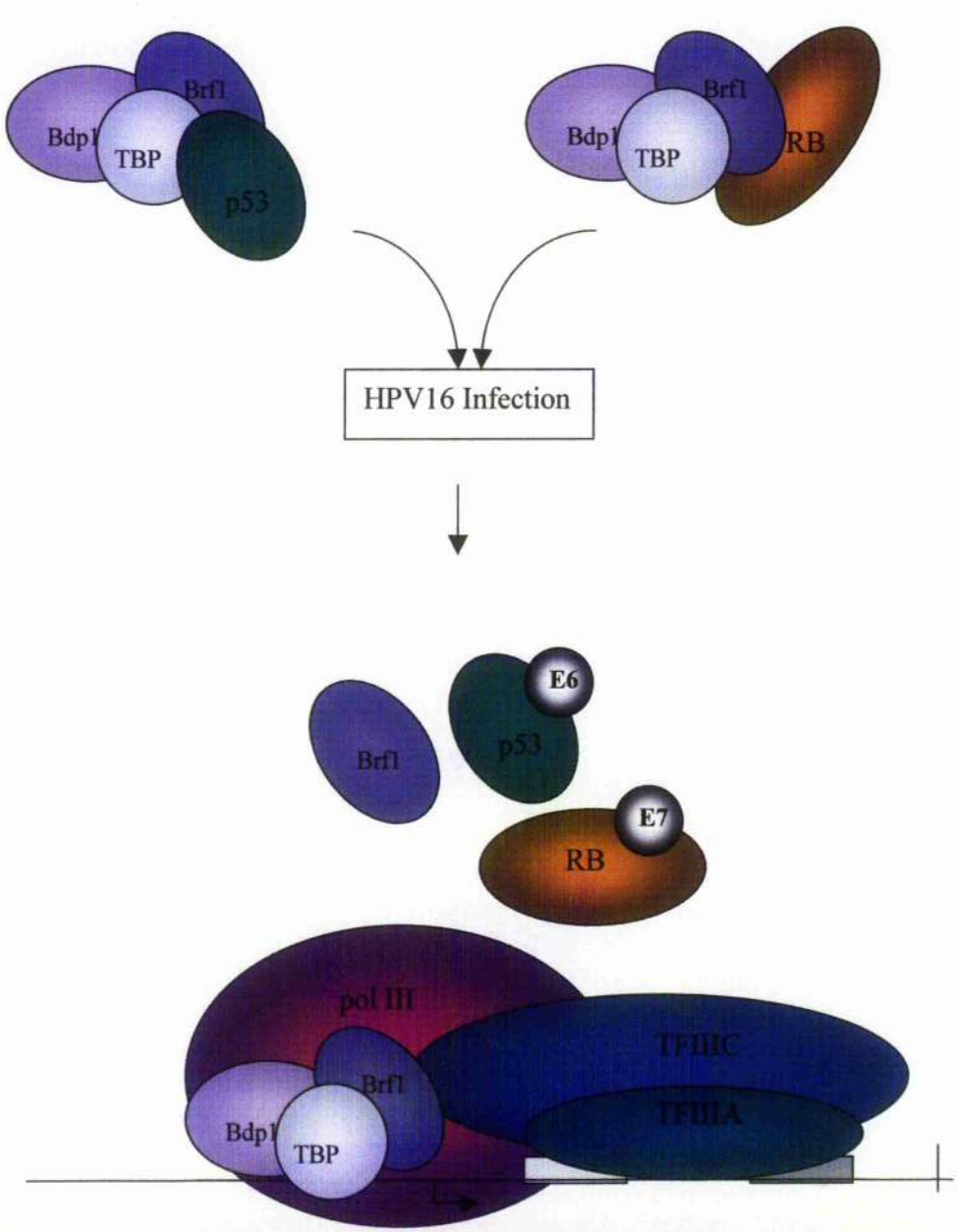
that it is limiting for tRNA and 5S rRNA synthesis in cervical cells. Clearly, the most effective transcriptional response can be obtained by regulating a limiting factor.

Activation of pols I and III was seen to occur as an early response that precedes transformation, as transcriptional upregulation is seen in premalignant cervical biopsy samples, relative to uninfected normal tissue. The high rates of synthesis of tRNA and rRNA may be advantageous to the virus, perhaps by raising translational capacity to facilitate production of viral proteins. It is striking that this mechanism is associated with the highly oncogenic HPV16, but is not apparent with HPV types that are less likely to promote malignant progression.

Figure 3.15

Model showing the effect of HPV16 on pol I and pol III transcription.

In uninfected cervical epithelial cells, rates of pol I and pol III transcription are repressed by the tumour suppressor proteins p53 and RB. Presence of HPV16 triggers an increase in the levels of the TFIIIB subunit Brf1, whilst the E6 and E7 viral oncoproteins bind to p53 and RB respectively, thereby derepressing TFIIIB and the pol I transcription factors SL1 and UBF.


Brf1
Bdp1
TBP
p53
Brf1
Bdp1
TBP
RB
HPV16 Infection
Brf1
E6
p53
E7
RB
pol III
TFIIIC
Brf1
TFIIIA
Bdp1
TBP
p53
SL1
UBF
RB
HPV16 Infection
E6
p53
E7
RB
CBP
UBF
Rrn3
TFIIH
pol I
SL1

Chapter 4

Breast Cancer

4.1 Introduction.

Breast cancer is by far the most common cancer for women. More than one in four of all female cancers in the UK occur in the breast. In 1999, there were approximately 41,000 new cases and 13,000 deaths from this cancer in UK women (Cancer Research UK, 2003a, b). Breast cancer can also occur in men. In the UK there are around 300 cases diagnosed in men each year (Cancer Research UK, 2003a). The development of breast cancer results from the interplay of dietary, lifestyle and environmental factors on the genetic background of the individual. Hormone levels have also been implicated in breast cancer. Recently, strong links between hormone replacement therapy and the development of such cancer have been established. A study involving a million women has concluded that women taking combination hormone replacement therapy may be twice as likely to develop breast cancer (Beral *et al.*, 2003).

Breast carcinoma arises from the epithelium of the mammary gland. The transition from normal to malignant breast epithelium involves a transformation of the breast epithelium from normal to hyperplastic, followed by the appearance of atypia in association with the hyperplasia, ultimately becoming malignant. Malignant cells continue to evolve from non-invasive carcinoma to invasive carcinoma and, ultimately, to cells with metastatic potential (Couch & Weber, 2002).

The best-studied risk factor in the development of breast cancer is a family history of the disease. It is estimated that 15 to 20 % of women with breast cancer fall into this category, with approximately 5% of breast cancers attributable to dominant susceptibility alleles. Two major breast cancer susceptibility genes (BRCA1 and BRCA2) have been identified (Miki *et al.*, 1994; Tavtigian *et al.*, 1996; Wooster *et*

al., 1995). These encode multifunctional proteins involved in maintaining genomic stability, the cellular response to DNA damage, transcriptional regulation, and cellular proliferation (Welcsh *et al.*, 2000).

BRCA1 is a highly penetrant breast cancer susceptibility gene on chromosome 17q21 (Miki *et al.*, 1994). It is thought to account for 20 to 30% of inherited breast cancers (Couch *et al.*, 1997; Frank *et al.*, 1998; Shattuck-Eidens *et al.*, 1997; Stoppa-Lyonnet *et al.*, 1997). Families with germ-line mutations in BRCA1 have an autosomal dominant inheritance pattern of breast cancer, as well as an increased incidence of ovarian cancer. Over 250 different disease-causing mutations, scattered throughout the gene have been discovered (Blackwood & Weber, 1998; Couch & Weber, 1996). These variants are distributed along the entire coding region of the gene.

BRCA2 is a highly penetrant breast cancer susceptibility gene on chromosome 13q12-13 (Wooster *et al.*, 1994). It is thought to account for 10 to 20% of inherited breast cancers (Szabo & King, 1997). Families with germ-line mutations in BRCA2 also have an autosomal dominant inheritance pattern of breast cancer, an increased incidence of ovarian cancer that is less striking than that with BRCA1, and an increased incidence of male breast cancer. Over 100 mutations have been described in BRCA2 (Couch *et al.*, 1996; Lancaster *et al.*, 1996; Miki *et al.*, 1996; Phelan *et al.*, 1996; Serova *et al.*, 1997; Wooster *et al.*, 1994). Like BRCA1, these are not confined to a particular region of the gene.

Other familial breast cancer diseases include the rare Li-Fraumeni syndrome, where mutations in *p53* are inherited (Malkin *et al.*, 1990; Srivastava *et al.*, 1990).

Individuals affected by this disease are susceptible to various types of cancer, predominantly breast carcinomas, soft tissue sarcomas, and brain tumours. Approximately half the members of Li-Fraumeni families who inherit a mutated *p53* allele develop cancer before they reach 30 years of age (Varley *et al.*, 1997). It has been found that pol III transcriptional activity is often highly elevated in primary fibroblasts from Li-Fraumeni patients, especially if germline p53 mutation is followed by loss of the remaining allele (Stein *et al.*, 2002a).

In addition to individuals with Li-Fraumeni syndrome, p53 is also found mutated in patients with sporadic breast cancer. In fact, alterations in this tumour suppressor gene are the most frequent genetic changes found in breast cancer, and have been detected in 15-45% of human breast cancer specimens in several studies (Andersen *et al.*, 1993; Deng *et al.*, 1994; Elledge *et al.*, 1993; Saitoh *et al.*, 1994). These changes often result in overexpression of mutant p53 protein, which is associated with poor prognosis (Allred *et al.*, 1993; Isola *et al.*, 1992; Silverstrini *et al.*, 1993; Thor *et al.*, 1992). Nuclear accumulation of this protein is found predominantly in the more advanced tumours (Domagala *et al.*, 1993; Isola *et al.*, 1992). This agrees with results obtained with transgenic *p53* knockout mice (Kemp *et al.*, 1993), which suggest that absence of wild-type p53 is important for progression, rather than initiation, of tumour development.

Rb also plays a role in breast cancer, but it has not yet been as well characterised as *p53*. Estimates suggest that *Rb* may be mutated in approximately 20% of breast cancers (Fung & T'Ang, 1992). Loss of RB expression correlates with the progression of the disease, and especially with the inability of cells to differentiate. A

study of 197 breast cancer specimens using immunohistochemistry suggested that loss of RB expression was correlated with the presence of lymph node metastasis (Sawan *et al.*, 1992).

As well as containing mutations in the tumour suppressor genes *p53* and *Rb*, sporadic breast cancers have also been shown to contain activated cellular oncogenes such as *myc*. Several reports indicate that genetic alterations of *c-myc* play an important role in the induction and progression of human breast cancer (Leder *et al.*, 1986; Roux-Dossetto *et al.*, 1992; Sierra *et al.*, 1999; Watson *et al.*, 1993, 1996). The presence of c-*myc* amplification has been demonstrated in 10-40% of breast cancers (Berns *et al.*, 1992, 1996; Chrzan *et al.*, 2001; Pertsouk *et al.*, 1993; Watson *et al.*, 1993, 1996). Overexpression of c-Myc has been related to poor prognosis (Berns *et al.*, 1992, 1996; Borg *et al.*, 1992), and is thought to occur at an early stage of breast cancer (Pietilainen *et al.*, 1995; Watson *et al.*, 1993). This overexpression to some extent correlates with gene amplification, but not all tumours with c-*myc* amplification display increased levels of this oncoprotein, and some tumours display c-Myc overexpression without gene amplification (Bruggers *et al.*, 1998; Pertsouk *et al.*, 1993).

Another proto-oncogene that is sometimes altered in breast cancer is *ras*. Although mutations in the coding region of *ras* occur in less than 10% of breast cancers, there is evidence that the Ras signalling pathway may be deregulated in breast cancer more frequently (reviewed in Couch & Weber, 2002). In a study of 85 breast cancer specimens, immunohistochemical staining was used to detect the presence of multiple oncogene products, including H-Ras (Bland *et al.*, 1995). Coexpression of c-Myc and

H-Ras with c-Fos correlated with an increased likelihood of recurrence, and decreased survival. Immediate or early co-expression of the three oncogene products in neoplastic transformation resulted in an aggressive phenotype in invasive carcinoma cells (Bland *et al.*, 1995). In addition to its role in sporadic tumours, there is evidence to suggest that H-*ras* alleles are associated with inherited susceptibility to breast cancer (Conway *et al.*, 1995). It has been suggested that these alleles may be associated with altered penetrance of the BRCA1 gene (Phelan *et al.*, 1996).

Analysis of breast cancer cells has shown frequent mutations in a number of genes, however, it is not yet clear which genetic changes are most important and whether they generally arise in a particular order. As a developing breast carcinoma grows and undergoes these changes in gene expression, the demands on the protein biosynthetic machinery are likely to be affected. This chapter therefore investigates transcription by pols I and III in breast tumours, to examine if deregulation occurs.

4.2 Results.

4.2.1 Pol III Transcripts are Overexpressed in Half of the Breast Tumour Biopsies Examined.

It has been previously shown that ovarian tumours overexpress some pol III transcripts (Winter *et al.*, 2000). To investigate pol III transcription in breast tumours, RT-PCR reactions were carried out with cDNAs generated using RNA extracted from the matched normal and tumour breast tissue samples. Using primers specific for tRNA ^{Leu}, ^{Sec}, 5S rRNA, and 7SL it was found that half of the tumour samples examined (#1, 5, 6) had elevated levels of these transcripts in comparison to the matched normal samples (Figure 4.1). The other three tumour samples displayed decreased levels of these transcripts. These changes are specific, as the levels of mRNA encoding the RNA polymerase II transcript ARPP P0 remained constant.

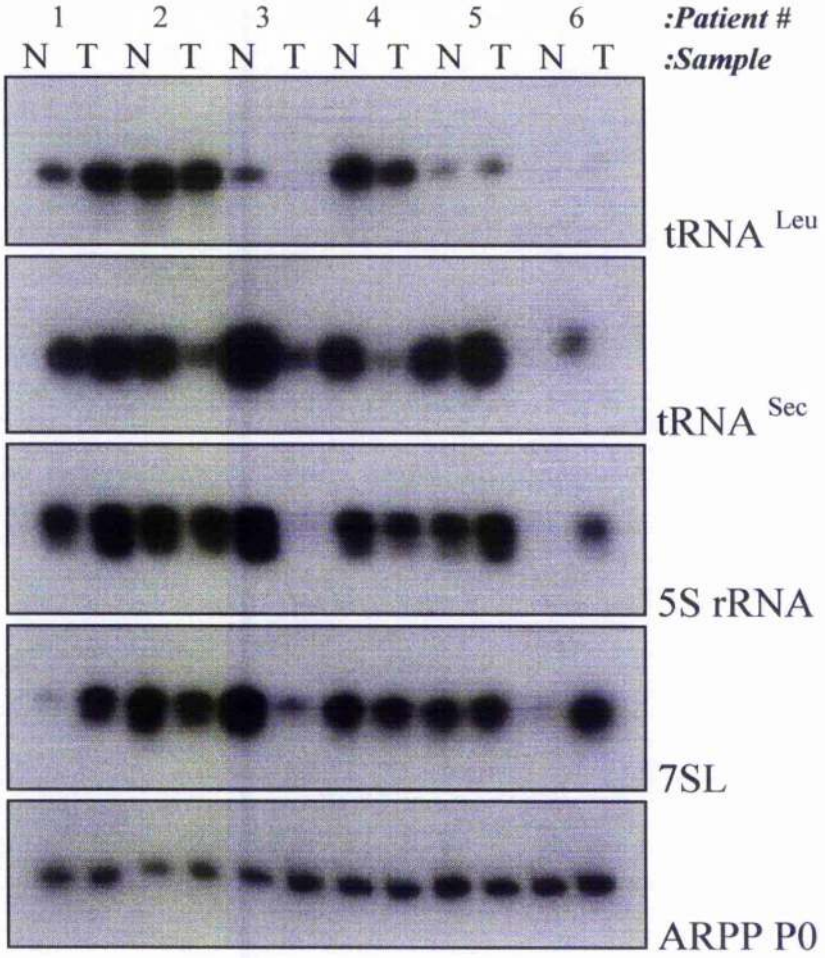
4.2.2 Genes with a Type III Promoter Structure are Overexpressed in Breast Tumour Biopsy Samples.

To examine if genes with type III promoter structures display the same expression pattern as observed with the type I and II promoters, MRP and 7SK transcripts were examined. As previously seen in cervical samples, it was found that in breast tumours these transcripts displayed quite different expression patterns than the other pol III genes examined (Figure 4.2). RT-PCRs revealed that both MRP and 7SK were upregulated in five of the six breast tumour samples examined in comparison with the

Figure 4.1

Pol III transcripts are overexpressed in half of the breast tumour biopsy samples examined.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour tissues. These cDNAs were PCR amplified using primers specific for $tRNA^{Leu}$, $tRNA^{Sec}$, 5S rRNA, 7SL and ARPP P0. N = normal, T = tumour.


1 2 3 4 5 6
:Patient #
N T N T N T N T N T N T
:Sample
tRNA Leu
tRNA Sec
5S rRNA
7SL
ARPP P0

matched normal breast samples. The exception to this upregulation was the tumour sample from patient #3, which displayed a decrease in the level of these pol III transcripts. Interestingly, the samples from this patient also showed a large decrease in the transcription of other pol III transcripts examined in Figure 4.1.

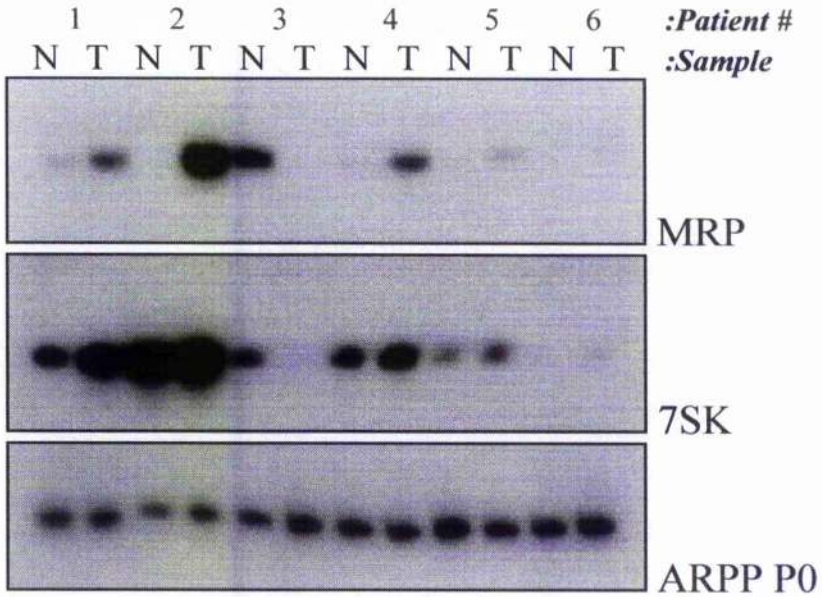
4.2.3 Transcription of the TFIIIC2 Subunits is Co-regulated in Breast Biopsy Samples.

To further investigate this deregulation, the assembly factors used to initiate transcription were investigated. The TFIIIC2 subunits which are utilized at type I and II promoters were examined by RT-PCR. It was observed that all the subunits of TFIIIC2 were upregulated in the breast tumour samples from patients #1 and 6, when compared to matched normal tissue (Figure 4.3). These samples displayed elevated levels of all of the pol III transcripts examined. The tumour sample from patient #5, which showed upregulation of pol III transcripts, did not display elevated levels of the TFIIIC2 subunits when compared to the normal tissue. The five subunits of TFIIIC2 were co-regulated in the biopsy samples, with the exception of patient #4. The relative levels of mRNAs for each subunit fluctuated by approximately the same amount when tumour biopsies were compared to normal breast tissue in each of the patients. In the biopsy samples from patients #2, 3 and 5, levels of TFIIIC2 were observed to be lower in the tumour samples relative to the matched normal tissue. The tissue samples from patient #4, did not display this co-regulation. Expression of TFIIIC110 and TFIIIC63 was found to be elevated in this breast tumour sample in comparison with the normal tissue. TFIIIC220 and TFIIIC102 remained relatively constant between the

Figure 4.2

Genes transcribed by pol III that have a type III promoter structure are overexpressed in breast tumour biopsy samples.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour tissues. These cDNAs were PCR amplified using primers specific for MRP, 7SK and ARPP P0. N = normal, T = tumour.


1 2 3 4 5 6
:Patient #
N T N T N T N T N T N T
:Sample
MRP
7SK
ARPP P0

normal and tumour samples, while levels of TFIIIC90 were seen to decrease in the tumour sample. Although an exact correlation between TFIIIC2 and pol III transcript levels was not found in these samples, it is possible that this transcription factor may be a limiting factor in some breast tumours. There is a fairly good correlation between TFIIIC2 mRNA and pol III transcript levels in four of the cases. However, both sets of transcripts behave inconsistently in patient #4, whereas in patient #5 there is a uniform decrease in TFIIIC2 mRNA that is not reflected by pol III transcript levels.

4.2.4 The Brf1 Subunit of TFIIIB is Upregulated in Breast Tumour Biopsy Samples.

To assess whether TFIIIB is deregulated in breast cancer, the three subunits of this transcription factor were examined by RT-PCR. It was found that the levels of Bdp1 mRNA remained relatively constant apart from an increase in patient #1 (Figure 4.4). TBP was found to be elevated in half of the tumour samples, in comparison with the matched normal breast tissue (patients #1, 4, 6). Again, this transcription factor did not correlate with the expression pattern of the pol III transcripts. Brf1 mRNA was found to be elevated in all except one (#2) of the tumour samples.

4.2.5 Increasing the Concentration of TFIIIB and TFIIIC2 in Breast Cells Stimulates Transcription of tRNA ^{Leu}.

To further examine the RT-PCR observations, it was investigated whether raising the level of TFIIIB or TFIIIC2 is sufficient to stimulate pol III transcription in extracts of

Figure 4.3

Transcription of the TFIIIC2 subunits is co-regulated in breast biopsy samples.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour tissues. These cDNAs were PCR amplified using primers specific for TFIIIC220, TFIIIC110, TFIIIC102, TFIIIC90, TFIIIC63 and ARPP P0. N = normal, T = tumour.

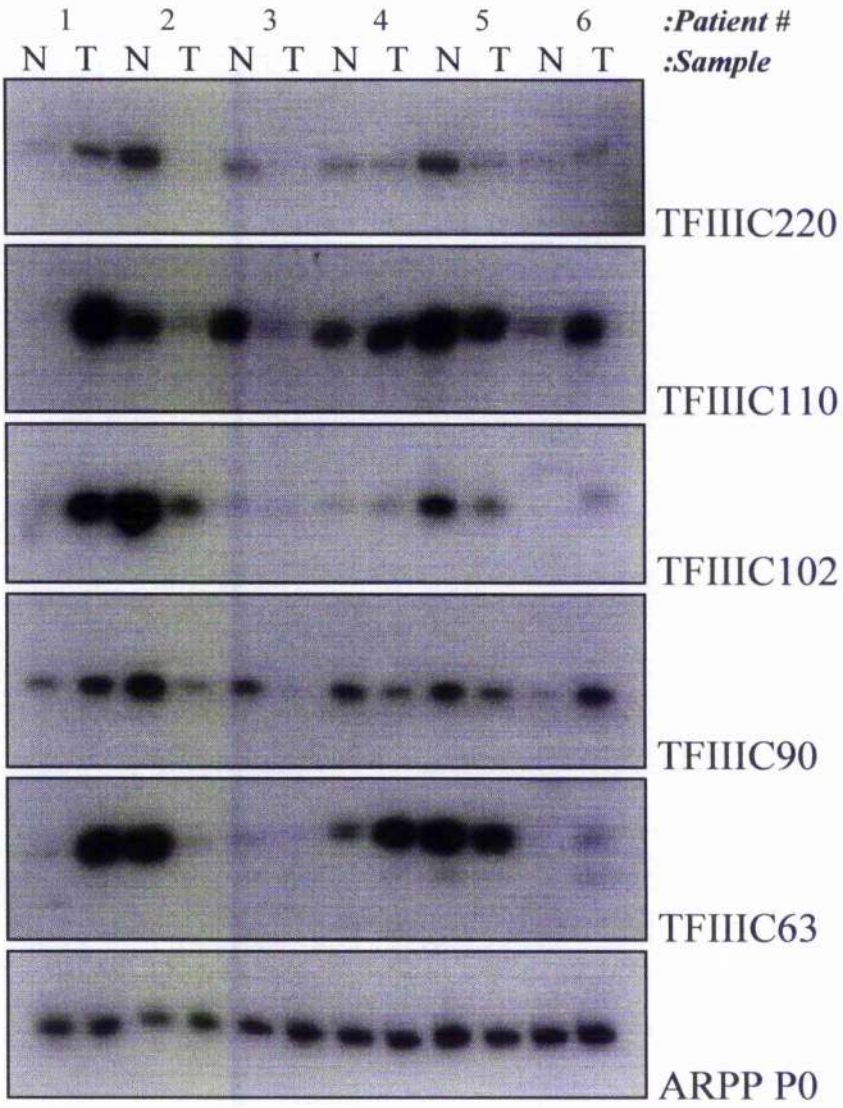
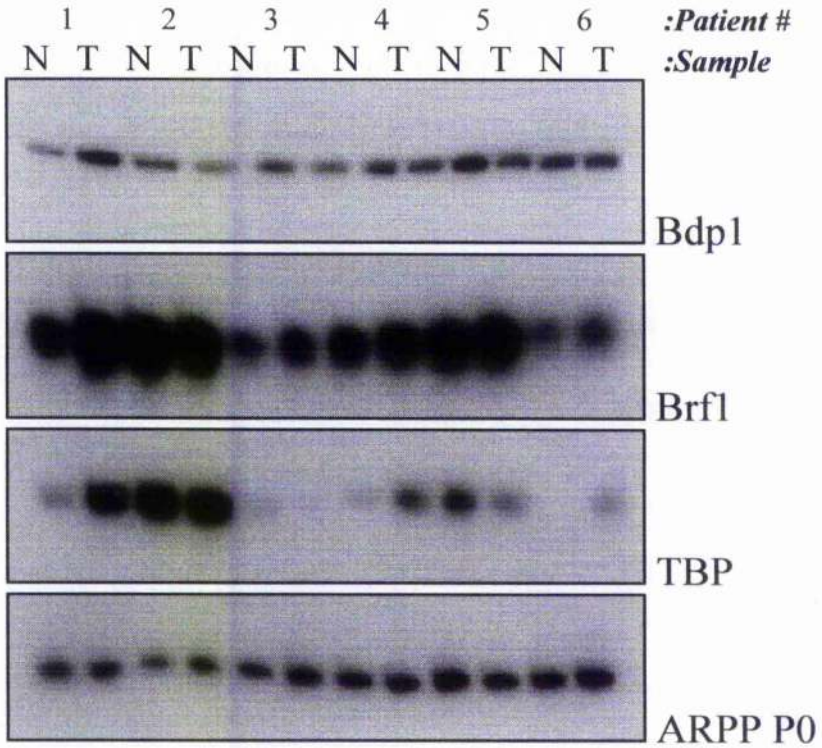

1 2 3 4 5 6
:Patient #
N T N T N T N T N T N T
:Sample
TFIIIC220
TFIIIC110
TFIIIC102
TFIIIC90
TFIIIC63
ARPP P0

Figure 4.4

The Brf1 subunit of TFIIIB is upregulated in breast tumour biopsy samples.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour tissues. These cDNAs were PCR amplified using primers specific for Brf1, Bdp1, TBP and ARPP P0. N = normal, T = tumour.


1 2 3 4 5 6
:Patient #
N T N T N T N T N T N T
:Sample
Bdp1
Brf1
TBP
ARPP P0

breast cells. MCF-7 whole cell extracts were incubated with increasing concentrations of TFIIIB or TFIIIC2 fractions. In initial experiments crude factions were used, prepared by phosphocellulose chromatography. *In vitro* transcription assays revealed that incubating the extracts with PC-B or PC-C resulted in increased transcription of the pol III template $tRNA^{Leu}$ (Figure 4.5A). To reduce the possibility of uncharacterised contaminants, the experiments were repeated with purer fractions. TFIIIB was further purified from PC-B by gradient chromatography on MonoQ, to give fraction 2Q-56. TFIIIC2 was further purified from PC-C by step chromatography on heparin, to give fraction Chep1-11, and on a B-block DNA affinity column to give fraction Oligo IIIC (White *et al.*, 1995a). Incubation of MCF-7 cell extracts with the TFIIIB fraction 2Q-56 or the TFIIIC2 fractions Oligo IIIC and Chep1-11 confirmed that transcription of $tRNA^{Leu}$ was indeed upregulated upon increasing the concentration of these transcription factors (Figure 4.5B).

4.2.6 Increasing the Concentration of TFIIIB and TFIIIC2 in Breast Cells Stimulates Transcription of VA1.

In vitro transcription assays were also carried out with the pol III template VA1 to investigate whether the previously observed increase in transcription occurs at other class III genes upon the addition of TFIIIB and TFIIIC2 fractions. Again it was found that increasing the concentrations of these transcription factors by the addition of PC-B or PC-C fractions increased VA1 transcription (Figure 4.6A). Increases in VA1 transcription were also observed upon addition of the TFIIIC fraction Oligo IIIC or a

Figure 4.5

Raising the concentration of TFIIIB and TFIIIC2 in breast cells stimulates pol III transcription of the gene encoding tRNA ^{Leu}.

MCF-7 whole cell extracts were prepared and incubated with (A) PC-B or PC-C fractions, or (B) the TFIIIB fraction 2Q-56 and the TFIIIC fractions Oligo IIIC and Chep1-11. *In vitro* transcription assays were carried out with the template tRNA ^{Leu}.

-
PC-B
PC-B
-
PC-C
PC-C
-
tRNA Leu
(A)
-
Oligo IIIC
Oligo IIIC
-
2Q-56
2Q-56
-
CHEP1-11
CHEP1-11
CHEP1-11
tRNA Leu
(B)

TFIIIB TAF fraction, 5B1K, prepared by affinity chromatography with immobilised TBP (White *et al.*, 1995a) (Figure 4.6B).

4.2.7 Raising the Concentration of TAFs, but not TBP, in Breast Cells Stimulates Pol III Transcription.

MCF-7 whole cell extracts were prepared and incubated with either a PC-B fraction or a denatured PC-B fraction, which had been heated to 47 degrees for fifteen minutes, inactivating TBP, but leaving the TAFs unaffected (HTPC-B). *In vitro* transcription assays revealed that when TBP is inactivated, the PC-B fraction containing TAFs still stimulates pol III transcription at the VA1 template, although slightly less than the untreated fraction (Figure 4.7A). Incubating 1.5-4.5 μg of recombinant TBP with the breast cell extracts depressed VA1 transcription (Figure 4.7B). However, no effects were seen when *in vitro* transcription assays were repeated with recombinant TBP at lower concentrations (0.01-1 μg) (Figure 4.7C). This squelching effect seen with high concentrations of TBP has been previously observed by White *et al.* (1995b). Thus, TBP is not limiting for pol III transcription in breast cell extracts.

4.2.8 Pol I Transcripts are Overexpressed in Breast Tumour Biopsy Samples.

To investigate if pol I transcription is also deregulated in breast tumours, RT-PCRs were carried out using primers that detect the processed transcripts. 5.8S rRNA, 18S

Figure 4.6

Raising the concentration of TFIIIB and TFIIIC2 in breast cells stimulates pol III transcription of the gene encoding VA1.

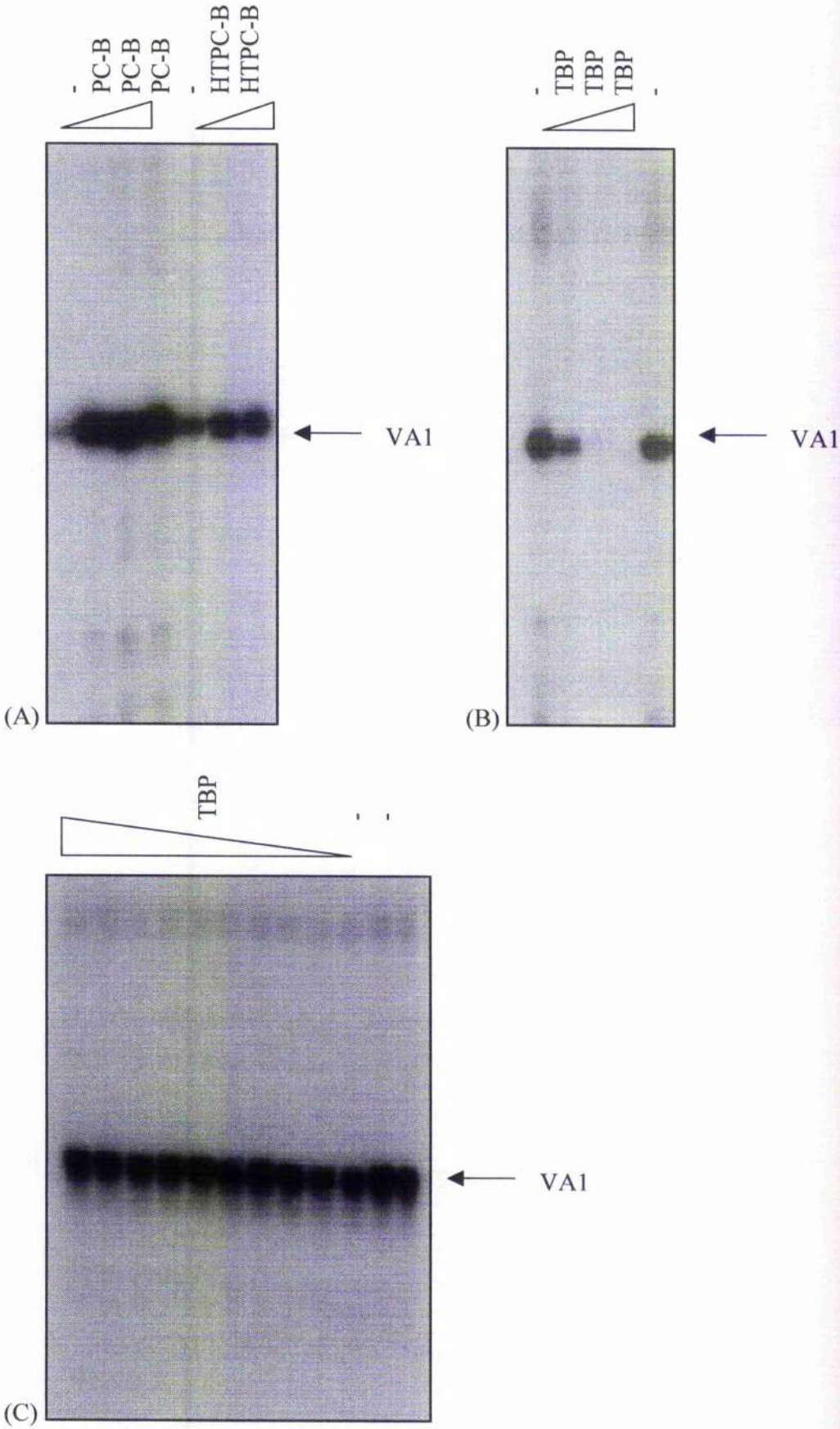
MCF-7 whole cell extracts were prepared and incubated with (A) PC-B or PC-C fractions, or (B) the TAF-containing fraction 5B1K and the TFIIIC fraction Oligo IIIC. *In vitro* transcription assays were carried out with the template VA1.

-
PC-B
PC-B
-
PC-C
PC-C
VA1
(A)
-
5B1K
5B1K
-
Oligo IIIC
Oligo IIIC
VA1
(B)

Figure 4.7

Raising the concentration of TAFs, but not TBP, in breast cells stimulates pol III transcription.

MCF-7 whole cell extracts were prepared and incubated with (A) 1.5-4.5 μg of PC-B fraction or 3-4.5 μg HTPC-B (PC-B fraction that has been heated to 47 degrees for fifteen minutes inactivating TBP, but leaving the TAFs unaffected), or recombinant TBP (B) 1.5-4.5 μg, (C) 1-0.01 μg. *In vitro* transcription assays were carried out with the pol III template VA1.


-
PC-B
PC-B
PC-B
-
HTPC-B
HTPC-B
VA1
(A)
-
TBP
TBP
TBP
-
VA1
(B)
TBP
- -
VA1
(C)

rRNA and 28S rRNA each showed overexpression in five of the six tumour samples examined, when compared to the matched normal tissue (Figure 4.8). The exception to this was the tumour sample from patient #3, which showed a decrease in transcription at these genes. This is the same tumour sample that consistently displayed down regulation of the genes transcribed by pol III. Upon investigation of the pol I-specific transcription factors Rrn3 and UBF, it was found that differences between the levels of these transcripts in normal and tumour samples fluctuated dramatically between patients, and did not correlate with pol I transcription (Figure 4.9). Availability of other factors (e.g. TAFs) may therefore be limiting for pol I transcription in breast epithelium.

4.2.9 c-Myc is Overexpressed in Breast Tumour Biopsy Samples.

Expression of the oncogene c-*myc* was also examined by RT-PCR in the breast biopsy samples. It was found that levels were elevated in four out of the six tumours examined in comparison with normal breast tissue from the same patient (Figure 4.10). The exceptions were tumour biopsy #3, which is the same sample that was consistently down regulated when the genes transcribed by pols I and III were examined, and #5.

Figure 4.8

Pol I transcripts are overexpressed in breast tumour biopsy samples.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour tissues. These cDNAs were PCR amplified using primers specific for 28S rRNA, 18S rRNA, 5.8S rRNA and ARPP P0. N = normal, T = tumour.

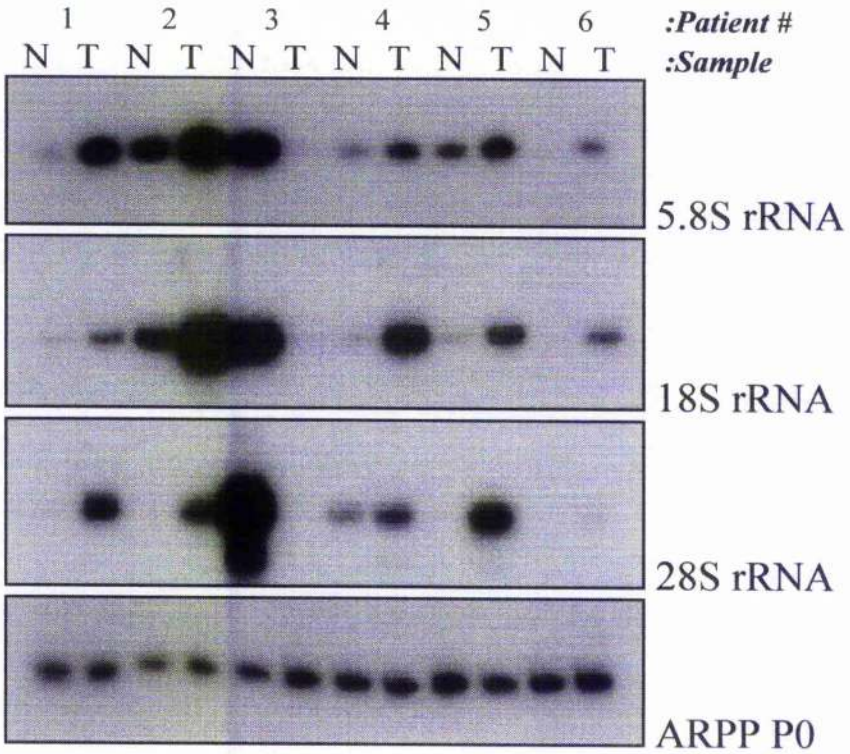

1 2 3 4 5 6
:Patient #
N T N T N T N T N T N T
:Sample
5.8S rRNA
18S rRNA
28S rRNA
ARPP P0

Figure 4.9

Rrn3 and UBF are only upregulated in two out of the six breast tumour biopsy samples examined.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour breast tissues. These cDNAs were PCR amplified using primers specific for Rrn3, UBF and ARPP P0. N = normal, T = tumour.

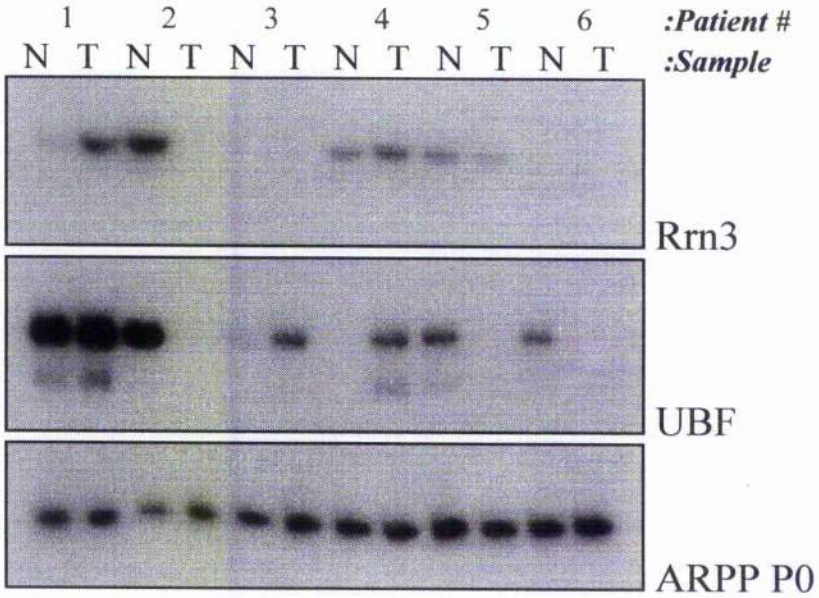
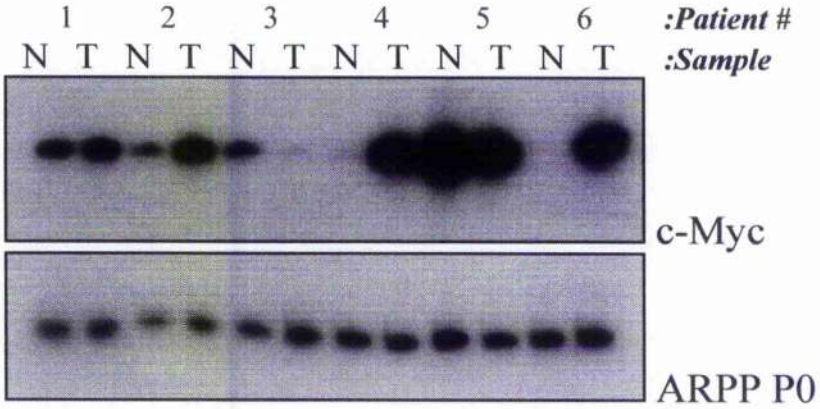

1 2 3 4 5 6
:Patient #
N T N T N T N T N T N T
:Sample
Rrn3
UBF
ARPP P0

Figure 4.10

c-Myc mRNA is overexpressed in breast tumour biopsy samples.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour breast tissues. These cDNAs were PCR amplified using primers specific for c-Myc and ARPP P0. N = normal, T = tumour.


1 2 3 4 5 6
:Patient #
N T N T N T N T N T N T
:Sample
c-Myc
ARPP P0

4.3 Discussion.

As breast cells undergo the numerous genetic changes that occur in the development of cancer, transcription by pols I and III is deregulated. These changes in gene expression are complicated by the fact that breast tumours consist of many different cell types, including carcinoma cells, but also additional epithelial cell types, stromal cells, adipose cells, endothelial cells, and infiltrating lymphocytes. In addition to this, breast carcinoma cells themselves are morphologically and genetically diverse (Perou *et al.*, 1999).

Examination of tRNA, 5S rRNA and 7SL transcripts by RT-PCR revealed that transcription of these genes was elevated in 50% of the tumour biopsy samples examined when compared to the matched normal tissue. 5S rRNA genes have a type I promoter arrangement, while tRNA has a type II structure. When the genes MRP and 7SK, which have a type III promoter structure, were examined, it was found that transcription was elevated at a higher frequency in these breast tumours, with five out of the six patients showing an increase in transcription when the tumour sample was compared to the matched normal breast tissue (Table 4.1).

When the transcription factors utilized by pol III were examined, it was found that all of the five subunits of TFIIIC2 were upregulated in two tumour samples (#1, 6). This correlated with an upregulation of type I and II genes transcribed by pol III. However, the tumour biopsy from patient #5, which displayed elevated levels of pol III transcription, did not have elevated levels of the TFIIIC2 subunits. To assess the role of this transcription factor further, whole breast cell extracts were made from MCF-7 cells, which were incubated with a PC-C fraction enriched with TFIIIC2. It was seen

that the addition of this crude fraction increased transcription at the pol III templates tRNA ^{Leu} and VA1. This was confirmed using the purer fractions Oligo IIIC and Chep1-11.

When the subunits of TFIIIB were investigated, it was found that Brf1 was upregulated in all except for one of the breast tumour biopsy samples, and that TBP was upregulated in half of them, while Bdp1 remained relatively constant. *In vitro* transcription assays revealed that addition of a PC-B fraction enriched with TFIIIB increased transcription at tRNA ^{Leu} and VA1 templates. Incubation of breast cells with the TAF fraction 5B1K or heat treated PC-B, in which TBP was denatured, also stimulated pol III transcription. The slight reduction in transcriptional stimulation observed when HTPC-B was added in comparison to untreated PC-B may be due to some denaturation of the TAFs, since addition of recombinant TBP had no stimulatory effect on pol III transcription in the breast cell extracts. Therefore, it is possible that TBP is in relative excess in breast epithelium and that TAFs are limiting along with TFIIIC2. However, although upregulation of Brf1 in most of the tumour samples was seen to correlate with upregulated transcription of MRP and 7SK, the correlation is unlikely to be causal, because these genes do not utilise Brf1. Given more time, it would have been interesting to investigate whether the level of Brf2 increases in parallel with Brf1, as this might be the limiting TAF for MRP and 7SK. Since these genes also require PTF, it would also be interesting to determine the expression level of this factor in the biopsy samples.

When RT-PCRs were carried out to examine pol I transcription, it was seen that the breast tumour biopsy samples were upregulated in five of the six patients. The

exception was a tumour from patient #3, which was also the only tumour that displayed a decrease in transcription with the MRP and 7SK genes. Examining mRNAs for two of the transcription factors utilized by pol I, UBF and Rrn3, did not reveal any correlation between these factors and expression of the processed pol I transcripts (Table 4.1). During the development of cancer, it is likely that the variety of transcription factors utilized by both pol I and III precludes a simplistic assignment of direct stimulatory capability to only one of them.

Analysis of c-*myc* transcription revealed that this gene was upregulated in four of the six tumour samples in comparison with the matched normal tissue. Apart from patient #5, this deregulation showed some correlation with the increased levels of pol I transcripts, as well as MRP and 7SK transcripts (Table 4.1). Since increased levels of c-Myc are thought to be involved in the induction and progression of human breast cancer (Leder *et al.*, 1986; Roux-Dosseto *et al.*, 1992; Sierra *et al.*, 1999; Watson *et al.*, 1993, 1996) it may be that c-Myc ensures that the components required for increased growth are available by increasing transcription of pols I and III. Indeed, in c-Myc null cells there is a marked decrease in net RNA synthesis (Mateyak *et al.*, 1997). It has also recently been demonstrated that TFIIIB is a target for c-Myc, enabling it to directly activate pol III transcription (Gomez-Roman *et al.*, 2003). It is unclear why the tumour sample from patient #3 displayed down regulation of most of the transcripts examined. However, it may be that a lot of necrotic material was present in this sample.

It is unsurprising, therefore, that pol I and III transcripts are frequently found to be upregulated in breast tumour cells, since these products are required for increased

protein synthesis, which is necessary to sustain the higher rates of proliferation of tumour cells. These data suggest that alterations in the transcription of c-Myc, pol I transcripts, and pol III transcripts with a type III promoter, are common changes found in breast carcinomas. However, since such a small number of samples were available for analysis in this study, a larger scale investigation would need to be carried out in order to confirm these results.

Table 4.1

Summary of changes in expression observed in breast tumour biopsy samples examined by RT-PCR.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour breast tissues. Arrows denote up or down regulation of a particular transcript. N = normal, T = tumour.

	1	2	3	4	5	6
	T	T	T	T	T	T
tRNA ^{Leu}	↑	↓	↓	↓	↑	↑
tRNA ^{Sec}	↑	↓	↓	↓	↑	↑
5S rRNA	↑	↓	↓	↓	↑	↑
7SL	↑	↓	↓	↓	↑	↑
MRP	↑	↑	↓	↑	↑	↑
7SK	↑	↑	↓	↑	↑	↑
TFIIIC 220	↑	↓	↓		↓	↑
TFIIIC 110	↑	↓	↓	↑	↓	↑
TFIIIC 102	↑	↓	↓		↓	↑
TFIIIC 90	↑	↓	↓	↓	↓	↑
TFIIIC 63	↑	↓	↓	↑	↓	↑
Bdp1	↑					
Brf1	↑	↓	↑	↑	↑	↑
TBP	↑			↑	↓	↑
5.8S rRNA	↑	↑	↓	↑	↑	↑
18S rRNA	↑	↑	↓	↑	↑	↑
28S rRNA	↑	↑	↓	↑	↑	↑
Rrn3	↑	↓	↑	↑		
UBF	↑	↓	↑	↑	↓	↓
c-Myc	↑	↑	↓	↑	↓	↑

Chapter 5

Colorectal Cancer

5.1 Introduction.

Colorectal cancer is the second commonest cancer in developed countries, but is much rarer in developing countries. In the UK, in the year 1999, there were approximately 36,000 new cases and 16,000 deaths from this cancer (Cancer Research UK, 2003a, b). The development of colorectal cancer results from an interplay of dietary, lifestyle and environmental factors on the genetic background of the individual (Fearon, 1997). Colorectal tumours progress through a series of clinical and histopathological stages, ranging from single crypt lesions (aberrant crypt foci), through small benign tumours (adenomatous polyps), to malignant cancers (carcinomas) (reviewed by Vogelstein & Kinzler, 2002). As the tumour progresses through these events (Fearon, 1992) it invades underlying layers of tissue, and can be staged according to how far it has penetrated the bowel wall and nodal status of the tumour. One of the most commonly used staging systems is the Astler-Coller modification of the original Dukes' system (Astler & Coller, 1954). "Dukes' A" is a cancer that is confined to the innermost lining of the bowel; "Dukes' B" means the cancer has invaded the muscle layer of the bowel; "Dukes' C" means the cancer has metastasised to at least one lymph node in the area, and "Dukes' D" means the cancer has metastasised to a distant site in the body, such as the liver or lungs.

A small proportion of cases of colorectal cancer are due to the inheritance of mutations in certain genes. Two of the best-characterized and most pronounced inherited predispositions are hereditary nonpolyposis colorectal cancer (HNPCC) (Ponz de Leon *et al.*, 1993) and familial adenomatous polyposis (FAP) (Bussey *et al.*, 1978). Unlike FAP patients, individuals with HNPCC do not have a marked increase in the number of adenomas; instead they inherit defective DNA mismatch repair

genes (Lynch *et al.*, 1995, 1996). However, the majority of cases of colorectal cancer are sporadic. Studies of cells from sporadic colorectal cancers have shown that the progression through the adenoma-carcinoma sequence of events is a multistep process in which genetic alterations accumulate to bring about the neoplastic phenotype (Fearon & Vogelstein, 1990) (Figure 5.1).

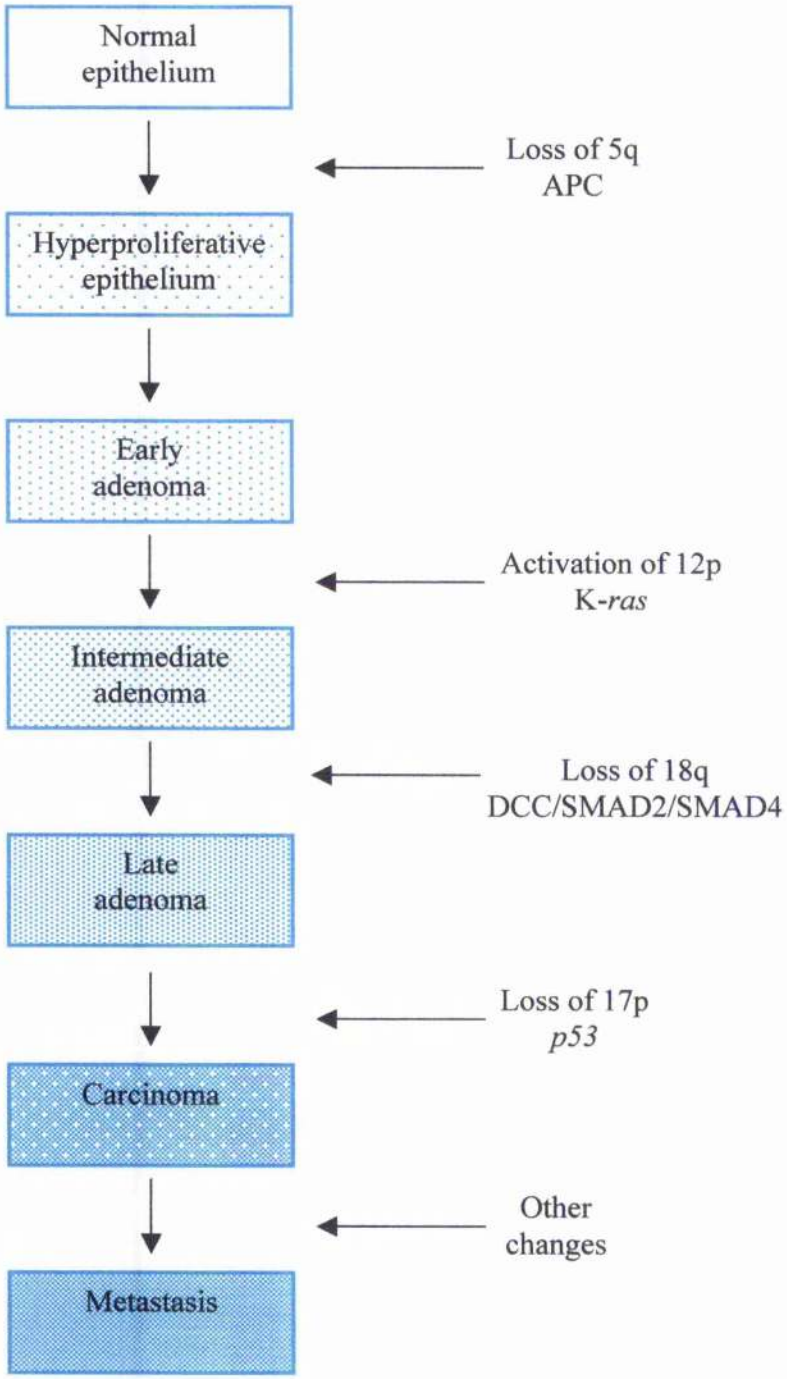
Mutations which inactivate the APC tumour suppressor gene on chromosome 5q appear to be the earliest genetic event in colorectal tumourigenesis (Vogelstein *et al.*, 1988). The frequency of APC mutations is just as high in adenomatous polyps as in carcinomas (Jen *et al.*, 1994; Miyoshi *et al.*, 1992; Powell *et al.*, 1992). In addition to causing FAP through germ-line transmission, mutations in the APC gene occur somatically in over 80% of sporadic colorectal tumours, whether benign or malignant (Miyoshi *et al.*, 1992; Powell *et al.*, 1992; Smith *et al.*, 1994). These mutations cause truncation of the APC protein (Nagase & Nakamura, 1993), and are largely the result of splice-site mutations, or nonsense mutations, or insertions/deletions that lead to frameshifts (Miyoshi *et al.*, 1992; Powell *et al.*, 1992). The truncated APC protein is unable to regulate ß-catenin/Tcf-mediated transcription and, therefore, transcription of downstream growth promoting genes is increased (Morin *et al.*, 1997; Sparks *et al.*, 1998).

In cancer cells, a generalised hypomethylation of the genome occurs relatively early during colorectal tumourigenesis, and can be observed even in small adenomas (el-Deiry *et al.*, 1991; Feinberg *et al.*, 1988; Goelz *et al.*, 1985). Although colorectal tumours are globally hypomethylated, a few specific regions of the chromosome may be hypermethylated (Silverman *et al.*, 1989). The cause of these changes in

Figure 5.1

A model for the molecular progression of colorectal tumourigenesis.

A series of genetic changes are necessary to convert the normal epithelial cell to a metastatic cancer.


Normal epithelium
Loss of 5q
APC
Hyperproliferative epithelium
Early adenoma
Activation of 12p
K-*ras*
Intermediate adenoma
Loss of 18q
DCC/SMAD2/SMAD4
Late adenoma
Loss of 17p
p53
Carcinoma
Other changes
Metastasis

methylation is not yet understood. The loss of DNA methylation can inhibit chromosome condensation (Schmid *et al.*, 1984), which, in turn, could result in abnormal chromosome segregation and in particular chromosome loss. This is one of the most common mechanisms for inactivating tumour suppressor genes (Vogelstein & Kinzler, 2002).

Activating mutations of *ras* oncogenes come a little later, occurring rarely in small polyps but quite commonly (about 50%) in colorectal cancers and in adenomas larger than 1.0cm in diameter (Bos *et al.*, 1987; Forrester *et al.*, 1987; Vogelstein *et al.*, 1988). The majority of the mutations identified (85%) are in codons 12 and 13 of K-*ras*, with the rest affecting codon 61 of K-*ras* or N-*ras* (Pretlow *et al.*, 1993; Shibata *et al.*, 1993). The lack of mutations in smaller adenomas suggests that *ras* mutations are acquired during adenoma progression. Microdissection studies have provided direct evidence for this by demonstrating subpopulations of adenoma cells that have acquired *ras* mutations (Shibata *et al.*, 1993). When malignant colorectal cells containing such *ras* mutations are grown in culture, they show typical features of transformed cells, such as the ability to proliferate without anchorage to a substratum; the cells revert to a nontransformed character and reduce their rate of proliferation if their activated *ras* gene is eliminated (Shirasawa *et al.*, 1993).

Three candidate tumour suppressor genes, DCC, SMAD2 and SMAD4 have been isolated from chromosome 18q (Eppert *et al.*, 1996; Fearon *et al.*, 1990; Hahn *et al.*, 1996). At least one copy of these genes is lost in 70% of colorectal carcinomas, and in almost 50% of late adenomas (Vogelstein *et al.*, 1988). Somatic alterations, including homozygous deletions, point mutations, or insertions, have been detected in all three

candidate genes, with mutations in SMAD4 being most frequent (Vogelstein & Kinzler, 20002).

Mutations in the *p53* gene on chromosome 17p come later still. This tumour suppressor gene is inactivated in at least 75% of colorectal cancers, but rarely in benign adenomatous polyps (Vogelstein *et al.*, 1988). Loss of *p53* seems to relieve mutant cells of their last inhibitions, and if normal *p53* is transfected back into colorectal carcinoma cells, their proliferation is suppressed (Baker *et al.*, 1990). p53 has been demonstrated to arrest cell growth in response to DNA damage. Cells with mutant p53 are only partially blocked and continue to divide (Kastan *et al.*, 1991, 1992; Kuerbitz *et al.*, 1992). Therefore, it has been suggested that p53 protects the integrity of the genome by preventing propagation of cells with DNA damage (Lane, 1992). Although mutations in *p53*, DCC, SMAD2, SMAD4, *ras* and APC may be critical rate-limiting steps in a large proportion of colorectal cancers, these mutations do not always occur in the same sequence, nor are they the only route to disease.

The increase in the rate of growth required during the adenoma-carcinoma sequence of events suggests the possibility that transcription would need to increase in order to sustain increased protein synthesis. Therefore, the aim of this chapter was to examine whether pols I and III are deregulated in colorectal tumours.

5.2 Results.

5.2.1 Pol III Activity in Colorectal Tumours.

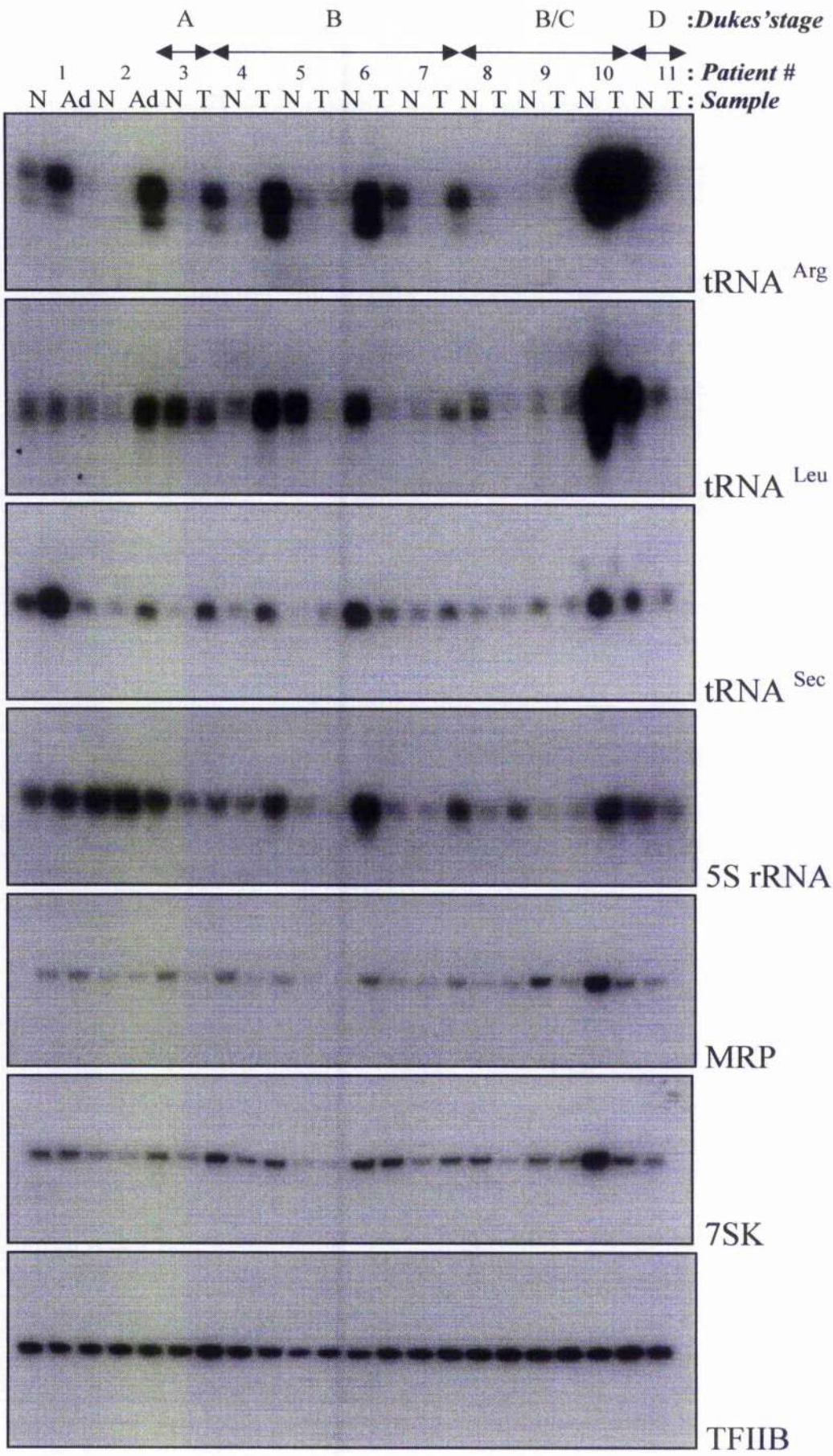
Total RNA, extracted from colorectal tumours and matched normal mucosa samples from eleven patients, was used to generate cDNAs by reverse transcription. Frequently used normalization controls, such as the housekeeping gene ARPP P0, have been shown to be upregulated during colorectal cancer; therefore, primers against another more suitable gene had to be designed. This proved problematic, as many of the potential control genes that were tested displayed elevated levels of mRNA in the colorectal tumour samples. RT-PCRs carried out with primers against TFIIB showed far less variation between normal and tumour colorectal samples; thus these primers were used as a control for subsequent PCR analyses.

To investigate the levels of pol III activity in colorectal tumour biopsy samples, RT-PCRs were carried out using primers specific for the pol III transcripts 5S rRNA, tRNA ^{Arg}, tRNA ^{Leu}, tRNA ^{Sec}, 7SK, and MRP. Out of the eleven colorectal tumour biopsies, it was found that four samples consistently overexpressed pol III transcripts in comparison with the matched normal tissue from the same individual (samples #1, 6, 9 & 10, Figure 5.2). This effect was specific, as no change was detected in the levels of mRNA encoding TFIIB, which is synthesized by pol II. In the other tumours examined, three of the samples displayed decreased levels of transcription at these class III genes, one remained relatively constant, and three showed a combination of equal and decreased levels, depending on the transcript.

Figure 5.2

Pol III transcripts are overexpressed in four out of the eleven colorectal tumour biopsy samples examined.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour colorectal tissues. These cDNAs were PCR amplified using primers specific for 5S rRNA, tRNA ^{Arg}, tRNA ^{Leu}, tRNA ^{Sec}, 7SK, MRP and TFIIB. Ad = adenoma, N = normal, T = tumour.


A
B
B/C
D
:Dukes'stage
1 2 3 4 5 6 7 8 9 10 11 : Patient #
N Ad N Ad N T N T N T N T N T N T N T N T N T : Sample
tRNA Arg
tRNA Leu
tRNA Sec
5S rRNA
MRP
7SK
TFIIB

5.2.2 Activity of Pol III Transcription Factors.

As deregulation is observed for a range of class III genes with distinct promoter sequences and structures, the general pol III transcription machinery that is utilised by each of these templates was investigated. First, the levels of the three subunits of TFIIIB were examined by RT-PCR to assess whether the upregulation of pol III activity in four of the colorectal tumours (samples #1, 6, 9 & 10) was due to an increase in the availability of transcription factors (Figure 5.3). Although upregulation of all three subunits occurred in the later stage Dukes' B and Dukes' B/C tumours (samples #6, 9 & 10), this was not the case with the earlier stage Dukes' A sample (#1). This tumour sample did, however, display elevated levels of the TFIIIB subunit Bdp1. Despite the fact that the colorectal tumour sample #2 displayed elevated levels of all of the TFIIIB subunits, overexpression of pol III templates examined was not observed. Tumour samples that displayed decreased levels of transcription at two or more of the pol III templates were also observed to have decreased levels of TBP and Brf1 RNA, and in some cases Bdp1.

Next, transcription of the TFIIIC2 subunits was investigated. Again, RT-PCRs showed that these subunits were expressed in a similar manner to TFIIIB. Upregulation of all five subunits occurred in the later stage colorectal tumours (Dukes' B and Dukes' B/C) that overexpressed pol III transcripts (samples #6, 9 & 10). This correlated upregulation of the five TFIIIC2 subunits was not observed in adenoma sample #1. Furthermore, sample #2 displayed elevated levels of the subunits TFIIIC110 and TFIIIC90 in comparison with the matched normal mucosa from the same individual (Figure 5.4). It was also observed that expression of at least one of

the TFIIIC2 subunits and, in sample #5 all of the subunits, were down regulated in the other tumour samples.

5.2.3 Pol I Activity in Colorectal Tumours.

As pols I and III are frequently regulated together, RT-PCRs were carried out using primers specific for the processed ribosomal RNAs 28S, 18S and 5.8S. These pol I transcripts were found to be overexpressed in over 50% of the colorectal tumour biopsy samples examined, in comparison with matched normal tissue from the same individual. Four of the samples that showed upregulation of pol I activity were the same samples that displayed upregulation of pol III transcripts (samples #1, 6, 9 & 10, Figure 5.5). The other two colorectal samples were a late stage Dukes' D tumour (sample #11) and another Dukes' B/C tumour (sample #8). Tumour samples #2 and 3 showed a decrease in the levels of the transcripts.

Upon investigation of the pol I-specific transcription factors Rrn3 and UBF, it was found that the levels of these transcripts are only upregulated in two out of the eleven colorectal tumour biopsy samples examined in comparison with matched normal tissue from the same individual (samples #6 & 10). These are two of the tumours that display elevated levels of pol I transcripts, but no evidence of coordinated overexpression was seen in the other four tumours that displayed increased levels of pol I activity (Figure 5.6). In the other tumour samples (excluding sample #9, which remained constant), decreased levels of one or both of these factors were observed.

Figure 5.3

The subunits of TFIIIB are upregulated in four out of the eleven colorectal tumour biopsy samples examined.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour colorectal tissues. These cDNAs were PCR amplified using primers specific for Brf1, Bdp1, TBP and TFIIB. Ad = adenoma, N = normal, T = tumour.

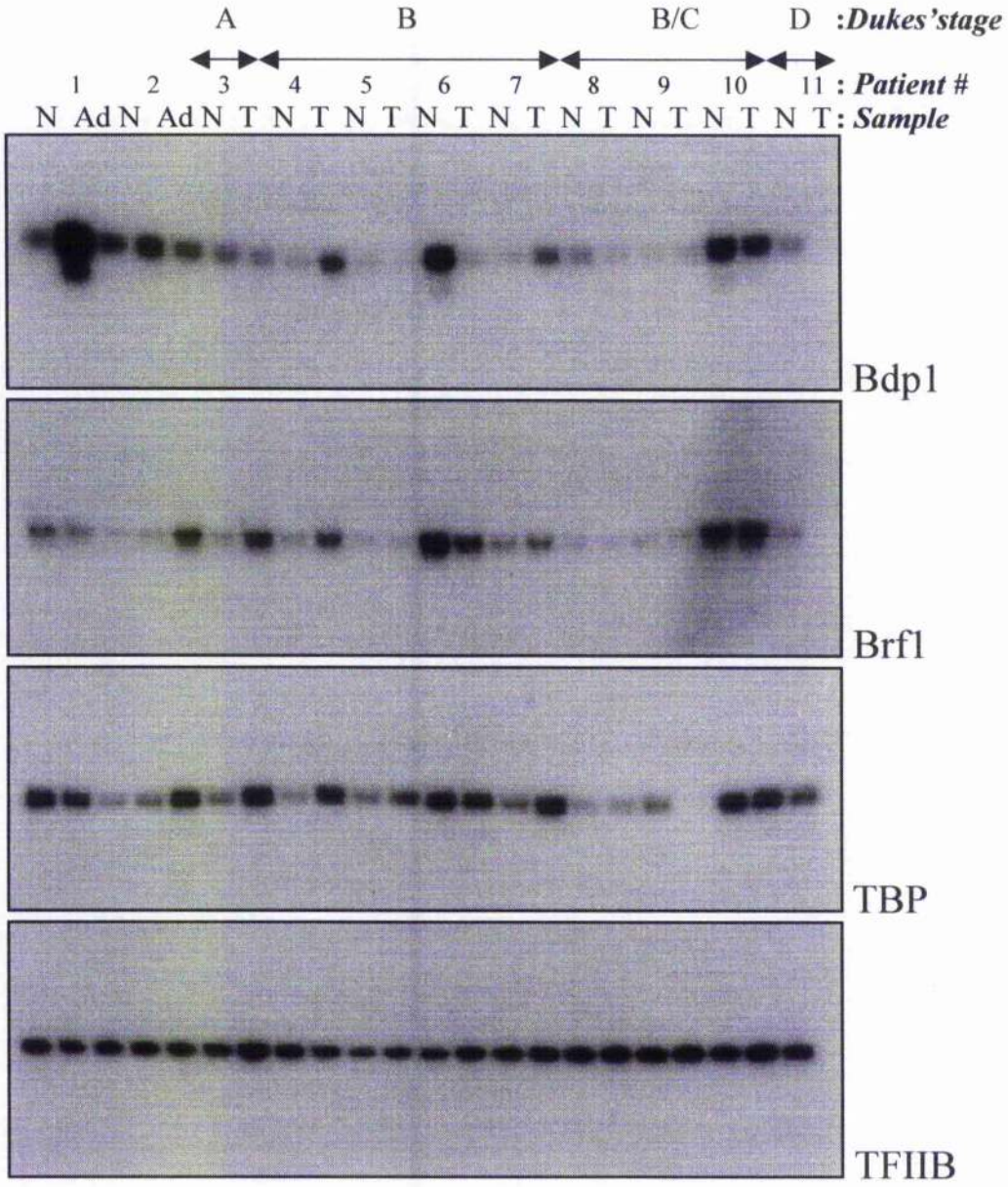

A
B
B/C
D
:Dukes'stage
1 2 3 4 5 6 7 8 9 10 11 : Patient #
N Ad N Ad N T N T N T N T N T N T N T : Sample
Bdp1
Brf1
TBP
TFIIB

Figure 5.4

Transcription of the TFIIIC2 subunits is upregulated in three out of the eleven colorectal tumour biopsy samples examined.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour colorectal tissues. These cDNAs were PCR amplified using primers specific for TFIIIC220, TFIIIC110, TFIIIC102, TFIIIC90, TFIIIC63 and TFIIB. Ad = adenoma, N = normal, T = tumour.

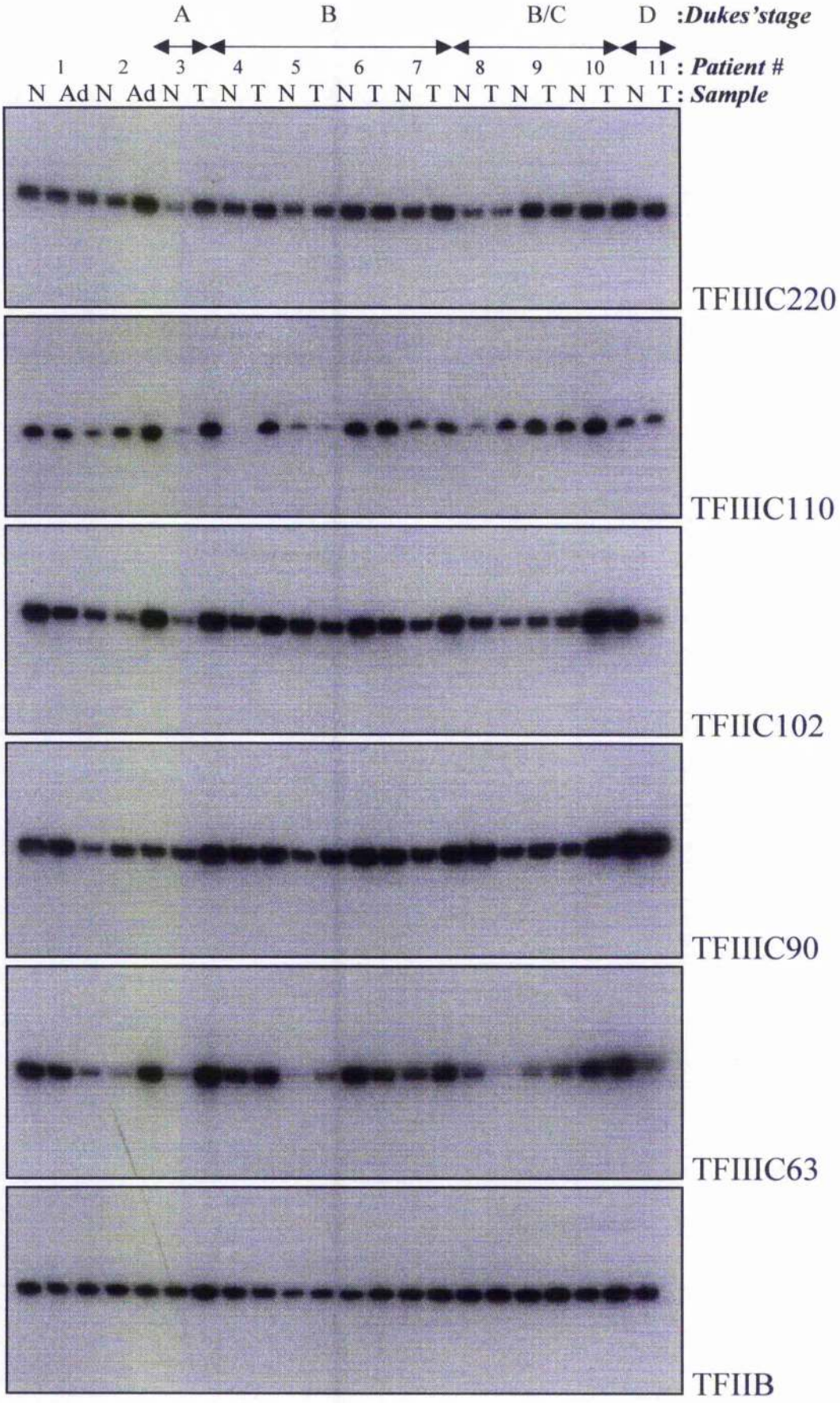

A
B
B/C
D
:Dukes'stage
1 2 3 4 5 6 7 8 9 10 11 : Patient #
N Ad N Ad N T N T N T N T N T N T N T N T N T : Sample
TFIIIC220
TFIIIC110
TFIIC102
TFIIIC90
TFIIIC63
TFIIB

Figure 5.5

Pol I transcripts are overexpressed in over half of the colorectal tumour biopsy samples examined.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour colorectal tissues. These cDNAs were PCR amplified using primers specific for 28S rRNA, 18S rRNA, 5.8SrRNA and TFIIB. Ad = adenoma, N = normal, T = tumour.

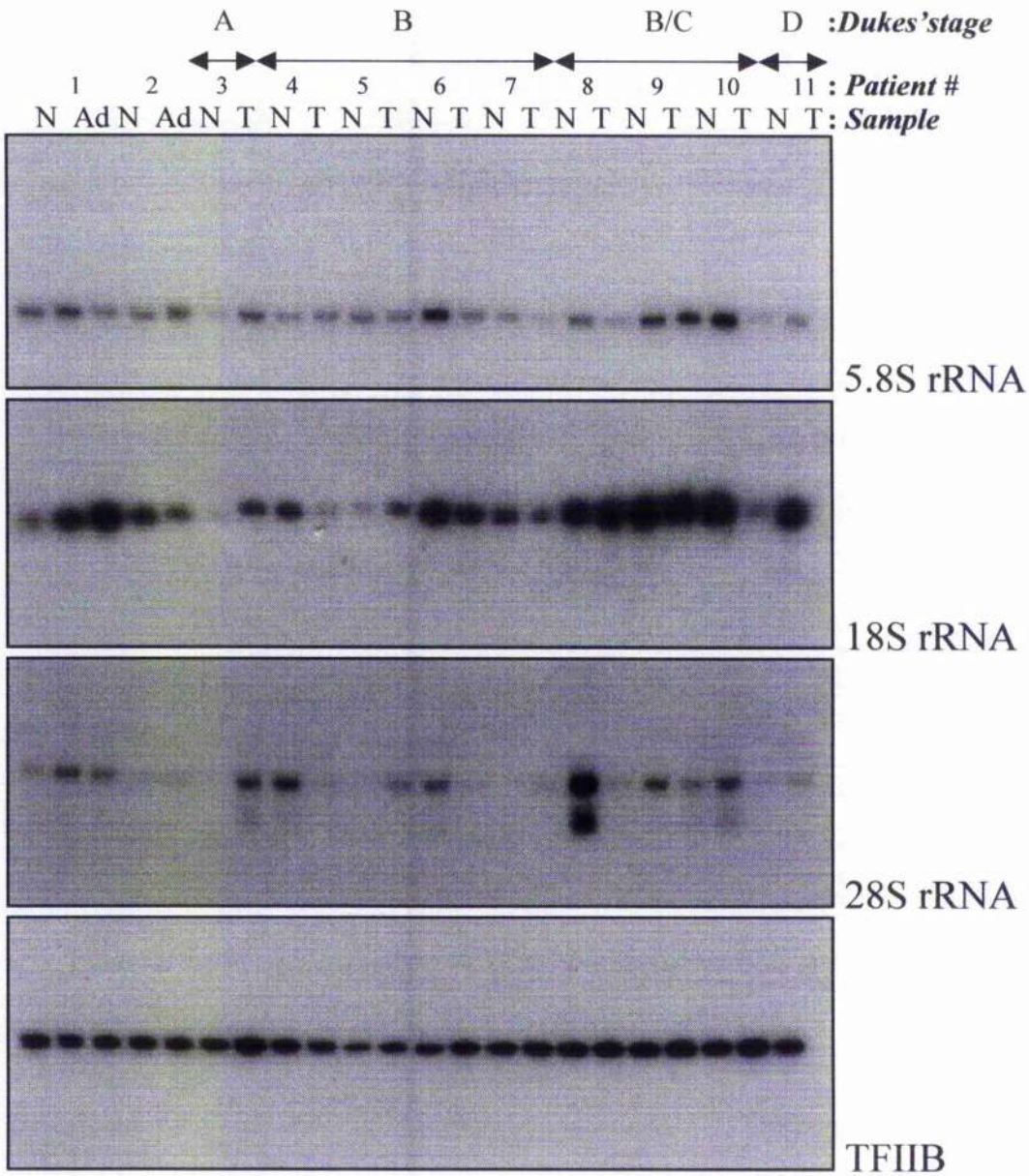
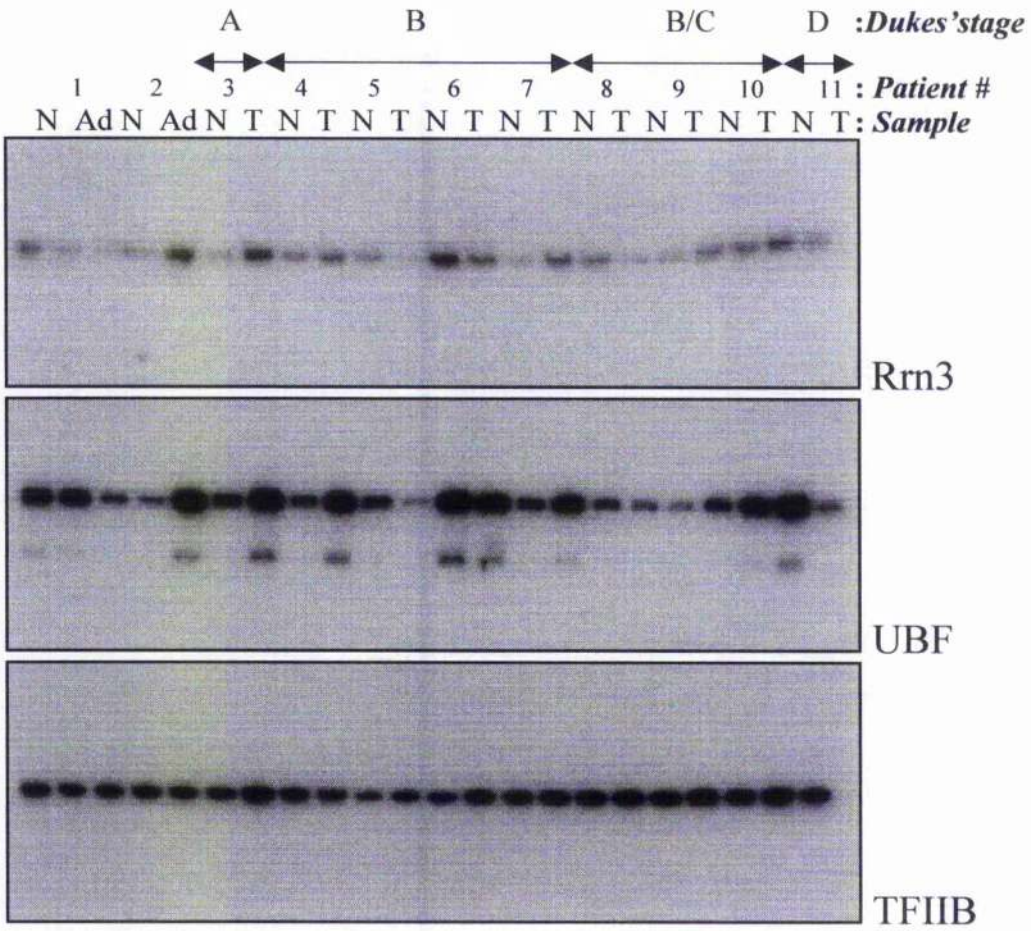

A
B
B/C
D
:Dukes'stage
1 2 3 4 5 6 7 8 9 10 11 : Patient #
N Ad N Ad N T N T N T N T N T N T N T N T N T : Sample
5.8S rRNA
18S rRNA
28S rRNA
TFIIB

Figure 5.6

Rrn3 and UBF are only upregulated in two out of the eleven colorectal tumour biopsy samples examined.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour colorectal tissues. These cDNAs were PCR amplified using primers specific for Rrn3, UBF and TFIIB. Ad = adenoma, N = normal, T = tumour.


A
B
B/C
D
:Dukes'stage
1 2 3 4 5 6 7 8 9 10 11 : Patient #
N Ad N Ad N T N T N T N T N T N T N T N T : Sample
Rrn3
UBF
TFIIB

5.2.4 rRNA Levels are Elevated in Colorectal Tumours.

The overexpression of pol I transcripts observed in colorectal tumours was also confirmed in another two Dukes' B matched normal and tumour colorectal samples (Figure 5.7). RNA from these two sets of samples was also assessed using an Agilent 2100 Bioanlayzer. This piece of equipment assesses the purity and integrity of the RNA and allows concentration to be measured very accurately. 250 ng of each of the colorectal RNA samples was loaded onto a RNA 6000 Nano microchip. When an electrical voltage is applied to the microchip, the sample migrates through microchannels etched in the chip surface. As the sample moves, ribosomal RNA and ribosomal transcripts of different sizes separate according to their mass. Intercalating dye within the sieving matrix allows the migrating RNA to be detected. The Agilent 2100 Bioanalyzer uses a laser for excitation of intercalating fluorescent dyes. The electropherograms for each matched normal/tumour pair were then overlaid (Figure 5.8). Levels of the two dominant ribosomal peaks representing 18S and 28S rRNA are a lot higher in the colorectal tumour samples in comparison with the matched normal mucosa from the same individual. The levels of the RNA apart from these two peaks are relatively constant between the normal and tumour samples. This confirms that the majority of transcripts present in these samples do not show the specific increase in expression observed in the tumour for rRNA.

5.2.5 c-Myc is Overexpressed in Colorectal Tumour Biopsy Samples.

As the APC gene is mutated early in colorectal tumourigenesis, downstream targets of ß-catenin were investigated. One ß-catenin target is the *c-myc* gene, which was found

Figure 5.7

Pol I transcripts are overexpressed in additional colon tumour biopsy samples.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour colorectal tissues. These cDNAs were PCR amplified using primers specific for 28S rRNA, 18S rRNA, 5.8S rRNA and TFIIB. N = normal, T = tumour.

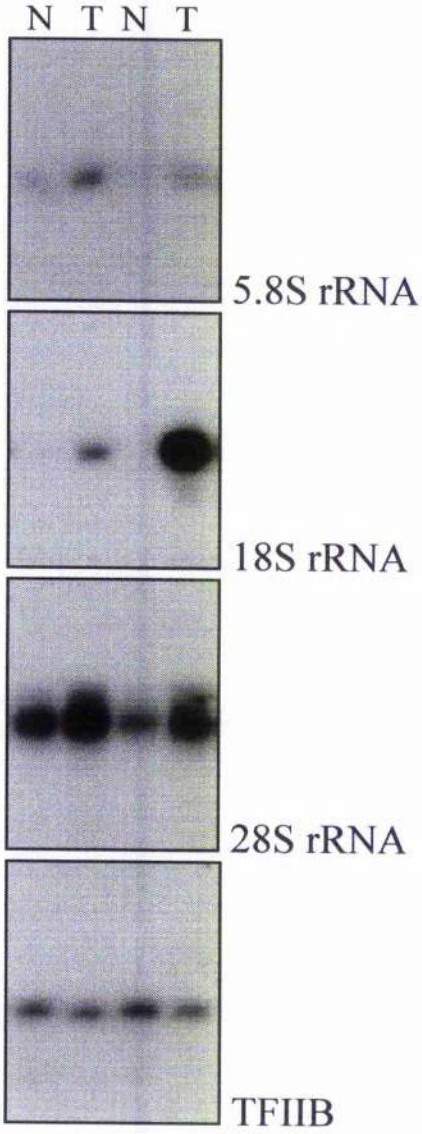
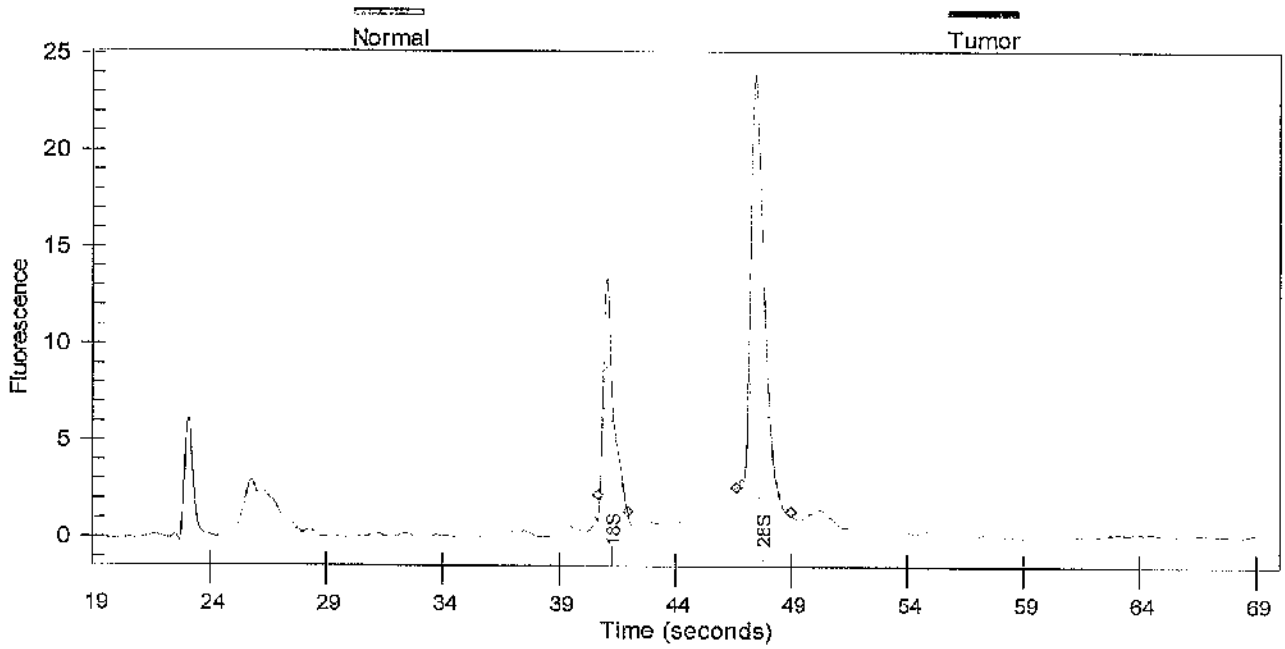
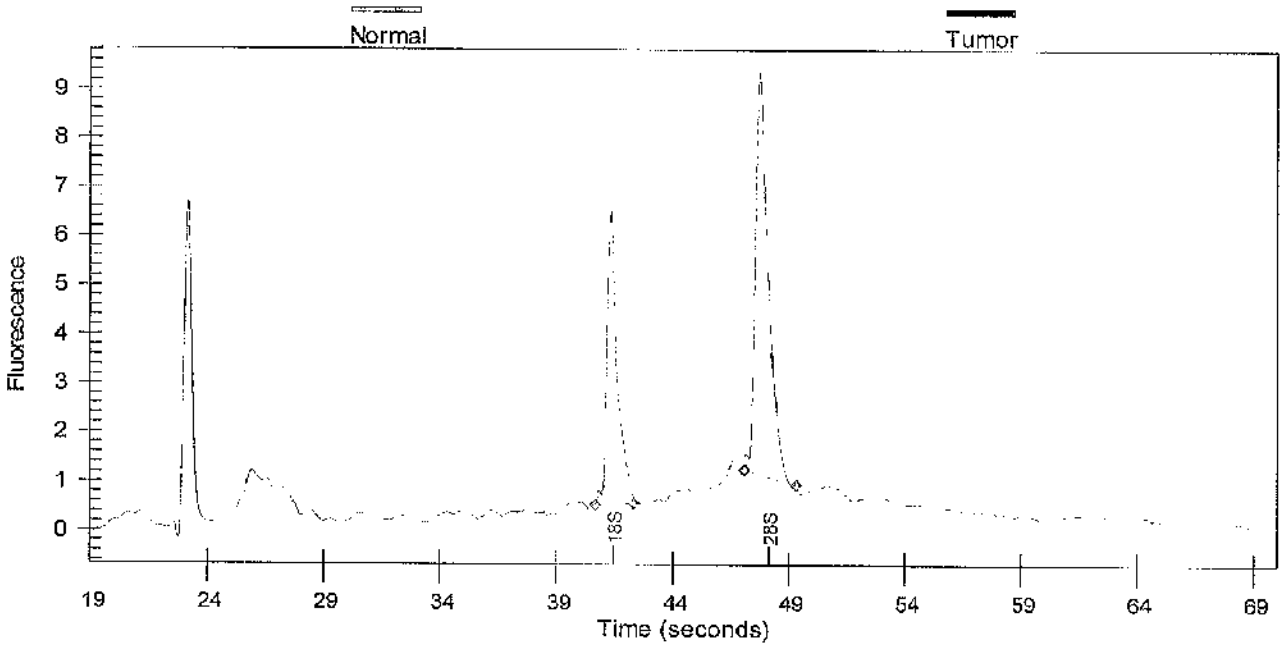

N T N T
5.8S rRNA
18S rRNA
28S rRNA
TFIIB

Figure 5.8

The levels of rRNA are specifically elevated in colorectal tumour biopsy samples compared to normal tissue from the same individuals.

RNA levels from matched normal and tumour colorectal tissues were assessed using an Agilent 2100 Bioanlayzer. 250 ng of each of the RNA samples were loaded onto a RNA 6000 Nano microchip. The electropherograms for each normal/tumour pair were then overlaid. The first peak is the marker. The next peak is a combination of the 5S rRNA, 5.8S rRNA and tRNA, and the following two peaks represent 18S and 28S rRNA.


Normal
Tumor
Fluorescence
25
20
15
10
5
0
18S
28S
19
24
29
34
39
44
49
54
59
64
69
Time (seconds)


Normal
Tumor
Fluorescence
9
8
7
6
5
4
3
2
1
0
18S
28S
19
24
29
34
39
44
49
54
59
64
69
Time (seconds)

to be overexpressed in all except one (sample #1) of the colorectal biopsy samples examined in comparison with matched normal tissue from the same individuals (Figure 5.9). ß-catenin also targets cyclin D1.

5.2.6 Levels of Cyclin D1 and D2 in Colorectal Tumours.

Various cyclins accumulate in a cell cycle-regulated manner and complex with cyclin-dependent kinases. Binding of a cyclin-dependent kinase to a specific cyclin partner activates the kinase, which in turn phosphorylates and activates downstream target proteins that are necessary to propel the cell into the next phase of the cell cycle (Buckley *et al.*, 1993). Overproduction of cyclins and cyclin-dependent kinases, or their presence at an inappropriate time, would be expected to cause unregulated cell division. Therefore, RT-PCRs were carried out to investigate the levels of cyclin D1 and cyclin D2 transcripts. Figure 5.10 shows that cyclin D1 is overexpressed in seven (samples #1, 5, 6, 7, 8, 9 & 10) of the eleven colorectal tumours in comparison with surrounding normal mucosa. In contrast, cyclin D2, which is a direct target of Myc, is overexpressed in four of these samples (#1, 6, 9 & 10). It is interesting to note that overexpression of cyclin D2 occurs in the same colorectal tumours that overexpress pol III transcripts.

Figure 5.9

c-Myc mRNA is overexpressed in colorectal tumour biopsy samples.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour colorectal tissues. These cDNAs were PCR amplified using primers specific for c-Myc and TFIIB. Ad = adenoma, N = normal, T = tumour.

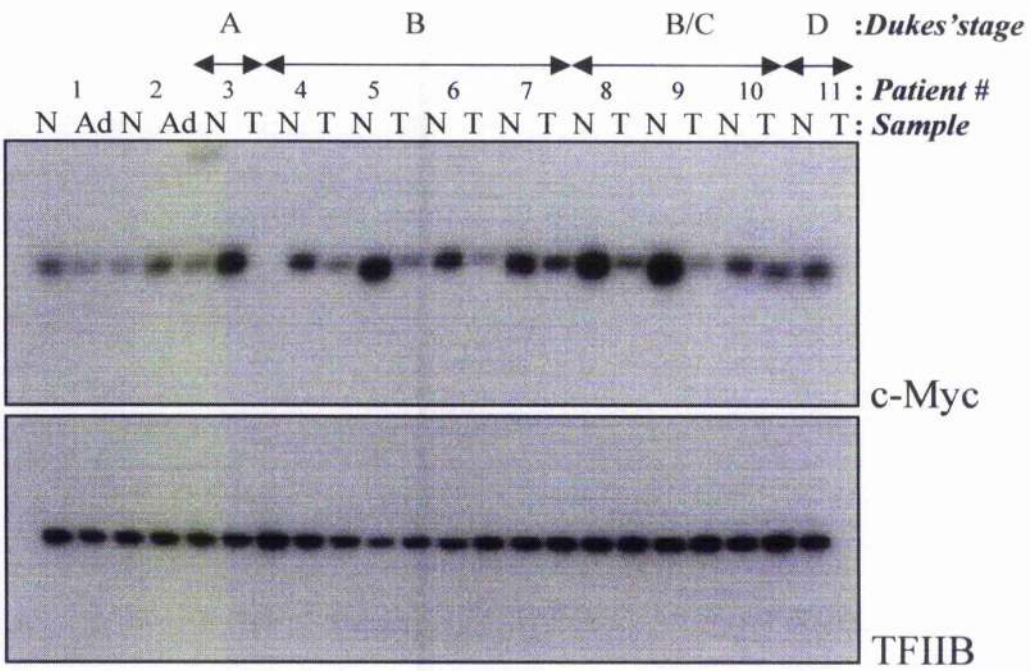
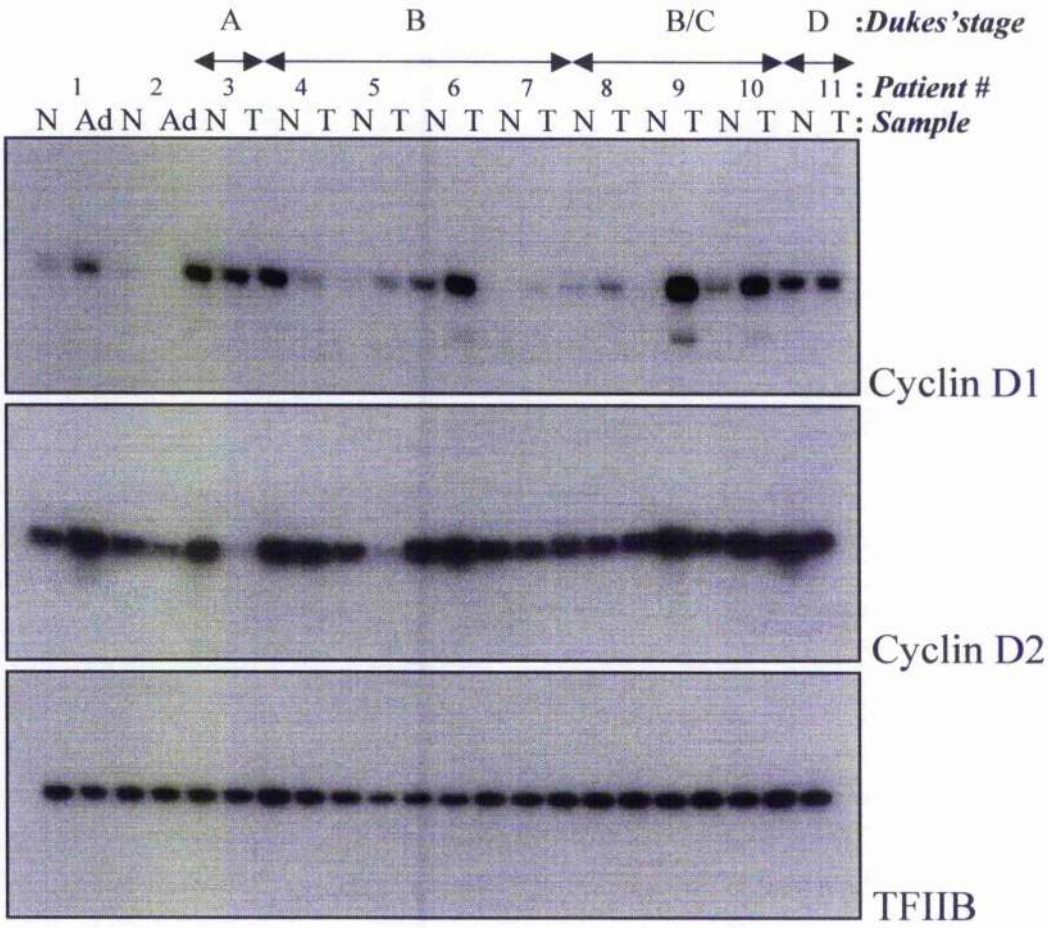

A
B
B/C
D
:Dukes'stage
1 2 3 4 5 6 7 8 9 10 11 : Patient #
N Ad N Ad N T N T N T N T N T N T N T : Sample
c-Myc
TFIIB

Figure 5.10

Cyclin D-dependent kinase mRNAs are overexpressed in many of the colorectal tumour biopsy samples examined.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour colorectal tissues. These cDNAs were PCR amplified using primers specific for cyclin D1, cyclin D2, and TFIIB. Ad = adenoma, N = normal, T = tumour.


A
B
B/C
D
:Dukes'stage
1 2 3 4 5 6 7 8 9 10 11 : Patient #
N Ad N Ad N T N T N T N T N T N T N T N T N T : Sample
Cyclin D1
Cyclin D2
TFIIB

5.3 Discussion.

It has been observed that relatively few changes in transcript expression are associated with colon cancer (Notterman *et al.*, 2001; Zhang *et al.*, 1997). Expression profiles obtained by oligonucleotide arrays from colorectal samples revealed that only nineteen transcripts out of the 4000 examined had elevated mRNA expression in colorectal tumours compared with paired normal samples, whereas 47 transcripts displayed lower expression in the tumour tissue compared with the normal samples (Notterman *et al.*, 2001).

The results from this study found that genes transcribed by pol III were elevated in over a third of the colorectal tumours examined. This upregulation of transcripts such as 5S rRNA and tRNAs agrees with previous tumour investigations carried out by Winter *et al.* (2000), who found that each of the four ovarian tumours examined displayed elevated levels of the aforementioned genes. In addition to upregulation observed in pol III transcription, pol I activity was found to be elevated in half of the colorectal tumour samples tested. Transcriptional down regulation by pols I and III was also observed in some tumour samples. These changes in transcript levels are summarised in table 5.1.

The variability in gene expression across different tumour and normal samples could be due to a number of factors. It is conceivable that the histopathologically normal appearing mucosa adjacent to a tumour is not normal at the molecular level. It is possible, therefore, that elevated transcription in these adjacent areas may be higher than normal tissue in mucosa distant from the tumour. Alternatively, tumour cells may secrete a factor(s) that affects gene regulation in the adjacent normal cells in a

paracrine fashion (Wang *et al.*, 2002). Differences could also be due to imbalances of cell types. This has been observed previously by groups working with colorectal tissue samples (Notterman *et al.*, 2001). Both normal and tumour tissues contain variable amounts of non-epithelial cells in which the normal expression of the investigated genes may outweigh the tumour associated alterations. Furthermore, the alterations in gene expression may not be homogeneous within a tumour. For example, overexpression of the gene encoding p21 is detected in mucinous carcinoma cell lines and can also be confirmed after analysis of tissue on a single cell level. However, this change is not detectable in tissue RNA, due to the inhomogeneity of p21 expression in the tumour (Backert *et al.*, 1999). The best way to overcome such problems might be to carry out *in situ* hybridisation of tissue sections.

With such a limited survey of tissue samples, the clinical stage of disease was not seen to be related to a specific pattern of gene expression. However, it was observed that elevation of transcription by pols I and III appeared to occur frequently in the later stages of tumour development (table 5.1). This is to be expected, as increases in the biosynthetic machinery are required to sustain rapidly increasing growth rates that occur in tumour progression. Indeed, oligonucleotide arrays have shown that a cluster of ribosomal protein genes is expressed at higher levels in colon tumour tissues than in normal colon tissues (Alon *et al.*, 1999).

Transcription by pols I and III was also seen to be upregulated in one of the adenoma samples. Since adenomas are considered to be the pathological precursors to colorectal carcinogenesis (Kinzler & Vogelstein, 1996), it may be that upregulation of pol I and III transcription occurs as an early event in tumour development. In

agreement with this, several ribosomal proteins (S6, S8, S12, L5 and ARPP P0) have been shown to be overexpressed in adenomas (Pogue-Geile *et al.*, 1991). With the furthest progressed Dukes' D tumour, there is seen to be no increase in the rate of transcription by either pol I or pol III. At this late stage, this lack of upregulation may be due to decreased growth rates in some regions of the tumour that are poorly vascularised and have become hypoxic. Indeed, it is well known that different areas of tumours express different genes according to the available oxygen supply. Therefore, tissue removed from the centre of a carcinogenic lesion may not reflect the active transcription at the perimeter of a tumour. Unfortunately, data regarding the precise location of tissue samples obtained was not available for analysis.

Analysis of some of the transcription factors utilized by pols I and III revealed that UBF and Rrn3 were not upregulated in all the tumour samples that displayed elevated pol I activity. In fact, they were only elevated in two of these samples and down regulation of these factors was more frequently observed. Therefore, pol I upregulation does not seem to require upregulation of these initiation factors, at least at the RNA level. This is in agreement with results from Huang *et al.* (2002) who previously observed that although upregulation of UBF was seen in eleven out of sixteen hepatocellular carcinomas tested, this is specific to this particular cancer and was not observed in lung, esophageous, breast and ovarian cancers. In fact, some of these tumour samples displayed lower levels of UBF in comparison with matched normal tissue. There appeared to be more correlation between TBP upregulation and increased transcription in both the pol I and III systems. However, in the colorectal adenoma sample #2, although TBP mRNA is elevated, there is no increase in the rate of transcription by pols I or III. The remaining TFIIIB subunits as well as TBP, and

all five of the TFIIIC2 subunits were found to be coordinately upregulated in samples #6, 9, and 10. The colorectal tumour sample #2 displayed elevated levels of TFIIIB and two of the subunits of TFIIIC2 (TFIIIC110 & TFIIIC90). However, transcription of pol III templates remained unchanged. The adenoma sample which displayed elevated levels of pol III transcripts (sample #1) also displayed upregulated Bdp1 transcription. Unfortunately, due to the limited amount of biopsy tissue available, protein could not be isolated to further analyse levels of these transcription factors. It therefore remains possible that some samples overexpress transcription factors without any change in their mRNA levels through translational control. Conversely, a change in mRNA level might not result in changed protein levels.

As no clear correlation was established between the level of expression of transcription factors utilized by pols I and III, and their activity, other regulators that are known to affect transcriptional activity were investigated. It has been shown that mutations in the tumour suppressor gene APC lead to aberrant nuclear accumulation of the β–catenin/Tcf transcription complex. This, in turn, leads to increased expression of target genes, such as *c-myc* (He *et al.*, 1998). In this study, c-Myc mRNA levels were found to be elevated in ten of the eleven colorectal tumours tested, in comparison with the adjacent normal mucosa. The high percentage of tumours in which c-Myc was upregulated is in agreement with results from other groups (Nesbit *et al.*, 1999; Rothberg, 1987; Wang *et al.*, 2002).

Cyclin D1 is another gene targeted by the β–catenin/Tcf transcription complex (Shtutman *et al.*, 1999; Tetsu & McCormick, 1999; Wang *et al.*, 2002). Cyclin D1 overexpression has been observed in a subset of neoplastic conditions, including

human colorectal cancers (Arber *et al.*, 1996, 1997; Sutter *et al.*, 1997). Arber *et al.* (1996) demonstrated that nuclear expression of cyclin D1 occurs in one third of the samples of colonic tumours, but this was not found to correlate with Dukes' staging. In addition, this group showed that inhibition of cyclin D1 expression causes growth arrest in colon carcinoma cell lines (Arber *et al.*, 1997). The constitutive activation of Ras further stimulates transcription of the cyclin D1 gene (Albanese *et al.*, 1995; Tetsu & McCormick, 1999). In keeping with these findings, many of the colorectal tumour samples examined displayed upregulated levels of cyclin D1 mRNA (seven out of the eleven tested). This occurred frequently in the later stage tumours (Table 5.1), presumably after activation of K-*ras*. Cyclin D2, which is controlled by different signalling pathways, was not seen to be upregulated as frequently. Although cyclin D1 is upregulated in many of the colorectal tumours, it is only when cyclin D2 is also elevated that increased pol III activity is observed. Overexpression of these products is expected to have a stimulating effect on transcription, as it would enable the phosphorylation of RB. Thus, in a hyperphosphorylated state RB would no longer be able to bind to UBF and TFIIIB, and therefore the transcriptional repression mediated by this tumour suppressor protein would be alleviated. Cyclin D2 is a well-characterized direct target for c-Myc. Elevated expression of cyclin D2 mRNA may therefore be taken as an indicator that c-Myc activity is raised. It is very striking that there is a perfect correlation between the induction of cyclin D2 mRNA and the induction of pol III transcripts. This suggests that Myc activity may be rate-limiting for pol III transcription in colorectal cells.

The results of this study suggest that activity of pol I and III is upregulated in a number of colorectal carcinomas, in one case occurring at an early stage in the

development of adenomas, with continued deregulation throughout the progression of the neoplastic disease. Further samples would need to be tested to gather more information regarding the frequencies of these overexpression events, but it appears that in colorectal carcinoma pol I is deregulated with a higher frequency than pol III.

A straightforward correlation is not apparent between the products of pols I and III and the mRNAs encoding any of the transcription factors that were investigated. This might be because regulation is occurring at a different level, such as phosphorylation of TFIIIB by Erk or CK2. Alternatively, individual tumours might use different mechanisms to achieve the same molecular end. For example, upregulation of Bdp1 might drive the activation of pol III transcription that is seen in adenoma #1, whereas the increase in tumours #6, 9 and 10 might be caused by increases in TFIIIC2 expression. However, one event that correlates perfectly with pol III activation is the induction of cyclin D2 mRNA. This might indicate that cyclin D2 has a direct effect on the pol III machinery, but it might also indicate a dependence on c-Myc activity. Although nearly all of the tumours express elevated c-Myc mRNA, only four of the samples show increased c-Myc function, as assessed by expression of its target cyclin D2. Perhaps c-Myc activation in these four samples is responsible for the induction of pol III transcription.

Table 5.1

Summary of changes in expression observed in colorectal tumour biopsy samples examined by RT-PCR.

cDNAs were generated by reverse transcription of total RNA extracted from matched normal and tumour colorectal tissues. Arrows denote up or down regulation of a particular transcript in the tumour relative to the matched healthy sample. Ad = adenoma, N = normal, T = tumour.

			Dukes' A	Dukes' B				Dukes' B/C			Dukes' D
	1	2	3	4	5	6	7	8	9	10	11
	Ad	Ad	T	T	T	T	T	T	T	T	T
tRNA ^{Arg}	↑		↓	↓	↓	↑	↓	↓	↑	↑	↓
tRNA ^{Leu}	↑			↓	↓	↑			↑	↑	↓
tRNA ^{Sec}	↑		↓	↓	↓	↑		↓	↑	↑	↓
5S rRNA	↑		↓	↓	↓	↑		↓	↑	↑	↓
MRP	↑		↓	↓	↓	↑		↓	↑	↑	↓
7SK	↑		↓	↓	↓	↑	↓		↑	↑	↓
Bdp1	↑	↑	↓		↓	↑		↓	↑	↑	↓
Brf1		↑	↓	↓	↓	↑	↓	↓	↑	↑	↓
TBP		↑	↓	↓	↓	↑	↓	↓	↑	↑	↓
TFIIIC220			↓		↓	↑		↓	↑	↑	↓
TFIIIC110		↑	↓	↓	↓	↑	↓	↓	↑	↑	
TFIIIC102	↓	↓	↓	↓	↓	↑	↓	↓	↑	↑	↓
TFIIIC90		↑			↓	↑			↑	↑	↓
TFIIIC63			↓	↓	↓	↑		↓	↑	↑	↓
5.8S rRNA	↑		↓			↑		↑	↑	↑	↑
18S rRNA	↑	↓	↓			↑		↑	↑	↑	↑
28S rRNA	↑	↓	↓			↑		↑	↑	↑	↑
Rrn3	↓		↓	↓		↑	↓	↓		↑	↓
UBF		↓	↓	↓	↓	↑	↓	↓		↑	↓
c-Myc		↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
Cyclin D1	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑	
Cyclin D2	↑	↓	↓		↓	↑			↑	↑	↓

Chapter 6

Trichostatin A

6.1 Introduction.

Gene expression is under the control of enzymes that regulate transcription by modifying the acetylation state of histones. Deregulation of the activity of these histone acetyltransferases (HATs) and histone deacetylases (HDACs) has been implicated as having a causative role in the generation of cancer (Wang *et al.*, 2001).

HATs transfer the acetyl moiety from acetyl coenzyme A to the ε-NH_3^+ groups of internal lysine residues (Kuo & Allis, 1998). Introduction of the acetyl group neutralizes the positive charge and increases the hydrophobicity. This acetylation of the histone H3 and H4 lysines is associated with destabilization of nucleosomes, as the DNA wrapped around the core histone becomes loosened (Grunstein, 1997). This DNA conformation enables transcription factors, regulatory complexes and RNA polymerases to gain access to the DNA. Therefore, expression of the corresponding genes is promoted (Bannister & Miska 2000; Bronwell & Allis, 1996; Tse *et al.*, 1998; Ura *et al.*, 1997).

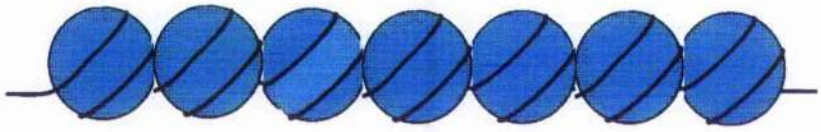
Histone acetylation is a reversible process (Figure 6.1) and, in the opposing reaction, HDACs remove the acetyl groups, re-establishing the positive charge in the histone tails and enabling condensation of the nucleosome structure (Kuo & Allis, 1998). This causes a tighter association between DNA and nucleosomes, which inhibits transcription by impairing the access of the transcription apparatus (Knoepfler & Eisenman, 1999).

In addition to modifying the acetylation state of histones, HATs and HDACs can regulate gene expression through their actions on transcription factors (Gu & Roeder,

Figure 6.1

Model depicting the regulation of gene expression by histone acetylation and deactetylation.

Recruitment of histone acetyltransferases (HATs) to silent regions of DNA causes the transfer of acetyl groups to the N-terminal tails of histones, thereby reducing the ability of nucleosomes to occlude the promoter region. The transcription machinery can then gain access to this area and transcription initiation can commence. Recruitment of histone deacetylases (HDACs) to active regions of DNA reverses this process. The box illustrates the molecular structure of trichostatin A (TSA), a specific inhibitor of HDACs.

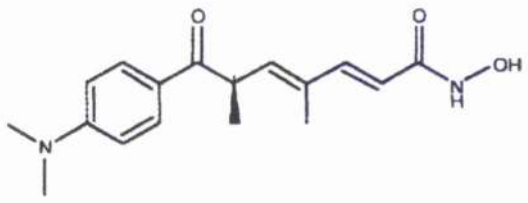


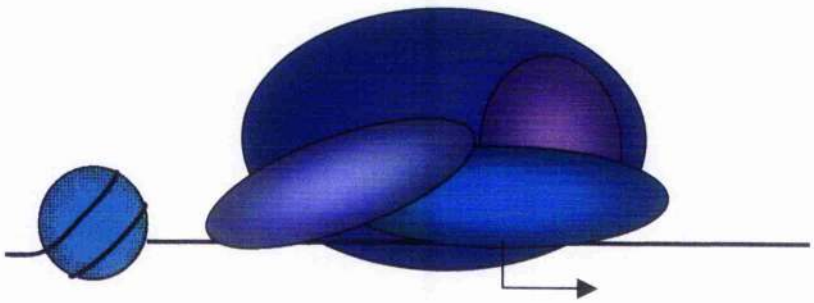
Silent

HATs

HDACs

TSA





Active

1997; Imhof *et al.*, 1997; Muth *et al.*, 2001; Smirnov *et al.*, 2000; Zhang & Bieker, 1998). In addition, some transcription factors have intrinsic acetyltransferase activity, such as TFIIIC2, which displays HAT activity that is intrinsic to the TFIIIC90 subunit, and probably also to the TFIIIC220 and TFIIC110 subunits (Hsieh *et al.*, 1999a; Kundu *et al.*, 1999).

HATs and HDACs also participate in cell cycle regulation. It has been demonstrated in the pol II system that transcriptional repression by the tumour suppressors p53 and RB, involves the recruitment of HDAC complexes (Brehm *et al.*, 1998; Luo & Postigo, 1998; Magnaghi-Jaulin *et al.*, 1998; Murphy *et al.*, 1999). RB binding to the transcription factor E2F involves recruitment of HDACs (Brehm *et al.*, 1998; Luo & Postigo, 1998; Magnaghi-Jaulin *et al.*, 1998). The importance of the binding of HDAC to RB is suggested by the fact that many tumour-specific mutations found in RB disrupt its association with the deacetylase (Brehm *et al.*, 1998). In addition, viral oncoproteins such as the HPV16 E7 protein or the SV40 T-antigen are able to displace deacetylase activity from RB (Brehm *et al.*, 1998; Magnahi-Jaulin *et al.*, 1998). These observations suggest a key role for HDACs in G1 arrest. Repression by RB of E2F can also occur because RB masks the activation domain of E2F (Ross *et al.*, 1999). These two distinct mechanisms used in the control of pol II transcription are selective, since many E2F-dependent promoters can be repressed by RB in the absence of HDAC activity (Luo & Postigo, 1998).

As deregulation of these enzymes would cause changes in the expression of a broad range of genes, it is unsurprising that mutations in HATs or aberrant recruitment of HDACs are associated with increased risks for cancer (Wang *et al.*, 2001).

Amplification and overexpression of the acetylase AIB1 has been demonstrated to be clearly associated with breast cancer (Anzick *et al.*, 1997). The p300/CBP family of acetylases is also implicated in cancer. It is found translocated in acute myelocytic leukaemia (Borrow *et al.*, 1996; Ida *et al.*, 1997; Sobulo *et al.*, 1997), and is found mutated in colorectal and gastric carcinomas (Muraoka *et al.*, 1996). Furthermore, interaction of p300/CBP with E1A is necessary for immortalisation by E1A (Moran, 1993). Aberrant recruitment of histone deacetylases has been found in cell lines derived from patients with acute promyelocytic leukaemia, acute myelogenous leukaemia, involving acute myelogenous leukaemia 1 and ETO genes, and non-Hodgkin lymphoma, with inappropriate expression of repressor BCL-6 (Marks *et al.*, 2000).

The use of drugs that inhibit the activity of HDACs has attracted considerable interest as a new therapeutic approach for cancer therapy. Low molecular weight molecules such as trichostatin A (TSA), suberoylanilide hydroxamic acid, and oxamflatin are potent HDAC inhibitors that block the growth of tumour cells (Marks *et al.*, 2001). The microbial metabolite TSA induces cell differentiation and cell-cycle arrest, at both G1 and G2 phases, and inhibits morphological transformation (Futamura *et al.*, 1995; Yoshida *et al.*, 1990, 1995).

TSA consists of a dimethylamino-phenyl group linked to hydroxamic acid (Yoshida *et al.*, 2001). Crystallographic studies have shown that this inhibitor mimics the substrate and is able to chelate the zinc in the catalytic pocket of HDACs through the actions of its functional hydroxamic acid group (Finnin *et al.*, 1999). Several studies have shown that the inhibition of HDACs with TSA induces the hyperacetylation of

histones (Van Lint *et al.*, 1996; Yoshida *et al.*, 1990, 1995) and regulates transcription of several genes (Chen *et al.*, 1997b; Chireux *et al.*, 1996; Van Lint *et al.*, 1996; Yoshida *et al.*, 1995).

Previously it has been demonstrated that histone acetylation can facilitate pol III transcription from a chromatin template *in vitro* (Lee *et al.*, 1993; Tse *et al.*, 1998; Ura *et al.*, 1997). Furthermore, acetylation can influence pol III activity *in vivo*. Pol III transcription of SINEs is heavily repressed by histones in chromatin from murine fibroblasts (Carey & Singh 1988; Russanova *et al.*, 1995). Treatment with TSA overcomes this repression, resulting in a strong induction of B2 transcripts in these untransformed cells (Sutcliffe *et al.*, 2000). Nevertheless, TSA does not block the ability of RB to regulate pol III genes *in vitro* or *in vivo*. This indicates that RB represses pol III transcription by an HDAC-independent method.

The aim of this chapter was to examine the effects of TSA on the expression of pol III-transcribed genes in matched transformed and untransformed cells in order to assess whether elevated transcription after transformation results from abnormal acetylation.

6.2 Results.

6.2.1 TSA may Interfere with Ribosomal Processing in Transformed Cells.

Previously it has been shown that TSA can upregulate expression of B2 genes, presumably by inhibiting HDACs (Sutcliffe *et al.*, 2000). To examine the effects of this drug on transformed cells in comparison with untransformed cells, the parental Balb/c 3T3 A31 and SV40-transformed Cl49 cell lines were cultured either without TSA, or in the presence of 100, 200 or 300 nM TSA. It was observed that culturing the transformed murine fibroblasts in the presence of TSA over a period of 48 hours caused significant apoptosis. This occurred in a dose dependent manner. Another SV40-transformed cell line, Cl38, was also initially examined in preliminary experiments. However, levels of apoptosis were extremely high, even at the lower concentrations of TSA; therefore this cell line was not used in further experiments. In the untransformed cells, TSA had no effect on cell viability.

RNA extracted from these cell lines was loaded onto a MOPS gel and stained with ethidium bromide. Upon analysis of this gel it was apparent that an additional band was present, which migrated in between 28S and 18S rRNA in the Cl49 samples that had been treated with TSA (Figure 6.2A). This additional band was also extremely prominent in the RNA extracted from Cl38 cells that had been treated with the HDAC inhibitor (Figure 6.2B). It was also observed that as the levels of this unidentified band increased with increasing TSA concentration, the intensity of 28S rRNA, and to a lesser extent 18S rRNA, decreased. In the untransformed cells, TSA had no such

effect on the RNA. This raises the possibility that TSA may be interfering with ribosomal processing in the transformed cells.

6.2.2 TSA Induces B2 Gene Expression in Untransformed and Transformed Cells.

Northern blot analysis was carried out with the same A31 and Cl49 RNA samples used in Figure 6.2A. The blot was first probed with a B2 gene, and then was stripped and reprobed with the control ARPP P0 gene. It was found that both the untransformed and SV40-transformed fibroblasts displayed increases in the expression of B2 with the HDAC inhibitor TSA. This occurred in a dose dependent manner (Figure 6.3). This response was seen to be specific, since the gene encoding ARPP P0 was unaffected by the presence of TSA. As previously observed by White *et al.* (1990), it was found that the transformed Cl49 fibroblasts expressed B2 at a higher rate than the untransformed A31 cells. Quantification of these RNA levels revealed that addition of TSA caused less of an increase in the transformed cells (approximately 1.5 fold) than in the untransformed cells (approximately 5 fold).

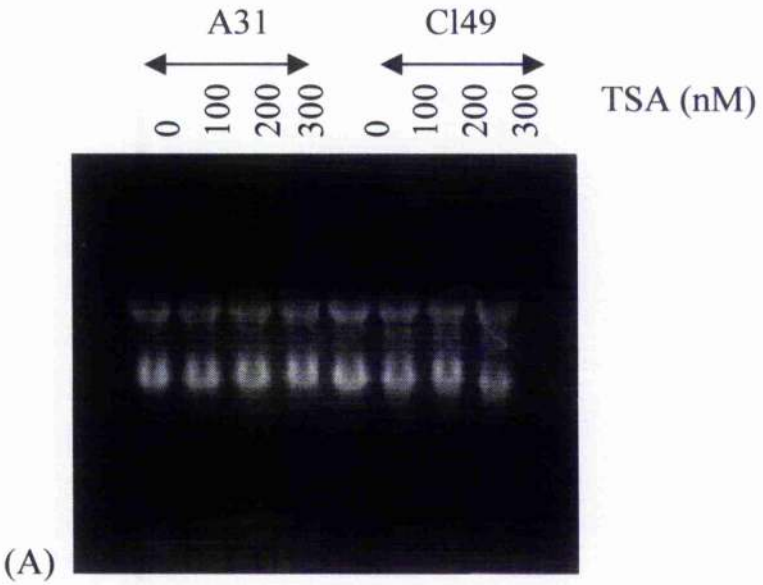
6.2.3 TSA Stimulates the Expression of other Pol III Templates.

To assess whether TSA causes increases in the expression of other pol III templates, RT-PCRs were carried out on RNA extracted from A31 and Cl49 fibroblasts cultured either without TSA or in the presence of 200 nM TSA. It was found that this drug caused increases in the expression of 5S rRNA, tRNA ^{Arg}, ^{Leu}, and ^{Sec}, as well as B2 in

Figure 6.2

TSA may interfere with ribosomal processing in transformed cells.

Total RNA extracted from A31 and (A) Cl49, or (B) Cl38 fibroblasts cultured either without TSA, or in the presence of 100, 200 or 300 nM TSA was loaded onto a MOPS gel and stained with ethidium bromide.


A31
Cl49
0
100
200
300
0
100
200
300
TSA (nM)
(A)

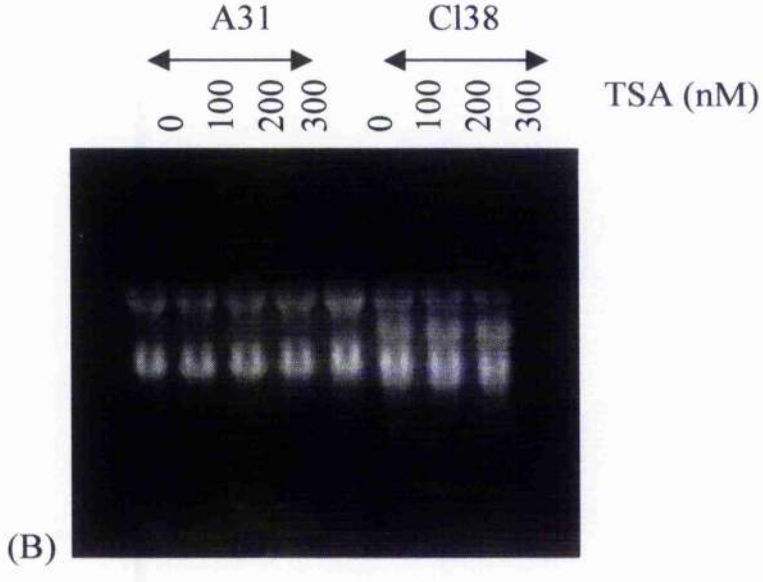
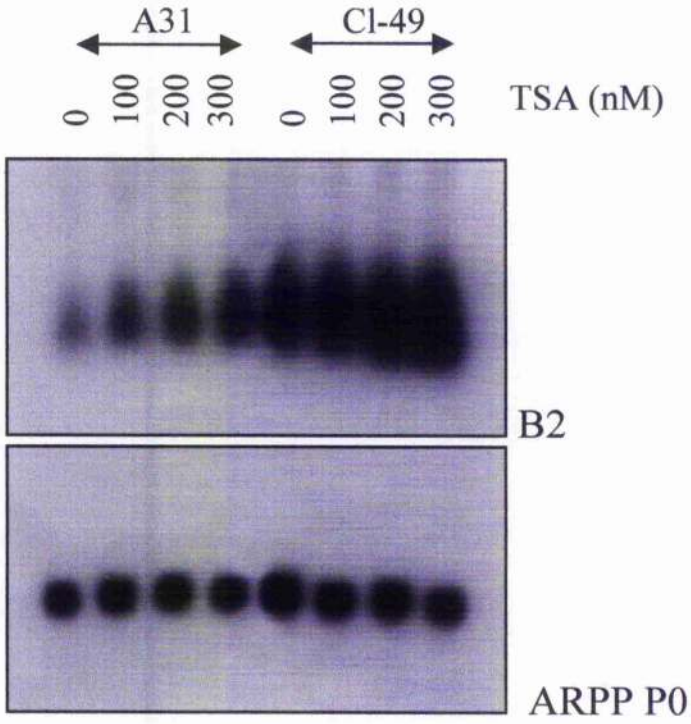
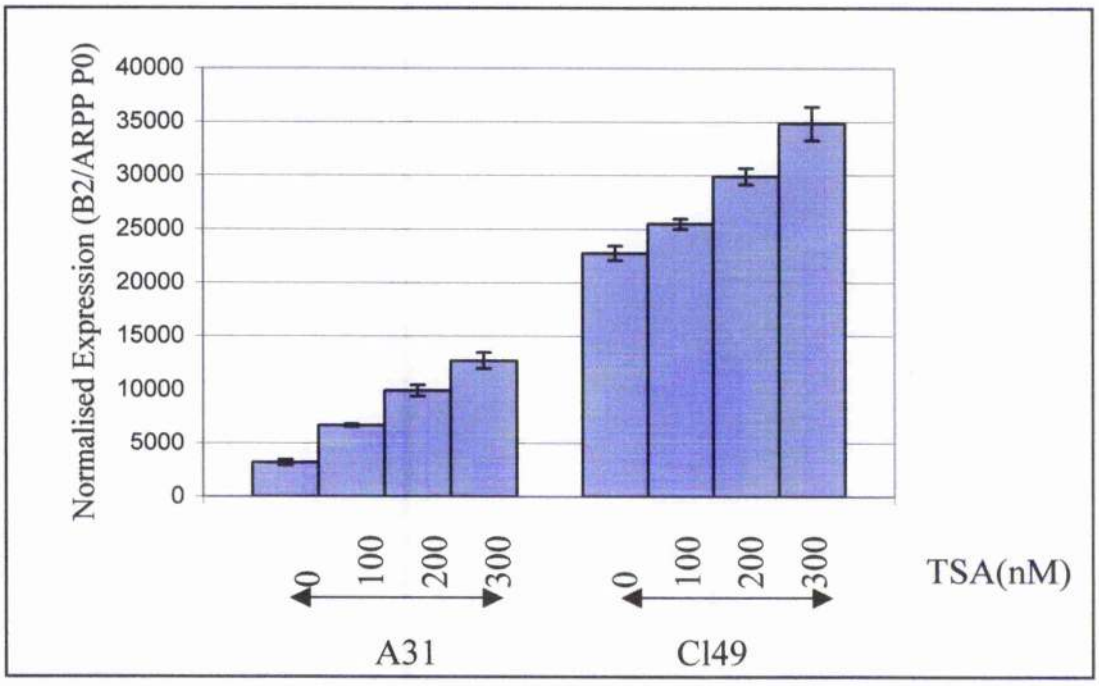

A31
Cl38
0
100
200
300
0
100
200
300
TSA (nM)
(B)

Figure 6.3

TSA induces B2 gene expression in untransformed and transformed cells.

Northern blot analysis of total RNA extracted from A31 and Cl49 fibroblasts cultured either without TSA, or in the presence of 100, 200 or 300 nM TSA. The blot was first probed with a B2 gene, and then was stripped and reprobed with the ARPP P0 gene. Levels of RNA were quantified using a phosphoimager. Values shown represent the means from two experiments.


A31
Cl-49
0
100
200
300
0
100
200
300
TSA (nM)
B2
ARPP P0


Normalised Expression (B2/ARPP P0)
40000
35000
30000
25000
20000
15000
10000
5000
0
0
100
200
300
0
100
200
300
TSA(nM)
A31
Cl49

both A31 and Cl49 fibroblasts (Figure 6.4). However, levels of expression of the pol III transcript 7SK, which has a type III promoter structure, did not appear to be affected by the presence of TSA in the transformed cells, and the levels of this transcript decreased in the untransformed A31 cells. When the subunits of TFIIIC2 were examined, it was found that TSA caused a decrease in expression in Cl49 cells, whereas it appeared to have no effect in A31 cells (data not shown).

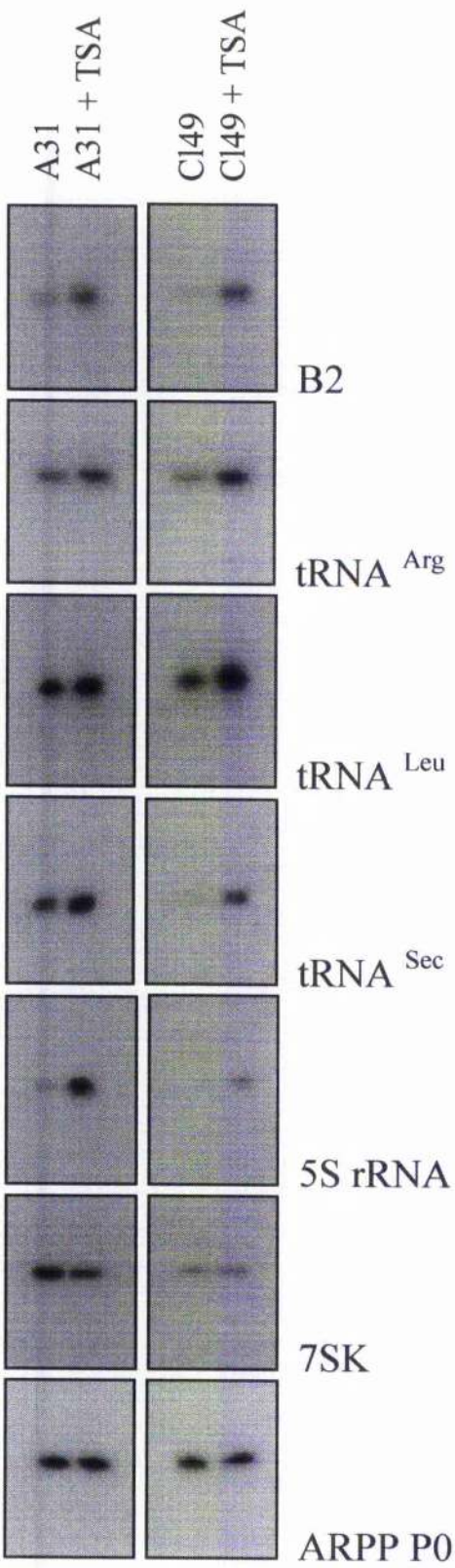
6.2.4 Inhibition of HDACs Increases the Occupancy of Pol III Transcription Machinery at Class III Gene Promoters in Untransformed and Transformed Murine Fibroblasts.

To examine whether TSA could affect the occupancy of transcriptional machinery on a pol III-transcribed gene, chromatin immunoprecipitation (ChIP) assays were carried out. Formaldehyde cross-linked soluble chromatin was prepared from A31 and Cl49 fibroblasts cultured either without TSA or in the presence of 200 nM TSA for a period of 48 hours. This was immunoprecipitated with antibodies against acetylated histone H4, the RPC155 subunit of pol III, the Brf1 subunit of TFIIIB, and the 110 kDa subunit of TFIIIC2. Various concentrations of DNA were used to ensure that the PCR reactions were in the linear range for each antibody used for the ChIP assays. Acetylated histone H4 was used as a positive control, and negative control ChIPs were carried out in the absence of antibody. In both the untransformed and transformed cells it was found that addition of TSA increased pol III, Brf1 and TFIIIC110 promoter occupancy at the promoters of B2, 5S rRNA and $tRNA^{Leu}$ genes (Figures 6.5 & 6.6).

Figure 6.4

TSA stimulates the expression of multiple pol III templates.

cDNAs were generated by reverse transcription of total RNA extracted from A31 and Cl49 fibroblasts cultured either without TSA or in the presence of 200 nM TSA. These cDNAs were PCR amplified using primers specific for B2, $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Sec}$, 5S rRNA, 7SK and ARPP P0.


A31
A31 + TSA
C149
C149 + TSA
B2
tRNA Arg
tRNA Leu
tRNA Sec
5S rRNA
7SK
ARPP P0

Immunoblot analysis demonstrated that the observed changes in pol III, Brf1, and TFIIIC110 occupancy did not result from altered protein levels (Figure 6.7). The levels of these proteins were comparable in the TSA treated and untreated extracts from both the transformed and untransformed cell lines. However, as expected, increases were observed in the acetylated histone H4. Thus, the significantly increased presence of these proteins on the chromatin-assembled genes that were investigated reflects changes in their recruitment to the promoter, and not the protein availability.

Figure 6.5

Repression of HDACs increases the occupancy of pol III transcription machinery at class III gene promoters in untransformed murine fibroblasts.

A31 fibroblasts were cultured either without TSA or in the presence of 200 nM TSA. Association of acetylated histone H4, pol III, Brf1, and TFIIIC110 with B2, $tRNA^{Leu}$, and 5S rRNA genes was determined by ChIPs, and quantitative PCR was performed on undiluted, 1:5 diluted, and 1:25 diluted input chromatin. Control ChIPs were carried out in the absence of antibody.

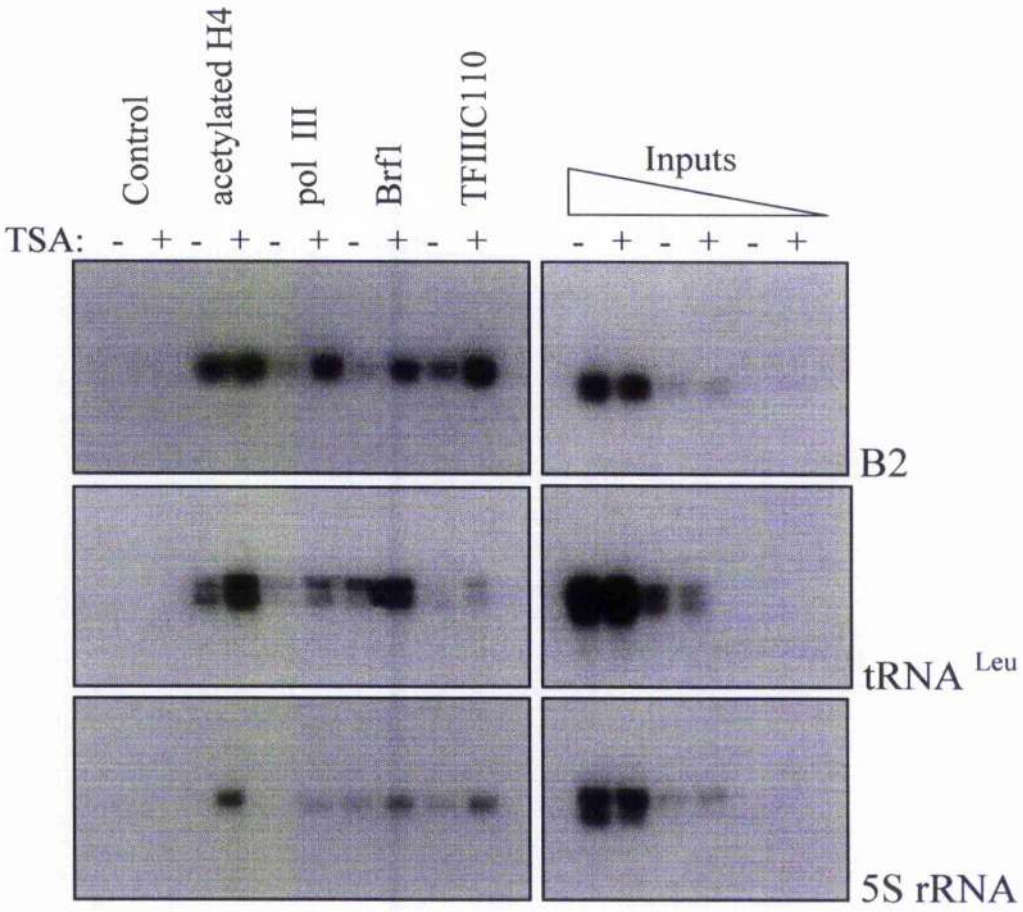

Control
acetylated H4
pol III
Brf1
TFIIIC110
Inputs
TSA: - + - + - + - + - + - + - + - +
B2
tRNA Leu
5S rRNA

Figure 6.6

Repression of HDACs increases the occupancy of pol III transcription machinery at class III gene promoters in transformed murine fibroblasts.

CI49 fibroblasts were cultured either without TSA or in the presence of 200 nM TSA. Association of acetylated histone H4, pol III, Brf1, and TFIIIC110 with B2, tRNA ^{Leu}, and 5S rRNA genes was determined by ChIPs, and quantitative PCR was performed on undiluted, 1:5 diluted, and 1:25 diluted input chromatin. Control ChIPs were carried out in the absence of antibody.

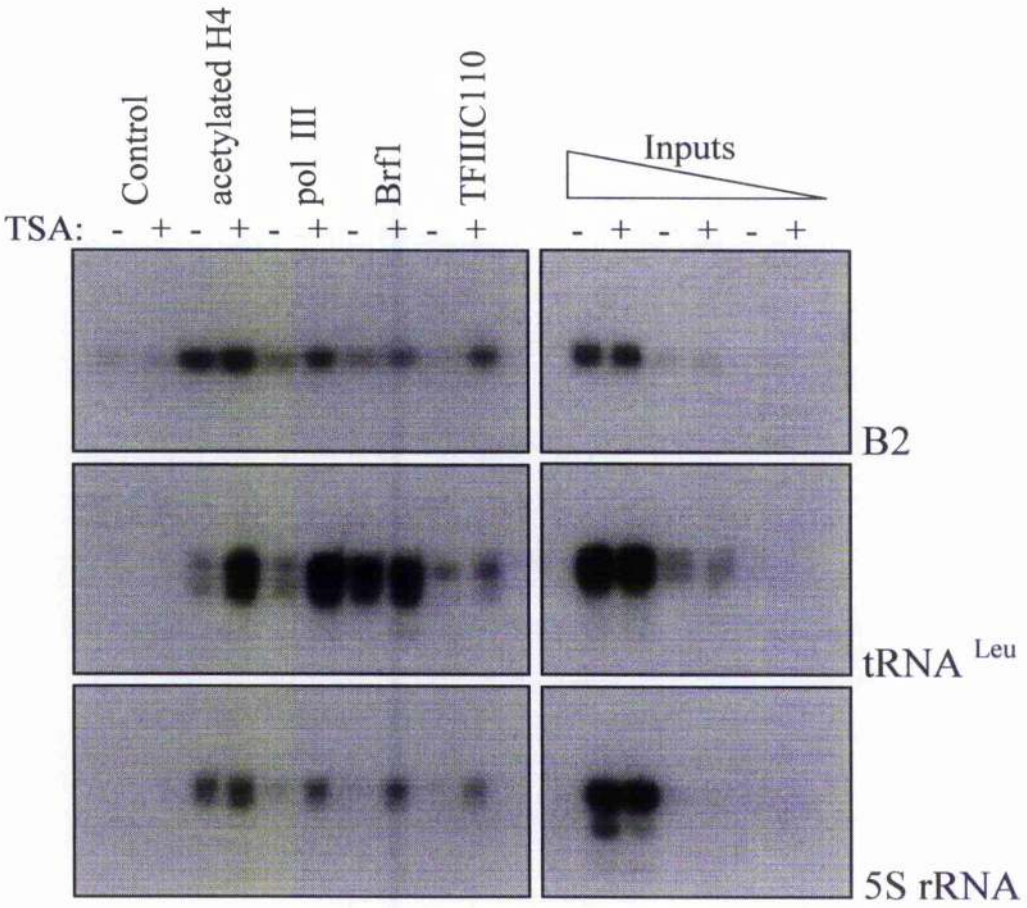
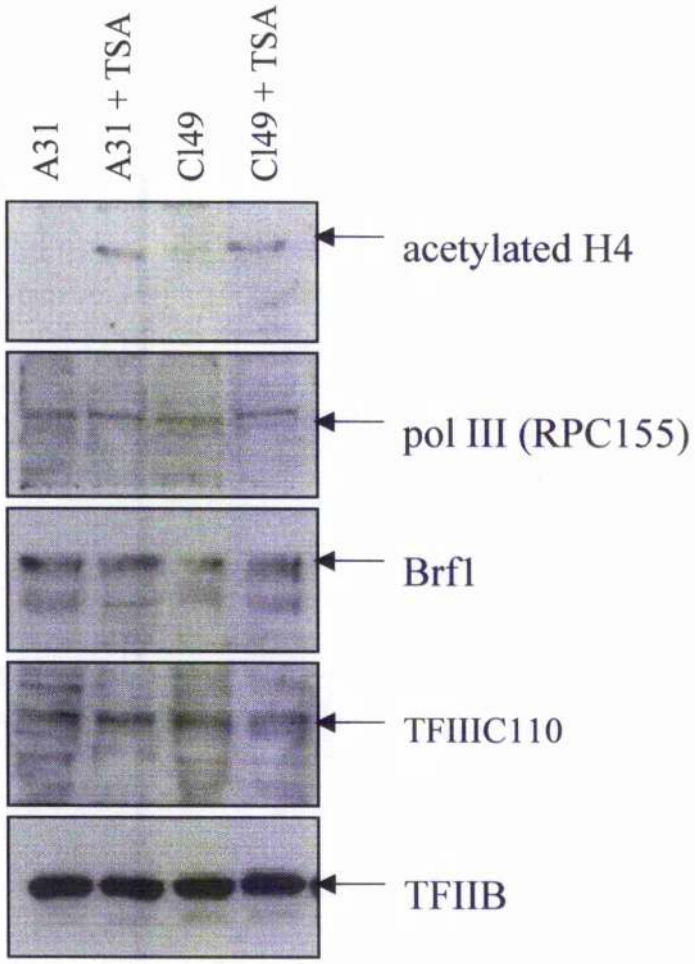

Control
acetylated H4
pol III
Brf1
TFIIIC110
Inputs
TSA: - + - + - + - + - + - + - + - +
B2
tRNA Leu
5S rRNA

Figure 6.7

TSA treatment does not change the levels of pol III, Brf1 and TFIIIC110 protein in transformed and untransformed murine fibroblasts.

Whole cell extracts were made from A31 and Cl49 fibroblasts cultured either without TSA or in the presence of 200 nM TSA. 50 μg of each protein sample was resolved on an SDS polyacrylamide gel and then analysed by Western blotting with antibodies directed against acetylated histone H4, pol III (RPC155 subunit), Brf1, TFIIC110 and TFIIB.


A31
A31 + TSA
C149
C149 + TSA
acetylated H4
pol III (RPC155)
Brf1
TFIIIC110
TFIIB

6.3 Discussion.

Transcription can be directly regulated through acetylation and deacetylation of chromatin. In many cases, recruitment of HATs to histones is associated with increased transcriptional activity, whereas recruitment of HDACs correlates with transcriptional repression. Pol III transcription may be regulated by such chromatin remodelling effects. In this study it was found that pol III transcription was increased in the presence of the HDAC inhibitor TSA. This occurred in both untransformed and transformed murine fibroblasts. As previously observed by White *et al.* (1990), transcription was seen to be higher in the SV40-transformed fibroblasts Cl49s than in the untransformed parental cell line A31, suggesting that expression is especially restrained in the untransformed cells. This is likely to be due, at least in part, to restraint by repressive chromatin structure, since TSA treatment has less effect on transcription in the transformed cells (approximately 1.5 fold, Figure 6.3) than in the untransformed cells (approximately 4 fold, Figure 6.3), suggesting that untransformed cells have less acetylation at their histones.

Addition of TSA to transformed cells was observed to cause significant apoptosis. TSA has been previously reported to cause apoptosis in human hepatoma, Jurkat lymphoid and colorectal carcinoma cells (Chen *et al.*, 2002; Medina *et al.*, 1997; Yamishita *et al.*, 2003). Examination of RNA from these samples revealed an unidentified band that migrated between 28S and 18S rRNA. This may be an indication that TSA is interfering with ribosomal processing in the transformed cells. It would be interesting in future work to develop primers that recognise such transcripts in the murine cells, such as MRP which is involved in rRNA splicing.

TSA was seen to inhibit HDACs at nanomolar concentrations, resulting in increased histone acetylation and modulation of gene expression at a selection of different pol III templates including B2, tRNA ^{Arg}, ^{Leu}, ^{Sec}, and 5S rRNA. However, 7SK, which has a type III promoter structure, was not seen to be affected by the presence of this drug. Boyd *et al.* (2000) have suggested that at 7SK genes a nucleosome positioned over the promoter region brings the DSE and PSE into juxtaposition. This facilitates interaction between the factors binding to these elements. This arrangement is very different from the type I and II promoters of the other genes investigated. Relaxation of the nucleosome structure upon addition of TSA, therefore could account for the observed decrease in transcription of 7SK seen in the untransformed A31 fibroblasts. Furthermore, the presence of binding sites for ZID, a transcription factor known to interact with a HDAC, have been identified upstream of the DSE (Boyd *et al.*, 2000), suggesting that deacetylation of histones may play a role in the regulation of the 7SK gene, perhaps by ensuring that a positioned nucleosome is stabilised. In the cell line Cl49, it is possible that transformation has caused aberrant acetylation of histones, which could account for the lack of effectiveness of TSA.

ChIPs and immunoblotting confirmed that the increases in expression of the pol III genes B2, tRNA ^{Leu}, and 5S rRNA upon addition of TSA, reflected increased promoter occupancy of the pol III transcriptional machinery. This was observed with the RPC155 subunit of pol III, Brf1, and TFIIIC110. At most of these genes this correlated with an increased presence of acetylated histone H4, and also elevated levels of acetylated H4 protein, upon the addition of TSA. However, at B2 genes it was surprising to observe that histone H4 is hyperacetylated without TSA, yet transcription still increases upon addition of the HDAC inhibitor. It is therefore

possible that TSA is affecting histone H3, or perhaps the increased transcription occurs as a result of histone-independent acetylation involving some other protein, for example Brf1.

These data, which suggest that tRNA ^{Leu}, and 5S rRNA are repressed by their chromatin structures, are not unprecedented. Previous studies have shown that B2 elements are sequestered by histone H1 in untransformed and SV40-transformed murine cells (Carey & Singh, 1988). It has also been shown that changes in both the chromatin structure and TFIIIA concentration regulate differential expression of *Xenopus* 5S rRNA genes during development (Brown & Schlissel., 1985; Schlissel & Brown, 1984). In addition, Lee *et al.* (1993) showed that acetylation of core histones improves the accessibility of the 5S rRNA gene to TFIIIA. tRNA genes are also thought to be repressed by nucleosome formation. TFIIIC, which has intrinsic HAT activity, has been found to relieve chromatin-mediated repression of this gene (Hsieh *et al.*, 1999a; Kundu *et al.*, 1999). However, it may be acetylation of a pol III factor that is crucial, rather than histone acetylation. Like phosphorylation, acetylation of transcription factors has been shown to regulate protein-DNA and protein-protein interactions (Boyes *et al.*, 1998; Gu & Roeder, 1997; Martinez-Balbas *et al.*, 2000; Waltzer & Bienz, 1998; Zhang & Bierker, 1998), and it may also increase protein stability (Martinez-Balbas *et al.*, 2000).

These results suggest that acetylation provides a link between chromatin structure and pol III transcriptional output. Class III genes with type I and II promoters may be repressed by their chromatin structure. This can be overcome by inhibition of HDACs, thus allowing the acetylation of histones, which in turn results in unfolding

of the nucleosome structure so that transcription factors and pol III can gain access. Alternatively, it may be that pol III transcription factors become acetylated. Since numerous studies (reviewed in Kouzarides, 1999) have implicated the involvement of acetylases in the control of cell proliferation, it may well be that acetylation provides an additional method for controlling pol III transcription. It would, therefore, be interesting to examine if tumours containing mutations in HATs and HDACs show deregulated pol III transcription.

Chapter 7

Final Discussion

Over the past 50 years much research has been devoted to unraveling the abnormalities that occur during the development of cancer. Despite this, many of the specific cellular and molecular processes of these complex events still remain to be elucidated. The genesis of malignancy requires the sequential accumulation of a number of genetic alterations (Fearon, 1992; Weinberg, 1991). These changes disrupt the regulatory mechanisms that limit growth potential and as a result, tumour cells are able to escape from senescence and display elevated proliferative activity. This would not be possible if the levels of transcription were limiting.

The main aim of this study was to investigate transcription by pols I and III in different tumours, to examine if deregulation occurs, and to identify transcription factors that may contribute to this. Expression studies of genes transcribed by these polymerases demonstrated that most of the tumour biopsies examined displayed deregulated transcriptional activity in comparison with matched normal tissue. However, the relationship between carcinogenesis and transcription was not found to be clear-cut and therefore, would not be suitable for diagnostic purposes.

Transcription by pol III in the cervical biopsy samples correlated with the presence of high risk HPV16, which caused a specific increase in the transcription of genes encoding tRNA ^{Arg}, ^{Leu}, ^{Sec} and 5S rRNA, and was irrespective of the degree of transformation. The presence of lower risk HPVs appeared to have little effect on pol III transcription. In addition, the physical status of the virus may also affect transcription by pol III, with integration correlating with an increase in the levels of pol III transcripts in comparison with cells where the virus remained episomal. In the breast and colon samples, where viral entities played no part in the development of

cancer, expression patterns of pol III-transcribed genes were more complex. However, examination of tRNA ^{Leu}, ^{Sec}, 5S rRNA and 7SL transcripts by RT-PCR revealed that transcription of these genes was elevated in 50% of the breast tumour biopsy samples examined, when compared to the matched normal tissue. It was found that genes transcribed by pol III were elevated in over a third of the colorectal tumours examined.

In addition to the tumour biopsy samples examined, it was observed that pol III transcription in a SV40-transformed cell line was higher than in the untransformed parental cell line. This suggests that expression in the untransformed cells is restrained. This is likely to be due, at least in part, to restraint by repressive chromatin structure, since treatment of transformed cells with the HDAC inhibitor TSA had less effect on transcription at a selection of different pol III templates than the observed effect of this drug on the untransformed cells. This suggests that histones associated with pol III-transcribed genes are less highly acetylated in untransformed cells. Differential acetylation of non-histone targets is also a possibility.

Transcription of MRP and 7SK genes was observed to behave very differently from the other pol III-transcribed genes examined. For example, in the breast biopsy samples it was found that transcriptional upregulation of these genes occurred more frequently in the tumour samples (five out of the six) when compared to the other pol III-transcribed genes that were examined. In the HPV16 infected cervical biopsy samples, 7SK and MRP RNA levels did not correlate with viral status, unlike tRNA and 5S rRNA. In the W12 system, the cells containing integrated HPV16 compared to cells that maintain HPV16 episomally did not display the large increase in

transcription of MRP and 7SK that was observed in the other pol III transcripts examined. The distinct response of 7SK and MRP might reflect their very different promoter arrangements from those of 5S rRNA and tRNA genes. Unlike tRNA and 5S rRNA genes, which have internal promoters, 7SK and MRP genes do not utilize TFIIIC2 or Brf1 (Schramm & Hernandez, 2002). Indeed, it was observed that raising the concentration of Brf1 in cervical epithelial cells had no effect on transcription of 7SK, in contrast to $tRNA^{Leu}$ and 5S rRNA.

In the untransformed and transformed fibroblasts it was also observed that genes with a type III promoter behaved differently than the other genes investigated. 7SK was not affected by the presence of the HDAC inhibitor TSA in transformed fibroblasts. However, upon addition of TSA to untransformed A31 fibroblasts, a decrease in transcription of 7SK was observed. This may be due to relaxation of the nucleosome structure, since it has been suggested that at 7SK genes a nucleosome brings the DSE and PSE into juxtaposition, which facilitates interaction between the factors binding to these elements (Boyd *et al.*, 2000). Indeed, the presence of binding sites for ZID, a transcription factor known to interact with a HDAC, have been identified upstream of the DSE (Boyd *et al.*, 2000), suggesting that deacetylation of histones may play a role in the regulation of the 7SK gene, perhaps by ensuring that a positioned nucleosome is stabilised.

Recruitment of pol III to particular DNA sequences requires the presence of transcription factors to position the enzyme over the promoter regions of specific genes. Investigation of these factors revealed a correlation between transcriptional output and the TFIIIB subunit Brf1. The increase in the levels of $tRNA^{Arg}$, ^{Leu}, ^{Sec} and

5S rRNA transcripts in HPV16 infected cervical biopsy samples were concomitant with an elevation of Brf1 mRNA. Furthermore, it was found that raising the concentration of Brf1 in cervical epithelial cells increased transcription of tRNA ^{Leu}, and 5S rRNA genes. In addition, upon integration of HPV16, the cervical cell line W12 displayed upregulated levels of Brf1, alongside an increase in pol III transcripts. In the breast biopsy samples, Brf1 was upregulated in all except for one of the breast tumours, and in the colorectal samples deregulation of Brf1 appeared to correlate with most of the changes in the levels of pol III transcripts.

Analysis of the expression of the other two subunits of TFIIIB in the colorectal samples did not reveal any straightforward correlations between the levels of Bdp1 and TBP mRNAs and the products transcribed by pol III. In the breast samples, Bdp1 remained relatively constant, while TBP was upregulated in half of the tumours. Previously, it has been reported that the HBV X oncoprotein stimulates pol III transcription by increasing TBP expression (Wang *et al.*, 1997), but this was not evident in the HPV infected cervical biopsies. Although the levels of TBP mRNA fluctuated dramatically in these samples, they did not correlate with any of the pol III transcripts examined. It has also been demonstrated that SV40 and polyomavirus transformed fibroblasts display increased levels of Bdp1 (Felton-Edkins & White, 2002), but in the cervical biopsy samples, like TBP, the levels of Bdp1 mRNA varied markedly, showing little correlation with expression of pol III templates. This suggests that TBP and Bdp1 may be in relative excess in the cervix. In one of the cervical tumour biopsy samples a dramatic overexpression of Bdp1 mRNA was found, which correlated with high levels of transcription at 7SK and MRP genes. This

raises the possibility that transcription of MRP and 7SK genes might be activated by very high levels of Bdp1, even if it is not normally limiting.

To further examine the effects of increased levels of TFIIIB, *in vitro* transcription assays were carried out with cervical and breast cell lines. It was found that raising the levels of TFIIIB increased transcription at tRNA ^{Leu} and VA1 templates. Furthermore, incubation of breast cells with a TFIIIB TAF-containing fraction and a TFIIIB fraction in which TBP was denatured, also stimulated pol III transcription. However, addition of recombinant TBP had no stimulatory effect. Therefore, it may be that in breast epithelium, TBP is in relative excess, and that TFIIIB TAFs are limiting. It would be interesting to investigate if this is also the case in colorectal cells. It is not unprecedented to suggest that subunits of the same transcription factor may be present in cells at different levels, since this has been demonstrated in virally transformed cells (Felton-Edkins & White, 2002; Wang *et al.*, 1997). By increasing the levels of TAFs, in particular Brf1, it may be that transcriptional repression by RB and p53 is overcome. These tumour suppressors exert their repressional effects on pol III transcription by disrupting key interactions between TFIIIB and the other components of the basal transcription apparatus (Crighton *et al.*, 2003; Sutcliffe *et al.*, 2000). It has been shown that binding of TFIIIB to TFIIIC2 can be blocked by RB. In addition, RB disrupts the interaction between TFIIIB and pol III (Sutcliffe *et al.*, 2000). In contrast, repression by p53 is mediated through direct contacts with the TBP subunit (Crighton *et al.*, 2003). p53 does not disrupt the direct interaction between TBP and Brf1, but prevents the association of Brf1 complexes with TFIIIC2 and pol III, thereby impeding the formation of functional transcription complexes (Crighton *et al.*, 2003). Therefore, by increasing the levels of Brf1 it may be that cancer cells are able to

overcome repression by promoting assembly of functional transcription complexes by mass action.

When the levels of TFIIIC2 were examined in the cervical biopsy samples, it was found that unlike SV40, polyomavirus and adenovirus (Felton-Edkins & White, 2002; Larminie *et al.*, 1999; Sinn *et al.*, 1995), the presence of HPV did not deregulate expression of the mRNAs encoding the TFIIIC2 subunits. The five subuits of this transcription factor have also been shown to be upregulated in ovarian cancer (Winter *et al.*, 2000). However, as observed in the cervical biopsy samples, it was found that in the colon and breast tumour biopsies deregulation of pol III was not always found to correlate with a corresponding change in the levels of TFIIIC2. But in whole cell breast extracts increasing the level of TFIIIC2 was found to elevate transcription of tRNA ^{Leu} and VA1. It is therefore possible that in some of the tumours TFIIIC2 may have been a limiting factor. Unfortunately, due to the limited amount of biopsy tissue available, protein could not be isolated to further analyse levels of these transcription factors. Therefore, in interpreting the RT-PCR results, the assumption is made that transcription factor levels in these tissue samples reflect the expression of their corresponding mRNAs. However, this might not be the case, as posttranscriptional controls may have a significant influence.

Investigations into the increased levels of pol III transcription observed in both untransformed and transformed fibroblasts that had been treated with TSA revealed increased promoter occupancy of pol III transcriptional machinery. This was observed by ChIP assays with the RPC155 subunit of pol III, Brf1 and TFIIIC110, and correlated with an increased presence of acetylated histone H4, and also elevated

levels of acetylated H4 protein. Since inhibition of HDACs is able to cause these changes, it may well be that the increases observed in the levels of pol III transcripts in some of the biopsy samples could be attributable to aberrant recruitment of HDACs. Indeed, such effects have been demonstrated in cell lines derived from patients with acute promyelocytic leukaemia, acute myelogenous leukaemia involving acute myelogenous leukaemia 1 and ETO genes, and non-Hodgkin lymphoma, with inappropriate expression of repressor BCL-6 (Marks *et al.*, 2000).

Investigation of pol I transcription revealed that it was deregulated with a higher frequency than pol III transcription. Indeed, in the breast biopsies upregulation of pol I was found in five out of the six tumours examined. In the cervical biopsy samples, deregulation of pol I transcription was seen to occur at the same frequency as pol III. Infection of cervical epithelium with HPV16 elevated the activity of pol I, but this was not the case in samples infected with HPV33 or HPV11. In the colorectal biopsies, pol I activity was found to be elevated in over half of the tumour samples examined. It is unlikely that the increased levels of transcripts involved in protein synthesis are simply due to the presence of a higher percentage of dividing cells in the tumour, since it has been shown that in colorectal carcinomas the proliferative index is not significantly different from that of normal colonic mucosa (Bleiberg & Galand, 1976; Hoffman & Post; 1967). Furthermore, the regulation of ribosomal protein levels does not generally involve increased mRNA stability (Pogue-Geile *et al.*, 1991). Instead, several investigations have demonstrated that growing cells more efficiently translate ribosomal protein mRNAs than do resting cells (Geyer *et al.*, 1982; Hammond & Bowman, 1988; Ignotz *et al.*, 1981).

Examination of the levels of the pol I transcription factors UBF and Rrn3 in breast and colorectal biopsy samples did not reveal any correlation with pol I deregulation. Therefore, pol I activation does not seem to be occurring as a result of upregulation of these iniatiation factors that it utilizes during transcriptional assembly. In fact, both factors were frequently seen to be down-regulated in the tumour samples in comparison with the matched normal tissue. In the case of UBF, this has previously been observed by Huang *et al.* (2002), who demonstrated that some lung, oesophageal, breast and ovarian tumour samples displayed lower levels of UBF than matched normal tissue. In addition to these pol I-specific transcription factors, TBP was also assessed to find out if expression patterns correlated with pol I activity. Previously it has been shown that promoter activation of TBP by the X oncoprotein of HBV stimulates pol I transcription (Wang *et al.*, 1998). However, in each of the three different types of biopsy samples analysed little correlation was observed between TBP mRNA and rRNA expression. During the development of cancer it is likely that that the great complexity and variety of transcription factors utilized by both pols I and III precludes a simplistic assignment of direct stimulatory capability to only one of them. Just as individual tumours show considerable heterogeneity, so there seem to be variable strategies that are used to increase transcriptional output.

The increase in pol I and III activity that was observed in some of the tumours examined is unsurprising since the products made by these polymerases are required for increased levels of the biosynthetic machinery. This is necessary to sustain the rapid growth rates which occur in tumour progression. What is surprising, however, are the decreases in the levels of transcripts that were observed in some tumours. The variability observed in expression of pol I and III-transcribed genes and their

transcription factors in different normal and tumour biopsy samples could be due to a number of factors. Changes in gene expression are complicated by the fact that tumours consist of many different cell types (Notterman *et al.*, 2001, Perou *et al.*, 1999). In addition to this, carcinoma cells themselves are morphologically and genetically diverse (Perou *et al.*, 1999). Furthermore, it may be that histopathologically normal appearing tissue surrounding a tumour is not normal at the molecular level. It is possible, therefore, that elevated transcription in these adjacent areas may be higher than in tissue distant from the tumour. Moreover, alterations in gene expression may be inhomogenous within a tumour (Backert *et al.*, 1999).

The biopsy samples were also investigated for changes in the levels of other regulators that are known to affect transcriptional activity. Analysis of the cellular oncogene c-*myc* in biopsy samples revealed that this gene was upregulated in five of the six breast tumour samples, and ten of the eleven colorectal tumours, in comparison with the surrounding normal tissue. This could be due to a number of alterations to c-*myc*, including gene amplification, chromosomal translocation, insertional mutations, altered transcriptional elongation rates, and prolonged mRNA half-life (Dang, 1999). In the breast samples, the upregulation of c-Myc was seen to closely correlate with the increased levels of pol I transcripts, as well as MRP and 7SK transcripts. Since increased levels of c-Myc are thought to be involved in the induction and progression of cancer (Leder *et al.*, 1986; Roux-Dosseto *et al.*, 1992; Sierra *et al.*, 1999; Watson *et al.*, 1993, 1996), it may be that c-Myc ensures that the components required for increased growth are available by increasing transcription by pols I and III. Indeed, in c-*myc* null cells there is a marked decrease in net RNA synthesis (Mateyak *et al.*,

1997). It has also recently been demonstrated that TFIIIB is a target for c-Myc, enabling it to directly activate pol III transcription (Gomez-Roman *et al.*, 2003).

In the colorectal samples, the high percentage of tumours in which c-Myc was upregulated is in agreement with results from other groups (Nesbit *et al.*, 1999; Rothberg, 1987; Wang *et al.*, 2002). This probably occurs as a consequence of mutations in the tumour suppressor gene APC that lead to aberrant nuclear accumulation of the β–catenin/Tcf transcription complex, which, in turn, leads to increased expression of target genes, such as *c-myc* (He *et al.*, 1998). Cyclin D1 is another gene targeted by the β–catenin/Tcf transcription complex (Shtutman *et al.*, 1999; Tetsu & McCormick, 1999; Wang *et al.*, 2002). Cyclin D1 overexpression has been observed in a subset of neoplastic conditions, including human colorectal cancers (Arber *et al.*, 1996, 1997; Sutter *et al.*, 1997). The constitutive activation of Ras further stimulates transcription of the cyclin D1 gene (Albanese *et al.*, 1995; Tetsu & McCormick, 1999). In keeping with these findings, many of the colorectal tumour samples examined displayed upregulated levels of cyclin D1 mRNA (seven out of the eleven tested). This occurred frequently in the later stage tumours, presumably after activation of K-*ras*. Cyclin D2, which is controlled by different signalling pathways, was not seen to be upregulated as frequently. Although cyclin D1 is upregulated in many of the colorectal tumours, it is only when cyclin D2 is also elevated that increased pol III activity is observed. Overexpression of cyclins D1 and D2 would be expected to have a stimulating effect on transcription, as it would enable the phosphorylation of RB. Thus, in a hyperphosphorylated state RB would no longer be able to bind to UBF and TFIIIB, and therefore the transcriptional repression mediated by this tumour suppressor protein would be alleviated. Cyclin D2 is a well-

characterized direct target for c-Myc. Elevated expression of cyclin D2 mRNA may therefore be taken as an indicator that c-Myc activity is raised. It is very striking that there is a perfect correlation between the induction of cyclin D2 mRNA and the induction of pol III transcripts. This suggests that Myc activity may be rate-limiting for pol III transcription in colorectal cells.

Numerous studies have found that in the majority of tumours the functions of p53 and/or RB are compromised. In the cervical biopsies examined in this study, inactivation of these tumour suppressor proteins would have occurred in some of the samples. Alterations in the levels of pol I and pol III transcription in HPV16 infected cervical samples may be explained by the fact that this high risk virus expresses the oncoproteins E6 and E7, which can inactivate tumour suppressors (Larminie et al., 1999; Stein *et al.*, 2002a; Sutcliffe *et al.*, 1999). E6 is able to inactivate p53, which causes the pol III transcription factor TFIIIB and the pol I transcription factor SL1 to be released from repression. E7 inactivates RB, which also causes the release of TFIIIB and the pol I transcription factor, UBF. Both these oncoproteins from high risk HPV16 bind to these tumour suppressors with a higher affinity than the proteins from lower risk HPVs, perhaps explaining why HPV33 and HPV11 do not elevate pol I or III transcription. Had protein samples been available for analysis from the biopsy samples it would have been interesting to investigate the levels of these tumour suppressors and their interaction with UBF, SL1 and TFIIIB, not only in the cervical tissue but also in the breast and colorectal biopsies, to examine if the samples displaying increased levels of pol I and III transcripts displayed decreased binding of tumour suppressor proteins to pol I and III factors.

The deregulation of pols I and III was seen to occur in a number of tumours in the three different types of carcinomas. In the colorectal samples the clinical stage of disease could not be directly correlated to a specific pattern of gene expression, perhaps due to a limited number of tissue samples. However, it was observed that elevated transcription by pols I and III appeared to occur frequently in the later stages of tumour development. Despite this, the furthest progressed colorectal tumour sample did not display increased rates of transcription by either pol I or pol III. At this late stage, the lack of upregulation may be due to decreased growth rates in some regions of the tumour that are poorly vascularised and have become hypoxic. Indeed, it is well known that different areas of tumours express different genes according to the available oxygen supply. Therefore, tissue removed from the centre of a carcinogenic lesion may not reflect the active transcription at the perimeter of a tumour. Transcription by pols I and III was also seen to be upregulated in one of the adenoma samples. Since adenomas are considered to be the pathological precursors to colorectal carcinogenesis (Kinzler & Vogelstein, 1996), it may be that upregulation of pol I and III transcription occurs as an early event in tumour development. In agreement with this, several ribosomal proteins (S6, S8, S12, L5 and ARPP P0) have been shown to be overexpressed in adenomas (Pogue-Geile *et al.*, 1991). Similarly, in the cervical samples infected with HPV16 it was observed that activation of pols I and III occurred as an early response preceding transformation, since transcriptional upregulation was observed in premalignant cervical biopsy samples, relative to uninfected normal tissue. Unfortunately, data regarding the staging of the breast samples was not available for analysis.

The data gathered during the course of this study was limited by numerous factors that would need to be addressed before commencing further studies. These include the unavailability of large numbers of biopsy samples, which would have allowed protein studies to be carried out; and the lack of clinical data regarding the disease state of the tumour, such as the staging. In addition, it would have been of interest to obtain follow-up information on the patients from which the biopsy samples were taken, so that any trends with regard to receptiveness to treatment and certain expression patterns of the genes investigated could have been examined.

From this study, it is apparent that the genetic changes which arise in tumours are dependent not only on the specific tissue type, but also on the individual tumour. Analysis of the biopsy samples provides clear evidence that deregulation of pols I and III is a frequent event during carcinogenesis. This appears to be a very complicated process, involving the co-operative interaction of many transcription factors. Understanding these changes that occur in transcription, and the mechanisms underlying this deregulation, may well provide opportunities for the design and construction of more effective therapeutics against cancer.

References

Ahlers, S. E. & Feldman, L. T. (1987). Effects of a temperature-sensitive mutation in the immediate-early gene of pseudorabies virus on class II and class III gene transcription. Journal of Virology, 61(4): 1103-1107.

Alazawi, W., Pett, M., Arch, B., Scott, L., Freeman, T., Stanley, M. A., & Coleman, N. (2002). Changes in cervical keratinocyte gene expression associated with integration of human papillomavirus 16. Cancer Research, 62: 6959-6965.

Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A., & Pestell, R. G. (1995). Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. Journal of Biological Chemistry, 270(40): 23589-23597.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., & Watson, J. D. (1994). Molecular Biology of the Cell. (3rd ed.). New York: Garland Publishing.

Allred, D. C., Clark, G. M., Elledge, R., Fuqua, S. A., Brown, R. W., Chamness, G. C., Osborne, C. K., & McGuire, W. L. (1993). Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. Journal of the National Cancer Institute, 85(3): 200-206.

Almouzni, G., Mechali, M., & Wolffe, A. P. (1991). Transcription complex disruption caused by a transition in chromatin structure. Molecular & Cellular Biology, 11(2): 655-665.

Alon, U., Barkai, N., Notterman, D. A., Gish, K., Ybarra, S., Mack, D., & Levine, A. J. (1999). Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues probed by oligonucleotide arrays. Proceedings of the National Academy of Sciences of the United States of America, 96(12): 6745-6750.

Andersen, T. I., Holm, R., Nesland, J. M., Heimdal, K. R., Ottestad, L., & Borresen, A. L. (1993). Prognostic significance of TP53 alterations in breast carcinoma. British Journal of Cancer, 68(3): 540-548.

Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., & Meltzer, P. S. (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science, 277(5328): 965-968.

Arber, N., Hibshoosh, H., Moss, S. F., Sutter, T., Zhang, Y., Begg, M., Wang, S., Weinstein, I. B., & Holt, P. R. (1996). Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis. Gastroenterology, 110(3): 669-674.

Arber, N., Doki, Y., Han, E. K., Sgambato, A., Zhou, P., Kim, N. H., Delohery, T., Klein, M. G., Holt, P. R., & Weinstein, I. B. (1997). Antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells. Cancer Research, 57(8): 1569-1574.

Astler, V. B. & Coller, F. A. (1954). The prognostic significance of direct extension of carcinoma of the colon and rectum. Annals of Surgery, 139: 846-851.

Aufiero, B. & Schneider, R. J. (1990). The hepatitis B virus X-gene product trans-activates both RNA polymerase II and III promoters. EMBO Journal, 9(2): 497-504.

Backert, S., Gelos, M., Kobalz, U., Hanski, M. L., Bohm, C., Mann, B., Lovin, N., Gratchev, A., Mansmann, U., Moyer, M. P., Riecken, E. O., & Hanski, C. (1999). Differential gene expression in colon carcinoma cells and tissues detected with a cDNA array. International Journal of Cancer, 82(6): 868-874.

Baker, R. E., Camier, S., Sentenac, A., & Hall, B. D. (1987). Gene size differentially affects the binding of yeast transcription factor tau to two intragenic regions. Proceedings of the National Academy of Sciences of the United States of America, 84(24): 8768-8772.

Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K., & Vogelstein, B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. Science, 249(4971): 912-915.

Baldwin, P., Laskey, R., & Coleman, N. (2003). Translational approaches to improving cervical screening. Nature Reviews Cancer, 3: 1-10.

Bannister, A. J. & Miska, E. A. (2000). Regulation of gene expression by transcription factor acetylation. Cell Mol Life Sci, 57: 1184-1192.

Barbacid, M. (1987). ras genes. Annual Review of Biochemistry, 56: 779-827.

Bartkova, J., Lukas, J., Muller, H., Lutzhoft, D., Strauss, M., & Bartek, J. (1994). Cyclin D1 protein expression and function in human breast cancer. International Journal of Cancer, 57(3): 353-361.

Bates, S. & Peters, G. (1995). Cyclin D1 as a cellular proto-oncogene. Seminars in Cancer Biology, 6(2): 73-82.

Baxter, G. C. & Stanners, C. P. (1978). The effect of protein degradation on cellular growth characteristics. Journal of Cellular Physiology, 96(2): 139-145.

Bazett-Jones, D. P., Leblanc, B., Herfort, M., & Moss, T. (1994). Short-range DNA looping by the Xenopus HMG-box transcription factor, xUBF. Science, 264(5162): 1134-1137.

Belenguer, P., Baldin, V., Mathieu, C., Prats, H., Bensaid, M., Bouche, G., & Amalric, F. (1989). Protein kinase NII and the regulation of rDNA transcription in mammalian cells. Nucleic Acids Research, 17(16): 6625-6636.

Bell, S. P., Learned, R. M., Jantzen, H. M., & Tjian, R. (1988). Functional cooperativity between transcription factors UBF1 and SL1 mediates human ribosomal RNA synthesis. Science, 241(4870): 1192-1197.

Bennett, K. L., Hill, R. E., Pietras, D. F., Woodworth-Gutai, M., Kane-Haas, C., Houston, J. M., Heath, J. K., & Hastie, N. D. (1984). Most highly repeated dispersed DNA families in the mouse genome. Molecular & Cellular Biology, 4(8): 1561-1571.

Beral, V. & Million Women Study, C. (2003). Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet, 362(9382): 419-427.

Bernard, H. A., Chan, S. K., Manos, M. M., Ong, C. K., Villa, L. L., Delius, H., Peyton, C. L., Bauer, H. M., & Wheeler, C. M. (1994). Identification and assesment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction length polymorphisms, nucleotide sequence, and phylogenetic algorithms. The Journal of Infectious Diseases, 170: 1077-1085.

Berns, E. M., Klijn, J. G., van Putten, W. L., van Staveren, I. L., Portengen, H., & Foekens, J. A. (1992). c-myc amplification is a better prognostic factor than HER2/neu amplification in primary breast cancer. Cancer Research, 52(5): 1107-1113.

Berns, E. M., Klijn, J. G., Smid, M., van Staveren, I. L., Look, M. P., van Putten, W. L., & Foekens, J. A. (1996). TP53 and MYC gene alterations independently predict poor prognosis in breast cancer patients. Genes, Chromosomes & Cancer, 16(3): 170-179.

Bieker, J. J., Martin, P. L., & Roeder, R. G. (1985). Formation of a rate-limiting intermediate in 5S RNA gene transcription. Cell, 40(1): 119-127.

Blackwood, M. A. & Weber, B. L. (1998). BRCA1 and BRCA2: from molecular genetics to clinical medicine. Journal of Clinical Oncology, 16(5): 1969-1977.

Bland, K. I., Konstadoulakis, M. M., Vezeridis, M. P., & Wanebo, H. J. (1995). Oncogene protein co-expression. Value of Ha-ras, c-myc, c-fos, and p53 as prognostic discriminants for breast carcinoma. Annals of Surgery, 221(6): 706-718; discussion 718-720.

Bleiberg, H. & Galand, P. (1976). In vitro autoradiographic determination of cell kinetic parameters in adenocarcinomas and adjacent healthy mucosa of the human colon and rectum. Cancer Research, 36(2 Pt 1): 325-328.

Borg, A., Baldetorp, B., Ferno, M., Olsson, H., & Sigurdsson, H. (1992). c-myc amplification is an independent prognostic factor in postmenopausal breast cancer. International Journal of Cancer, 51(5): 687-691.

Borrow, J., Stanton, V. P., Jr., Andresen, J. M., Becher, R., Behm, F. G., Chaganti, R. S., Civin, C. I., Disteche, C., Dube, I., Frischauf, A. M., Horsman, D., Mitelman, F., Volinia, S., Watmore, A. E., & Housman, D. E. (1996). The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. Nature Genetics, 14(1): 33-41.

Bos, J. L., Fearon, E. R., Hamilton, S. R., Verlaan-de Vries, M., van Boom, J. H., van der Eb, A. J., & Vogelstein, B. (1987). Prevalence of ras gene mutations in human colorectal cancers. Nature, 327(6120): 293-297.

Bosc, D. G., Luscher, B., & Litchfield, D. W. (1999). Expression and regulation of protein kinase CK2 during the cell cycle. Molecular & Cellular Biochemistry, 191(1-2): 213-222.

Bosch, F. X., Manos, M. M., Munoz, N., Sherman, M., Jansen, A. M., Peto, J., Schiffman, M. H., Moreno, V., Kurman, R., & Shah, K. V. (1995). Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. Journal of the National Cancer Institute, 87(11): 796-802.

Boshart, M., Gissmann, L., Ikenberg, H., Kleinheinz, A., Scheurlen, W., & zur Hausen, H. (1984). A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO Journal, 3(5): 1151-1157.

Boyd, D. C., Greger, I. H., & Murphy, S. (2000). In vivo footprinting studies suggest a role for chromatin in transcription of the human 7SK gene. Gene, 247(1-2): 33-44.

Boyer, S. N., Wazer, D. E., & Band, V. (1996). E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Research, 56(20): 4620-4624.

Boyes, J., Byfield, P., Nakatani, Y., & Ogryzko, V. (1998). Regulation of activity of the transcription factor GATA-1 by acetylation. Nature, 396: 594-598.

Bredow, S., Kleinert, H., & Benecke, B. J. (1990). Sequence and factor requirements for faithful in vitro transcription of 7SL DNA. Gene, 86: 217-225.

Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., & Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature, 391(597-601).

Brooks, R. F. (1977). Continuous protein synthesis is required to maintain the probability of entry into S phase. Cell, 12(1): 311-317.

Brown, D. D. & Schlissel, M. S. (1985). A positive transcription factor controls the differential expression of two 5S RNA genes. Cell, 42(3): 759-767.

Brownell, J. E. & Allis, C. D. (1996). Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. Current Opinion in Genetics & Development, 6(2): 176-184.

Bruggers, C. S., Tai, K. F., Murdock, T., Sivak, L., Le, K., Perkins, S. L., Coffin, C. M., & Carroll, W. L. (1998). Expression of the c-Myc protein in childhood medulloblastoma. Journal of Pediatric Hematology/Oncology, 20(1): 18-25.

Buckley, M. F., Sweeney, K. J., Hamilton, J. A., Sini, R. L., Manning, D. L., Nicholson, R. I., deFazio, A., Watts, C. K., Musgrove, E. A., & Sutherland, R. L. (1993). Expression and amplification of cyclin genes in human breast cancer. Oncogene, 8(8): 2127-2133.

Budde, A. & Grummt, I. (1999). p53 represses ribosomal gene transcription. Oncogene, 18(4): 1119-1124.

Bullock, A. N., Henckel, J., & Fersht, A. R. (2000). Quantitative analysis of residual folding and DNA binding in mutant p53 core domain: definition of mutant states for rescue in cancer therapy. Oncogene, 19(10): 1245-1256.

Burlingame, R. W., Love, W. E., Wang, B. C., Hamlin, R., Nguyen, H. X., & Moudrianakis, E. N. (1985). Crystallographic structure of the octameric histone core of the nucleosome at a resolution of 3.3 A. Science, 228(4699): 546-553.

Bussey, H. J., Veale, A. M., & Morson, B. C. (1978). Genetics of gastrointestinal polyposis. Gastroenterology, 74(6): 1325-1330.

Čabart, P. & Murphy, S. (2001). BRFU, a TFIIB-like factor, is directly recruited to the TATA-box of polymerase III small nuclear RNA gene promoters through its interaction with TATA-binding protein. Journal of Biological Chemistry, 276(46): 43056-43064.

Čabart, P. & Murphy, S. (2002). Assembly of human small nuclear RNA gene-specific transcription factor IIIB complex de novo on and off promoter. Journal of Biological Chemistry, 277(30): 26831-26838.

Cairns, C. A. & White, R. J. (1998). p53 is a general repressor of RNA polymerase III transcription. EMBO Journal, 17(11): 3112-3123.

Cancer Research UK (April 2003a). Incidence - UK. www.cancerresearchuk.org

Cancer Research UK (February 2003b). Mortality - UK. www.cancerresearchuk.org

Cancer Research UK (January 2003c). Cervical Cancer - UK. www.cancerresearchuk.org

Carey, M. F., Gerrard, S. P., & Cozzarelli, N. R. (1986a). Analysis of RNA polymerase III transcription complexes by gel filtration. Journal of Biological Chemistry, 261(9): 4309-4317.

Carey, M. F., Singh, K., Botchan, M., & Cozzarelli, N. R. (1986b). Induction of specific transcription by RNA polymerase III in transformed cells. Molecular & Cellular Biology, 6(9): 3068-3076.

Carey, M. F. & Singh, K. (1988). Enhanced B2 transcription in simian virus 40-transformed cells is mediated through the formation of RNA polymerase III transcription complexes on previously inactive genes. Proceedings of the National Academy of Sciences of the United States of America, 85(19): 7059-7063.

Cavanaugh, A. H., Hempel, W. M., Taylor, L. J., Rogalsky, V., Todorov, G., & Rothblum, L. I. (1995). Activity of RNA polymerase I transcription factor UBF blocked by Rb gene product. Nature, 374(6518): 177-180.

Chambon, P. (1975). Eukaryotic nuclear RNA polymerases. Annual Review of Biochemistry, 44: 613-638.

Chao, Y. & Pellegrini, M. (1993). In vitro transcription of Drosophila rRNA genes shows stimulation by a phorbol ester and serum. Molecular & Cellular Biology, 13(2): 934-941.

Chen, W., Bocker, W., Brosius, J., & Tiedge, H. (1997a). Expression of neural BC200 RNA in human tumours. Journal of Pathology, 183(3): 345-351.

Chen, W. Y., Bailey, E. C., McCune, S. L., Dong, J. Y., & Townes, T. M. (1997b). Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase. Proceedings of the National Academy of Sciences of the United States of America, 94(11): 5798-5803.

Chen, Z., Clark, S., Birkeland, M., Sung, C. M., Lago, A., Liu, R., Kirkpatrick, R., Johanson, K., Winkler, J. D., & Hu, E. (2002). Induction and superinduction of growth arrest and DNA damage gene 45 (GADD45) alpha and beta messenger RNAs by histone deacetylase inhibitors trichostatin A (TSA) and butyrate in SW620 human colon carcinoma cells. Cancer Letters, 188: 127-140.

Chesnokov, I., Chu, W. M., Botchan, M. R., & Schmid, C. W. (1996). p53 inhibits RNA polymerase III-directed transcription in a promoter-dependent manner. Molecular & Cellular Biology, 16(12): 7084-7088.

Chireux, M., Espinos, E., Bloch, S., Yoshida, M., & Weber, M. J. (1996). Histone hyperacetylating agents stimulate promoter activity of human choline acetyltransferase gene in transfection experiment. Brain Research Molecular Brain Research, 39(1-2): 68-78.

Cho, K. R. (2002). Cervical Cancer. In B. Vogelstein & K. W. Kinzler (Eds.), The Genetic Basis of Human Cancer, 2nd ed.: Chapter 43. USA: The McGraw-Hill Companies Inc.

Chrzan, P., Skokowski, J., Karmolinski, A., & Pawelczyk, T. (2001). Amplification of c-myc gene and overexpression of c-Myc protein in breast cancer and adjacent non-neoplastic tissue. Clinical Biochemistry, 34(7): 557-562.

Chu, W. M., Wang, Z., Roeder, R. G., & Schmid, C. W. (1997). RNA polymerase III transcription repressed by Rb through its interactions with TFIIIB and TFIIIC2. Journal of Biological Chemistry, 272(23): 14755-14761.

Ciarmatori, S., Scott, P. H., Sutcliffe, J. E., McLees, A., Alzuherri, H. M., Dannenberg, J. H., te Riele, H., Grummt, I., Voit, R., & White, R. J. (2001).

Overlapping functions of the pRb family in the regulation of rRNA synthesis. Molecular & Cellular Biology, 21(17): 5806-5814.

Clark, D. J. & Wolffe, A. P. (1991). Superhelical stress and nucleosome-mediated repression of 5S RNA gene transcription in vitro. EMBO Journal, 10(11): 3419-3428.

Clayton, D. A. (1994). A nuclear function for RNase MRP. Proceedings of the National Academy of Sciences of the United States of America, 91(11): 4615-4617.

Clemens, K. R., Liao, X., Wolf, V., Wright, P. E., & Gottesfeld, J. M. (1992). Definition of the binding sites of individual zinc fingers in the transcription factor IIIA-5S RNA gene compex. Proceedings of the National Academy of Sciences of the United States of America, 89: 10822-10826.

Comai, L., Tanese, N., & Tjian, R. (1992). The TATA-binding protein and associated factors are integral components of the RNA polymerase I transcription factor, SL1. Cell, 68(5): 965-976.

Conway, K., Edmiston, S., Fried, D. B., Hulka, B. S., Garrett, P. A., & Liu, E. T. (1995). Ha-ras rare alleles in breast cancer susceptibility. Breast Cancer Research & Treatment, 35(1): 97-104.

Couch, F. J., Farid, L. M., DeShano, M. L., Tavtigian, S. V., Calzone, K., Campeau, L., Peng, Y., Bogden, B., Chen, Q., Neuhausen, S., Shattuck-Eidens, D., Godwin, A. K., Daly, M., Radford, D. M., Sedlacek, S., Rommens, J., Simard, J., Garber, J., Merajver, S., & Weber, B. L. (1996). BRCA2 germline mutations in male breast cancer cases and breast cancer families. Nature Genetics, 13(1): 123-125.

Couch, F. J. & Weber, B. L. (1996). Mutations and polymorphisms in the familial early-onset breast cancer (BRCA1) gene. Breast Cancer Information Core. Human Mutation, 8(1): 8-18.

Couch, F. J., DeShano, M. L., Blackwood, M. A., Calzone, K., Stopfer, J., Campeau, L., Ganguly, A., Rebbeck, T., & Weber, B. L. (1997). BRCA1 mutations in women attending clinics that evaluate the risk of breast cancer. New England Journal of Medicine, 336(20): 1409-1415.

Couch, F. J. & Weber, B. L. (2002). Breast Cancer. In B. Vogelstein & K. W. Kinzler (Eds.), The Genetic Basis of Human Cancer, 2nd ed.: Chapter 33. USA: The McGraw-Hill Companies Inc.

Cox, L. S. & Lane, D. P. (1995). Tumour suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage. Bioessays, 17(6): 501-508.

Crane-Robinson, C. (1997). Where is the globular domain of linker histone located on the nucleosome? Trends in Biochemical Sciences, 22(3): 75-77.

Crighton, D., Woiwode, A., Zhang, C., Mandavia, N., Morton, J. P., Warnock, L. J., Milner, J., White, R. J., & Johnson, D. L. (2003). p53 represses RNA polymerase III

transcription by targeting TBP and inhibiting promoter occupancy by TFIIIB. EMBO Journal, 22(11): 2810-2820.

Cripe, T. P., Haugen, T. H., Turk, J. P., Tabatabai, F., Schmid, P. G., 3rd, Durst, M., Gissmann, L., Roman, A., & Turek, L. P. (1987). Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and repressor gene products: implications for cervical carcinogenesis. EMBO Journal, 6(12): 3745-3753.

Crook, T., Wrede, D., & Vousden, K. H. (1991). p53 point mutation in HPV negative human cervical carcinoma cell lines. Oncogene, 6(5): 873-875.

Crook, T. & Vousden, K. H. (1992). Properties of p53 mutations detected in primary and secondary cervical cancers suggest mechanisms of metastasis and involvement of environmental carcinogens. EMBO Journal, 11(11): 3935-3940.

Cullen, A. P., Reid, R., Campion, M., & Lorincz, A. T. (1991). Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. Journal of Virology, 65(2): 606-612.

Dang, C. V. (1999). C-Myc target genes involved in cell growth, apoptosis, and metabolism. Molecular & Cellular Biology, 19(1): 1-11.

Daniels, G. R. & Deininger, P. L. (1985). Repeat sequence families derived from mammalian tRNA genes. Nature, 317(6040): 819-822.

Das, G., Henning, D., Wright, D., & Reddy, R. (1988). Upstream regulatory elements are necessary and sufficient for transcription of a U6 RNA gene by RNA polymerase III. EMBO Journal, 7(2): 503-512.

Daum, G., Eisenmann-Tappe, I., Fries, H. W., Troppmair, J., & Rapp, U. R. (1994). The ins and outs of Raf kinases. Trends in Biochemical Sciences, 19(11): 474-480.

Davies, R., Hicks, R., Crook, T., Morris, J., & Vousden, K. (1993). Human papillomavirus type 16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. Journal of Virology, 67(5): 2521-2528.

de Villiers, E. M. (1989). Heterogeneity of the human papillomavirus group. Journal of Virology, 63(11): 4898-4903.

Dean, N. & Berk, A. J. (1988). Ordering promoter binding of class III transcription factors TFIIIC1 and TFIIIC2. Molecular & Cellular Biology, 8(8): 3017-3025.

Deininger, P. L., Jolly, D. J., Rubin, C. M., Friedmann, T., & Schmid, C. W. (1981). Base sequence studies of 300 nucleotide renatured repeated human DNA clones. Journal of Molecular Biology, 151(1): 17-33.

del Mar Peña, L. M. & Laimins, L. A. (2001). Differentiation-dependent chromatin rearrangement coincides with activation of human papillomavirus type 31 late gene expression. Journal of Virology, 75(20): 10005-10013.

Deng, G., Chen, L. C., Schott, D. R., Thor, A., Bhargava, V., Ljung, B. M., Chew, K., & Smith, H. S. (1994). Loss of heterozygosity and p53 gene mutations in breast cancer. Cancer Research, 54(2): 499-505.

DePinho, R. A., Schreiber-Agus, N., & Alt, F. W. (1991). myc family oncogenes in the development of normal and neoplastic cells. Advances in Cancer Research, 57: 1-46.

Domagala, W., Harezga, B., Szadowska, A., Markiewski, M., Weber, K., & Osborn, M. (1993). Nuclear p53 protein accumulates preferentially in medullary and high-grade ductal but rarely in lobular breast carcinomas. American Journal of Pathology, 142(3): 669-674.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., & Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature, 356(6366): 215-221.

Dürst, M., Kleinheinz, A., Hotz, M., & Gissman, L. (1985). The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumours. Journal of General Virology, 66(Pt 7): 1515-1522.

Dyson, N., Howley, P. M., Munger, K., & Harlow, E. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science, 243(4893): 934-937.

Dyson, N., Guida, P., Munger, K., & Harlow, E. (1992). Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. Journal of Virology, 66(12): 6893-6902.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. Genes & Development, 12(15): 2245-2262.

el-Deiry, W. S., Nelkin, B. D., Celano, P., Yen, R. W., Falco, J. P., Hamilton, S. R., & Baylin, S. B. (1991). High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. Proceedings of the National Academy of Sciences of the United States of America, 88(8): 3470-3474.

el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., & Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell, 75(4): 817-825.

Elgin, S. C. (1988). The formation and function of DNase I hypersensitive sites in the process of gene activation. Journal of Biological Chemistry, 263(36): 19259-19262.

Elledge, R. M., Fuqua, S. A., Clark, G. M., Pujol, P., & Allred, D. C. (1993). William L. McGuire Memorial Symposium. The role and prognostic significance of p53 gene alterations in breast cancer. Breast Cancer Research & Treatment, 27(1-2): 95-102.

Eppert, K., Scherer, S. W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L. C., Bapat, B., Gallinger, S., Andrulis, I. L., Thomsen, G. H., Wrana, J. L., & Attisano, L. (1996). MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. Cell, 86(4): 543-552.

Epstein, P., Reddy, R., Henning, D., & Busch, H. (1980). The nucleotide sequence of nuclear U6 (4.7 S) RNA. The Journal of Biological Chemistry, 255(18): 8901-8906.

Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z., & Hancock, D. C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell, 69(1): 119-128.

Fabrizio, P., Coppo, A., Fruscoloni, P., Benedetti, P., Di Segni, G., & Tocchini-Valentini, G. P. (1987). Comparative mutational analysis of wild-type and stretched tRNA3(Leu) gene promoters. Proceedings of the National Academy of Sciences of the United States of America, 84(24): 8763-8767.

Fearon, E. R., Cho, K. R., Nigro, J. M., Kern, S. E., Simons, J. W., Ruppert, J. M., Hamilton, S. R., Preisinger, A. C., Thomas, G., Kinzler, K. W., & et al. (1990). Identification of a chromosome 18q gene that is altered in colorectal cancers. Science, 247(4938): 49-56.

Fearon, E. R. & Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell, 61(5): 759-767.

Fearon, E. R. (1992). Genetic alterations underlying colorectal tumorigenesis. Cancer Surveys, 12: 119-136.

Fearon, E. R. (1997). Human cancer syndromes: clues to the origin and nature of cancer. Science, 278(5340): 1043-1050.

Feinberg, A. P., Gehrke, C. W., Kuo, K. C., & Ehrlich, M. (1988). Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Research, 48(5): 1159-1161.

Felton-Edkins, Z. A. & White, R. J. (2002). Multiple mechanisms contribute to the activation of RNA polymerase III transcription in cells transformed by papovaviruses. The Journal of Biological Chemistry, 277(50): 48182-48191.

Felton-Edkins, Z. A., Fairley, J. A., Graham, E. L., Johnston, I. M., White, R. J., & Scott, P. H. (2003). The mitogen-activated protein (MAP) kinase ERK induces tRNA synthesis by phosphorylating TFIIIB. EMBO Journal, 22(10): 2422-2432.

Felton-Edkins, Z. A., Kenneth, N. S., Brown, T. R., Daly, N. L., Gomez-Roman, N., Grandori, C., Eisenman, R. N., & White, R. J. (2003). Direct Regulation of RNA Polymerase III Transcription by RB, p53 and c-Myc. Cell Cycle, 2(3): 181-184.

Filmus, J., Robles, A. I., Shi, W., Wong, M. J., Colombo, L. L., & Conti, C. J. (1994). Induction of cyclin D1 overexpression by activated ras. Oncogene, 9(12): 3627-3633.

Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R., & Pavletich, N. P. (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature, 401(6749): 188-193.

Ford, E., Strubin, M., & Hernandez, N. (1998). The Oct-1 POU domain activates snRNA gene transcription by contacting a region in the SNAPc largest subunit that bears sequence similarities to the Oct-1 coactivator OBF-1. Genes & Development, 12(22): 3528-3540.

Forrester, K., Almoguera, C., Han, K., Grizzle, W. E., & Perucho, M. (1987). Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. Nature, 327(6120): 298-303.

Foster, M. P., Wuttke, D. S., Radhakrishnan, I., Case, D. A., Gottesfeld, J. M., & Wright, P. E. (1997). Domain packing and dynamics in the DNA complex of the N-terminal zinc fingers of TFIIIA. Nature Structural Biology, 4(8): 605-608.

Fradkin, L. G., Yoshinaga, S. K., Berk, A. J., & Dasgupta, A. (1987). Inhibition of host cell RNA polymerase III-mediated transcription by poliovirus: inactivation of specific transcription factors. Molecular & Cellular Biology, 7(11): 3880-3887.

Frank, T. S., Manley, S. A., Olopade, O. I., Cummings, S., Garber, J. E., Bernhardt, B., Antman, K., Russo, D., Wood, M. E., Mullineau, L., Isaacs, C., Peshkin, B., Buys, S., Venne, V., Rowley, P. T., Loader, S., Offit, K., Robson, M., Hampel, H., Brener, D., Winer, E. P., Clark, S., Weber, B., Strong, L. C., & Thomas, A. (1998). Sequence analysis of BRCA1 and BRCA2: correlation of mutations with family history and ovarian cancer risk. Journal of Clinical Oncology, 16(7): 2417-2425.

Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., & Dryja, T. P. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature, 323(6089): 643-646.

Fujita, M., Inoue, M., Tanizawa, O., Iwamoto, S., & Enomoto, T. (1992). Alterations of the p53 gene in human primary cervical carcinoma with and without human papillomavirus infection. Cancer Research, 52(19): 5323-5328.

Fung, Y. K. & T'Ang, A. (1992). The role of the retinoblastoma gene in breast cancer development. Cancer Treatment & Research, 61: 59-68.

Futamura, M., Monden, Y., Okabe, T., Fujita-Yoshigaki, J., Yokoyama, S., & Nishimura, S. (1995). Trichostatin A inhibits both ras-induced neurite outgrowth of PC12 cells and morphological transformation of NIH3T3 cells. Oncogene, 10(6): 1119-1123.

Galli, G., Hofstetter, H., & Birnstiel, M. L. (1981). Two conserved sequence blocks within eukaryotic tRNA genes are major promoter elements. Nature, 294(5842): 626-631.

Garber, M., Panchanathan, S., Fan, R. S., & Johnson, D. L. (1991). The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, induces specific transcription by RNA

polymerase III in Drosophila Schneider cells. Journal of Biological Chemistry, 266(31): 20598-20601.

Garber, M. E., Vilalta, A., & Johnson, D. L. (1994). Induction of Drosophila RNA polymerase III gene expression by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is mediated by transcription factor IIIB. Molecular & Cellular Biology, 14(1): 339-347.

Garel, J. (1976). Quantitative adaption of isoacceptor tRNAs to mRNA codons of alanine, glycine and serine. Nature, 260: 805-806.

Gaynor, R. B., Feldman, L. T., & Berk, A. J. (1985). Transcription of class III genes activated by viral immediate early proteins. Science, 230(4724): 447-450.

Geiduschek, E. P. & Kassavetis, G. A. (2001). The RNA polymerase III transcription apparatus. Journal of Molecular Biology, 310(1): 1-26.

Geyer, P. K., Meyuhas, O., Perry, R. P., & Johnson, L. F. (1982). Regulationof ribosomal protein mRNA content and translation in growth-stimulated mouse fibroblasts. Molecular & Cellular Biology, 2: 685-693.

Ghavidel, A. & Schultz, M. C. (1997). Caesin kinase II regulation of yeast TFIIIB is mediated by the TATA-binding protein. Genes & Development, 11: 2780-2789.

Ghavidel, A. & Schultz, M. C. (2001). TATA binding protein-associated CK2 transduces DNA damage signals to the RNA polymerase III transcriptional machinery. Cell, 106(5): 575-584.

Giaccia, A. J. & Kastan, M. B. (1998). The complexity of p53 modulation: emerging patterns from divergent signals. Genes & Development, 12(19): 2973-2983.

Gibson, A. W., Ye, R., Johnston, R. N., & Browder, L. W. (1992). A possible role for c-Myc oncoproteins in post-transcriptional regulation of ribosomal RNA. Oncogene, 7(11): 2363-2367.

Gillett, C., Fantl, V., Smith, R., Fisher, C., Bartek, J., Dickson, C., Barnes, D., & Peters, G. (1994). Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Research, 54(7): 1812-1817.

Goelz, S. E., Vogelstein, B., Hamilton, S. R., & Feinberg, A. P. (1985). Hypomethylation of DNA from benign and malignant human colon neoplasms. Science, 228(4696): 187-190.

Goh, H. S., Yao, J., & Smith, D. R. (1995). p53 point mutation and survival in colorectal cancer patients. Cancer Research, 55(22): 5217-5221.

Gomez-Roman, N., Grandorf, C., Eisenman, R. N., & White, R. J. (2003). Direct activation of RNA polymerase III transcription by c-myc. Nature, 421: 290-294.

Goodrich, D. W., Wang, N. P., Qian, Y. W., Lee, E. Y., & Lee, W. H. (1991). The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell, 67(2): 293-302.

Gottesfeld, J. M. (1987). DNA sequence-directed nucleosome reconstitution on 5S RNA genes of Xenopus laevis. Molecular & Cellular Biology, 7(5): 1612-1622.

Gottesfeld, J. M., Wolf, V. J., Dang, T., Forbes, D. J., & Hartl, P. (1994). Mitotic repression of RNA polymerase III transcription in vitro mediated by phosphorylation of a TFIIIB component. Science, 263(5143): 81-84.

Gottesfeld, J. M., Johnson, D. L., & Nyborg, J. K. (1996). Transcriptional activation of RNA polymerase III-dependent genes by the human T-cell leukemia virus type 1 tax protein. Molecular & Cellular Biology, 16(4): 1777-1785.

Grafia, X., Garriga, J., & Mayol, X. (1998). Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. Oncogene, 17: 3365-3383.

Grunstein, M. (1997). Histone acetylation in chromatin structure and transcription. Nature, 389: 349-352.

Gu, W. & Roeder, R. G. (1997). Activation of p53 sequence -specific DNA binding by acetylation of the p53 C-terminal domain. Cell, 90: 595-606.

Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., & Kern, S. E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science, 271(5247): 350-353.

Hammond, M. L. & Bowman, L. H. (1988). Insulin stimulates the translation of ribosomal proteins and the transcription of rDNA in mouse myoblasts. Journal of Biological Chemistry, 263(33): 17785-17791.

Hannan, R. D., Hempel, W. M., Cavanaugh, A., Arino, T., Dimitrov, S. I., Moss, T., & Rothblum, L. I. (1998). Affinity purification of mammalian RNA polymerase I. The Journal of Biological Chemistry, 273(2): 1257-1267.

Hannan, K. M., Hannan, R. D., Smith, S. D., Jefferson, L. S., Lun, M., & Rothblum, L. I. (2000a). Rb and p130 regulate RNA polymerase I transcription: Rb disrupts the interaction between UBF and SL-1. Oncogene, 19(43): 4988-4999.

Hannan, K. M., Kennedy, B. K., Cavanaugh, A. H., Hannan, R. D., Hirschler-Laszkiewicz, I., Jefferson, L. S., & Rothblum, L. I. (2000b). RNA polymerase I transcription in confluent cells: Rb downregulates rDNA transcription during confluence-induced cell cycle arrest. Oncogene, 19(31): 3487-3497.

Hansen, J. C. & Wolffe, A. P. (1992). Influence of chromatin folding on transcription initiation and elongation by RNA polymerase III. Biochemistry, 31(34): 7977-7988.

Hansen, J. C. & Wolffe, A. P. (1994). A role for histones H2A/H2B in chromatin folding and transcriptional repression. Proceedings of the National Academy of Sciences of the United States of America, 91(6): 2339-2343.

Hateboer, G., Timmers, H. T., Rustgi, A. K., Billaud, M., van't Veer, L. J., & Bernards, R. (1993). TATA-binding protein and the retinoblastoma gene product bind to overlapping epitopes on c-Myc and adenovirus E1A protein. Proceedings of the National Academy of Sciences of the United States of America, 90(18): 8489-8493.

Hayashi, K. (1981). Organization of sequences related to U6 RNA in the human genome. Nucleic Acids Research, 9(14): 3379-3388.

Hayes, J. J., Clark, D. J., & Wolffe, A. P. (1991). Histone contributions to the structure of DNA in the nucleosome. Proceedings of the National Academy of Sciences of the United States of America, 88(15): 6829-6833.

Hayes, J. J. & Wolffe, A. P. (1993). Preferential and asymmetric interaction of linker histones with 5S DNA in the nucleosome. Proceedings of the National Academy of Sciences of the United States of America, 90(14): 6415-6419.

Hayes, J. J., Pruss, D., & Wolffe, A. P. (1994). Contacts of the globular domain of histone H5 and core histones with DNA in a "chromatosome". Proceedings of the National Academy of Sciences of the United States of America, 91(16): 7817-7821.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., & Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. Science, 281(5382): 1509-1512.

Heix, J., Vente, A., Voit, R., Budde, A., Michaelidis, T. M., & Grummt, I. (1998). Mitotic silencing of human rRNA synthesis: inactivation of the promoter selectivity factor SL1 by cdc2/cyclin B-mediated phosphorylation. EMBO Journal, 17(24): 7373-7381.

Helland, A., Holm, R., Kristensen, G., Kaern, J., Karlsen, F., Trope, C., Nesland, J. M., & Borresen, A. L. (1993). Genetic alterations of the TP53 gene, p53 protein expression and HPV infection in primary cervical carcinomas. Journal of Pathology, 171(2): 105-114.

Hermeking, H. & Eick, D. (1994). Mediation of c-Myc-induced apoptosis by p53. Science, 265(5181): 2091-2093.

Herwig, S. & Strauss, M. (1997). The retinoblastoma protein: a master regulator of cell cycle, differentiation and apoptosis. European Journal of Biochemistry, 246(3): 581-601.

Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I., & Weinberg, R. A. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell, 70(6): 993-1006.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. Journal of Molecular Biology, 26(2): 365-369.

Hockmann, D. J. & Schultz, M. C. (1996). Caesin kinase II is required for efficient transcription by RNA polymerase III. Molecular & Cellular Biology, 16(3): 892-898.

Hoeffler, W. K. & Roeder, R. G. (1985). Enhancement of RNA polymerase III transcription by the E1A gene product of adenovirus. Cell, 41(3): 955-963.

Hoeffler, W. K., Kovelman, R., & Roeder, R. G. (1988). Activation of transcription factor IIIC by the adenovirus E1A protein. Cell, 53(6): 907-920.

Hoffman, J. & Post, J. (1967). In vivo studies of DNA synthesis in human normal and tumor cells. Cancer Research, 27(5): 898-902.

Hollstein, M., Sidransky, D., Vogelstein, B., & Harris, C. C. (1991). p53 mutations in human cancers. Science, 253(5015): 49-53.

Hollstein, M., Rice, K., Greenblatt, M. S., Soussi, T., Fuchs, R., Sorlie, T., Hovig, E., Smith-Sorensen, B., Montesano, R., & Harris, C. C. (1994). Database of p53 gene somatic muations in human tumors and cell lines. Nucleic Acids Research, 22(17): 3551-3555.

Howard, B. H. & Sakamoto, K. (1990). Alu interspersed repeats: selfish DNA or a functional gene family? New Biologist, 2(9): 759-770.

Hsieh, C. L., Donlon, T. A., Darras, B. T., Chang, D. D., Topper, J. N., Clayton, D. A., & Francke, U. (1990). The gene for the RNA component of the mitochondrail RNA-processing endoribonuclease is located on human chromosome 9p and on mouse chromosome 4. Genomics, 6: 540-544.

Hsieh, Y. J., Kundu, T. K., Wang, Z., Kovelman, R., & Roeder, R. G. (1999a). The TFIIIC90 subunit of TFIIIC interacts with multiple components of the RNA polymerase III machinery and contains a histone-specific acetyltransferase activity. Molecular & Cellular Biology, 19(11): 7697-7704.

Hsieh, Y. J., Wang, Z., Kovelman, R., & Roeder, R. G. (1999b). Cloning and characterization of two evolutionarily conserved subunits (TFIIIC102 and TFIIIC63) of human TFIIIC and their involvement in functional interactions with TFIIIB and RNA polymerase III. Molecular & Cellular Biology, 19(7): 4944-4952.

Hu, Q. J., Dyson, N., & Harlow, E. (1990). The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. EMBO Journal, 9(4): 1147-1155.

Huang, R., Wu, T., Xu, L., Liu, A., Ji, Y., & Hu, G. (2002). Upstream binding factor up-regulated in hepatocellular carcinoma is related to the survival and cisplatin-sensitivity of cancer cells. FASEB Journal, 16: 293-301.

Huibregtse, J. M., Scheffner, M., & Howley, P. M. (1991). A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. EMBO Journal, 10(13): 4129-4135.

Huibregtse, J. M., Scheffner, M., & Howley, P. M. (1993). Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. Molecular & Cellular Biology, 13(2): 775-784.

Hunter, T. & Pines, J. (1994). Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. Cell, 79(4): 573-582.

Iben, S., Tschochner, H., Bier, M., Hoogstraten, D., Hozak, P., Egly, J. M., & Grummt, I. (2002). TFIIH plays an essential role in RNA polymerase I transcription. Cell, 109: 297-306.

Ida, K., Kitabayashi, I., Taki, T., Taniwaki, M., Noro, K., Yamamoto, M., Ohki, M., & Hayashi, Y. (1997). Adenoviral E1A-associated protein p300 is involved in acute myeloid leukemia with t(11;22)(q23;q13). Blood, 90(12): 4699-4704.

Ignotz, G. G., Hokari, S., DePhilip, R. M., Tsukada, K., & Lieberman, I. (1981). Lodish model and regulation of ribosomal protein synthesis by insulin-deficient chick embryo fibroblasts. Biochemistry, 20(9): 2550-2558.

Imhof, A., Yang, X. J., Ogryzko, V. V., Nakatani, Y., Wolffe, A. P., & Ge, H. (1997). Acetylation of general transcription factors by histone acetyltransferases. Current Biology, 7(9): 689-692.

International Human Genome Sequencing, C. (2001). Initial sequencing and analysis of the human genome. Nature, 409(6822): 860-921.

Ishii, S., Merlino, G. T., & Pastan, I. (1985). Promoter region of the human Harvey ras proto-oncogene: similarity to the EGF receptor proto-oncogene promoter. Science, 230(4732): 1378-1381.

Ishii, S., Kadonaga, J. T., Tjian, R., Brady, J. N., Merlino, G. T., & Pastan, I. (1986). Binding of the Sp1 transcription factor by the human Harvey ras1 proto-oncogene promoter. Science, 232(4756): 1410-1413.

Isola, J., Visakorpi, T., Holli, K., & Kallioniemi, O. P. (1992). Association of overexpression of tumor suppressor protein p53 with rapid cell proliferation and poor prognosis in node-negative breast cancer patients. Journal of the National Cancer Institute, 84(14): 1109-1114.

Jelinek, W. R. & Schmid, C. W. (1982). Repetitive sequences in eukaryotic DNA and their expression. Annual Review of Biochemistry, 51: 813-844.

Jen, J., Powell, S. M., Papadopoulos, N., Smith, K. J., Hamilton, S. R., Vogelstein, B., & Kinzler, K. W. (1994). Molecular determinants of dysplasia in colorectal lesions. Cancer Research, 54(21): 5523-5526.

Jeon, S., Allen-Hoffmann, B. L., & Lambert, P. F. (1995). Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. Journal of Virology, 69(5): 2989-2997.

Jeon, S. & Lambert, P. F. (1995). Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. Proceedings of the National Academy of Sciences of the United States of America, 92(5): 1654-1658.

Johnson, S. A., Mandavia, N., Wang, H. D., & Johnson, D. L. (2000). Transcriptional regulation of the TATA-binding protein by Ras cellular signaling. Molecular & Cellular Biology, 20(14): 5000-5009.

Johnston, I. J., Allison, S. J., Morton, J. P., Schramm, L., Scott, P. H., & White, R. J. (2002). CK2 forms a stable complex with TFIIIB and activates RNA polymerase III transcription in human cells. Molecular & Cellular Biology, 22(11): 3757-3768.

Kassavetis, G. A., Braun, B. R., Nguyen, L. H., & Geiduschek, E. P. (1990). S. cerevisiae TFIIIB is the transcription initiation factor proper of RNA polymerase III, while TFIIIA and TFIIIC are assembly factors. Cell, 60(2): 235-245.

Kassavetis, G. A., Bartholomew, B., Blanco, J. A., Johnson, T. E., & Geiduschek, E. P. (1991). Two essential components of the Saccharomyces cerevisiae transcription factor TFIIIB: transcription and DNA-binding properties. Proceedings of the National Academy of Sciences of the United States of America, 88(16): 7308-7312.

Kassavetis, G. A., Letts, G. A., & Geiduschek, E. P. (2001). The RNA polymerase III transcription initiation factor TFIIIB participates in two steps of promoter opening. EMBO Journal, 20(11): 2823-2834.

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., & Craig, R. W. (1991). Participation of p53 protein in the cellular response to DNA damage. Cancer Research, 51(23 Pt 1): 6304-6311.

Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., & Fornace, A. J., Jr. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell, 71(4): 587-597.

Kemp, C. J., Donehower, L. A., Bradley, A., & Balmain, A. (1993). Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors. Cell, 74(5): 813-822.

Kerkhoff, E. & Rapp, U. R. (1997). Induction of cell proliferation in quiescent NIH 3T3 cells by oncogenic c-Raf-1. Molecular & Cellular Biology, 17(5): 2576-2586.

Kerkhoff, E. & Rapp, U. R. (1998). Cell cycle targets of Ras/Raf signalling. Oncogene, 17(11 Reviews): 1457-1462.

Kessis, T. D., Slebos, R. J., Han, S. M., Shah, K., Bosch, X. F., Munoz, N., Hedrick, L., & Cho, K. R. (1993). p53 gene mutations and MDM2 amplification are uncommon in primary carcinomas of the uterine cervix. American Journal of Pathology, 143(5): 1398-1405.

Kessis, T. D., Connolly, D. C., Hedrick, L., & Cho, K. R. (1996). Expression of HPV16 E6 or E7 increases integration of foreign DNA. Oncogene, 13(2): 427-431.

Keyomarsi, K., O'Leary, N., Molnar, G., Lees, E., Fingert, H. J., & Pardee, A. B. (1994). Cyclin E, a potential prognostic marker for breast cancer. Cancer Research, 54(2): 380-385.

Khoo, B., Brophy, B., & Jackson, S. P. (1994). Conserved functional domains of the RNA polymerase III general transcription factor BRF. Genes & Development, 8(23): 2879-2890.

Kihm, A. J., Hershey, J. C., Haystead, T. A. J., Madsen, C. S., & Owens, G. K. (1998). Phosphorylation of the rRNA transcription factor upstream binding factor promotes its association with TATA biding protein. Proceedings of the National Academy of Sciences of the United States of America, 95: 14816-14820.

Kim, C. M., Koike, K., Saito, I., Miyamura, T., & Jay, G. (1991). HBx gene of hepatitus B virus induces liver cancer in transgenic mice. Nature, 351: 317-320.

Kinzler, K. W. & Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. Cell, 87(2): 159-170.

Klein, J. & Grummt, I. (1999). Cell cycle-dependent regulation of RNA polymerase I transcription: the nucleolar transcription factor UBF is inactive in mitosis and early G1. EMBO Journal, 96: 6096-6101.

Knoepfler, P. S. & Eisenman, R. N. (1999). Sin meets NuRD and other tails of repression. Cell, 99(5): 447-450.

Knudsen, E. & Wang, J. (1997). Dual mechanisms for the inhibition of E2F binding to RB by cyclin- dependent kinase-mediated RB phosphorylation. Molecular & Cellular Biology, 17(10): 5771-5783.

Ko, L. J. & Prives, C. (1996). p53: puzzle and paradigm. Genes & Development, 10(9): 1054-1072.

Kouzarides, T. (1995). Transcriptional control by the retinoblastoma protein. Seminars in Cancer Biology, 6(2): 91-98.

Kouzarides, T. (1999). Histone acetylases and deacetylases in cell proliferation. Current Opinion in Genetics & Development, 9: 40-48.

Kovelman, R. & Roeder, R. G. (1992). Purification and characterization of two forms of human transcription factor IIIC. Journal of Biological Chemistry, 267(34): 24446-24456.

Krayev, A. S., Kramerov, D. A., Skryabin, K. G., Ryskov, A. P., Bayev, A. A., & Georgiev, G. P. (1980). The nucleotide sequence of the ubiquitous repetitive DNA sequence B1 complementary to the most abundant class of mouse fold-back RNA. Nucleic Acids Research, 8(6): 1201-1215.

Kruger, W. & Benecke, B. J. (1987). Structural and functional analysis of a human 7 S K RNA gene. Journal of Molecular Biology, 195(1): 31-41.

Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., & Kastan, M. B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proceedings of the National Academy of Sciences of the United States of America, 89(16): 7491-7495.

Kuhn, A., Vente, A., Dorée, M., & Grummt, I. (1998). Mitotic phosphorylation of the TBP-containing factor SL1 represses ribosomal gene transcription. Journal of Molecular Biology, 284: 1-5.

Kundu, T. K., Wang, Z., & Roeder, R. G. (1999). Human TFIIIC relieves chromatin-mediated repression of RNA polymerase III transcription and contains an intrinsic histone acetyltransferase activity. Molecular & Cellular Biology, 19(2): 1605-1615.

Kunkel, G. R. & Pederson, T. (1989). Transcription of a human U6 small nuclear RNA gene in vivo withstands deletion of intragenic sequences but not of an upstream TATATA box. Nucleic Acids Research, 17(18): 7371-7379.

Kuo, M. H. & Allis, C. D. (1998). Roles of histone acetyltransferases and deacetylases in gene regulation. BioEssays, 20: 615-626.

Lagna, G., Kovelman, R., Sukegawa, J., & Roeder, R. G. (1994). Cloning and characterization of an evolutionarily divergent DNA-binding subunit of mammalian TFIIIC. Molecular & Cellular Biology, 14(5): 3053-3064.

Lammie, G. A., Fantl, V., Smith, R., Schuuring, E., Brookes, S., Michalides, R., Dickson, C., Arnold, A., & Peters, G. (1991). D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. Oncogene, 6(3): 439-444.

Lancaster, J. M., Wooster, R., Mangion, J., Phelan, C. M., Cochran, C., Gumbs, C., Seal, S., Barfoot, R., Collins, N., Bignell, G., Patel, S., Hamoudi, R., Larsson, C., Wiseman, R. W., Berchuck, A., Iglehart, J. D., Marks, J. R., Ashworth, A., Stratton, M. R., & Futreal, P. A. (1996). BRCA2 mutations in primary breast and ovarian cancers. Nature Genetics, 13(2): 238-240.

Lane, D. P. (1992). Cancer. p53, guardian of the genome. Nature, 358(6381): 15-16.

Larminie, C. G., Cairns, C. A., Mital, R., Martin, K., Kouzarides, T., Jackson, S. P., & White, R. J. (1997). Mechanistic analysis of RNA polymerase III regulation by the retinoblastoma protein. EMBO Journal, 16(8): 2061-2071.

Larminie, C. G., Sutcliffe, J. E., Tosh, K., Winter, A. G., Felton-Edkins, Z. A., & White, R. J. (1999). Activation of RNA polymerase III transcription in cells transformed by simian virus 40. Molecular & Cellular Biology, 19(7): 4927-4934.

Lassar, A. B., Martin, P. L., & Roeder, R. G. (1983). Transcription of class III genes: formation of preinitiation complexes. Science, 222(4625): 740-748.

Leder, A., Pattengale, P. K., Kuo, A., Stewart, T. A., & Leder, P. (1986). Consequences of widespread deregulation of the c-myc gene in transgenic mice: multiple neoplasms and normal development. Cell, 45(4): 485-495.

Lee, D. Y., Hayes, J. J., Pruss, D., & Wolffe, A. P. (1993). A positive role for histone acetylation in transcription factor access to nucleosomal DNA. Cell, 72(1): 73-84.

Leresche, A., Wolf, V. J., & Gottesfeld, J. M. (1996). Repression of RNA polymerase II and III transcription during M phase of the cell cycle. Experimental Cell Research, 229(282-8).

L'Etoile, N. D., Fahnestock, M. L., Shen, Y., Acbersold, R., & Berk, A. J. (1994). Human transcription factor IIIC box B binding subunit. Proceedings of the National Academy of Sciences of the United States of America, 91: 1652-1656.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. Cell, 88(3): 323-331.

Liebhaber, S. A., Wolf, S., & Schlessinger, D. (1978). Differences in rRNA metabolism of primary and SV40-transformed human fibroblasts. Cell, 13(1): 121-127.

Liu, J. J., Chao, J. R., Jiang, M. C., Ng, S. Y., Yen, J. J., & Yang-Yen, H. F. (1995). Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. Molecular & Cellular Biology, 15(7): 3654-3663.

Lobo, S. M. & Hernandez, N. (1989). A 7 bp mutation converts a human RNA polymerase II snRNA promoter into an RNA polymerase III promoter. Cell, 58(1): 55-67.

Long, E. O. & Dawid, I. B. (1980). Repeated genes in eukaryotes. Annual Review of Biochemistry, 49: 727-764.

Lorincz, A., Reid, R., Jenson, A., Greenburg, M., Lancaster, W., & Kurman, R. (1992). Human Papillomavirus Infection of the Cervix: Relative Risk Associations of 15 Common Angenital Types. Obstetrics & Gynecology, 79(3): 328-337.

Low, S. C. & Berry, M. J. (1996). Knowing when not to stop: selenocysteine incorporation in eukaryotes. Trends in Biochemical Sciences, 21(6): 203-208.

Lozano, G. & Elledge, S. J. (2000). p53 sends nucleotides to repair DNA. Nature, 404(24-5).

Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., & Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature, 389(6648): 251-260.

Luo, R. X. & Postigo, A. A. (1998). Rb interacts with histone deacetylase to repress transcription. Cell, 92: 463-473.

Lygerou, Z., Allmang, C., Tollervey, D., & Seraphin, B. (1996). Accurate processing of a eukaryotic precursor ribosomal RNA by ribonuclease MRP in vitro. Science, 272(5259): 268-270.

Lynch, H. T., Smyrk, T., & Jass, J. R. (1995). Hereditary nonpolyposis colorectal cancer and colonic adenomas: aggressive adenomas? Seminars in Surgical Oncology, 11(6): 406-410.

Lynch, H. T., Smyrk, T., & Lynch, J. F. (1996). Overview of natural history, pathology, molecular genetics and management of HNPCC (Lynch Syndrome). International Journal of Cancer, 69(1): 38-43.

Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J. P., Troalen, F., Trouche, D., & Harel-Bellan, A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature, 391: 601-604.

Maheswaran, S., McCormack, J. E., & Sonenshein, G. E. (1991). Changes in phosphorylation of myc oncogene and RB antioncogene protein products during growth arrest of the murine lymphoma WEHI 231 cell line. Oncogene, 6(11): 1965-1971.

Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Jr., Nelson, C. E., Kim, D. H., Kassel, J., Gryka, M. A., Bischoff, F. Z., & Tainsky, M. A. (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science, 250(4985): 1233-1238.

Marcu, K. B., Bossone, S. A., & Patel, A. J. (1992). myc function and regulation. Annual Review of Biochemistry, 61: 809-860.

Marks, P. A., Richon, V. M., & Rifkind, R. A. (2000). Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. Journal of the National Cancer Institute, 92(15): 1210-1216.

Marks, P. A., Richon, V. M., Breslow, R., & Rifkind, R. A. (2001). Histone deactetylase inhibitors as new cancer drugs. Current Opinion in Oncology, 13: 477-483.

Martinez-Balbas, M., Bauer, U. M., Nielsen, S. J., Brehm, A., & Kouzarides, T. (2000). Regulation of E2F1 activity by acetylation. EMBO Journal, 19: 662-671.

Mateyak, M. K., Obaya, A. J., Adachi, S., & Sedivy, J. M. (1997). Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. Cell Growth & Differentiation, 8(10): 1039-1048.

Mathis, D., Oudet, P., & Chambon, P. (1980). Structure of transcribing chromatin. Progress in Nucleic Acid Research & Molecular Biology, 24: 1-55.

Matsukura, T., Koi, S., & Sugase, M. (1989). Both episomal and integrated forms of human papillomavirus type 16 are involved in invasive cervical cancers. Virology, 172(1): 63-72.

Mattaj, I. W., Tollervey, D., & Seraphin, B. (1993). Small nuclear RNAs in messenger RNA and ribosomal RNA processing. FASEB Journal, 7(1): 47-53.

Mauck, J. C. & Green, H. (1974). Regulation of pre-transfer RNA synthesis during transition from resting to growing state. Cell, 3(2): 171-177.

Medina, V., Edmonds, B., Young, G. P., James, R., Appleton, S., & Zalewski, P. D. (1997). Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway. Cancer research, 57(17): 3697-3707.

Mighell, A. J., Markham, A. F., & Robinson, P. A. (1997). Alu sequences. FEBS Letters, 417(1-5).

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, A. P., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, M. L., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Tran, T., McClure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher, H., Yakumo, K., Gholami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C., Bogden, R., Dayananth, P., Ward, J., Tonin, P., Narod, S., Bristow, P. K., Norris, F. H., Helvering, L., Morrison, P., Rosteck, P., Lai, M., Barrett, J. C., Lewis, C., Neuhausen, S., Cannon-Albright, L., Goldgar, D., Wiseman, R., Kamb, A., & Skolnick, M. H. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science, 266(5182): 66-71.

Miki, Y., Katagiri, T., Kasumi, F., Yoshimoto, T., & Nakamura, Y. (1996). Mutation analysis in the BRCA2 gene in primary breast cancers. Nature Genetics, 13(2): 245-247.

Miller, O. L. & Beatty, B. R. (1969). Visualisation of nucleolar genes. Science, 164: 955-957.

Miller, J., McLachlan, A. D., & Klug, A. (1985). Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. EMBO Journal, 4(6): 1609-1614.

Miller, G., Panov, K. I., Friedrich, J. K., Trinkle-Mulcahy, L., & Zomerdijk, J. C. B. M. (2001). hRRN3 is essential in the SL1-mediated recruitment of RNA polymerase I to rRNA gene promoters. EMBO Journal, 20(6): 1373-1382.

Mital, R., Kobayashi, R., & Hernandez, N. (1996). RNA polymerase III transcription from the human U6 and adenovirus type 2 VAI promoters has different requirements

for human BRF, a subunit of human TFIIIB. Molecular & Cellular Biology, 16(12): 7031-7042.

Mittal, V., Cleary, M. A., Herr, W., & Hernandez, N. (1996). The Oct-1 POU-specific domain can stimulate small nuclear RNA gene transcription by stabilizing the basal transcription complex SNAPc. Molecular & Cellular Biology, 16(5): 1955-1965.

Mittal, V. & Hernandez, N. (1997). Role for the amino-terminal region of human TBP in U6 snRNA transcription. Science, 275(5303): 1136-1140.

Mittnacht, S. (1998). Contol of pRB phosphorylation. Current Opinion in Genetics & Development, 8: 21-27.

Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., & Nakamura, Y. (1992). Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Human Molecular Genetics, 1(4): 229-233.

Momand, J., Jung, D., Wilczynski, S., & Niland, J. (1998). The MDM2 gene amplification database. Nucleic Acids Research, 26(15): 3453-3459.

Moran, E. (1993). DNA tumor virus transforming proteins and the cell cycle. Current Opinion in Genetics & Development, 3(1): 63-70.

Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., & Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science, 275(5307): 1787-1790.

Moss, T. & Stefanovsky, V. Y. (2002). At the center of eukaryotic life. Cell, 109: 545-548.

Mulligan, G. & Jacks, T. (1998). The retinoblastoma gene family: cousins with overlapping interests. Trends in Genetics, 14(6): 223-229.

Münger, K., Phelps, W. C., Bubb, V., Howley, P. M., & Schlegel, R. (1989a). The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. Journal of Virology, 63(10): 4417-4421.

Münger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E., & Howley, P. M. (1989b). Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO Journal, 8(13): 4099-4105.

Muraoka, M., Konishi, M., Kikuchi-Yanoshita, R., Tanaka, K., Shitara, N., Chong, J. M., Iwama, T., & Miyaki, M. (1996). p300 gene alterations in colorectal and gastric carcinomas. Oncogene, 12(7): 1565-1569.

Murphy, S., Altruda, F., Ullu, E., Tripodi, M., Silengo, L., & Melli, M. (1984). DNA sequences complementary to human 7SK RNA show structural similarities to the

short mobile elements of the mammalian genome. Journal of Molecular Biology, 177(4): 575-590.

Murphy, S., Tripodi, M., & Melli, M. (1986). A sequence upstream from the coding region is required for the transcription of the 7SK RNA genes. Nucleic Acids Research, 14(23): 9243-9260.

Murphy, S., Pierani, A., Scheidereit, C., Melli, M., & Roeder, R. G. (1989). Purified octamer binding transcription factors stimulate RNA polymerase III--mediated transcription of the 7SK RNA gene. Cell, 59(6): 1071-1080.

Murphy, S., Yoon, J. B., Gerster, T., & Roeder, R. G. (1992). Oct-1 and Oct-2 potentiate functional interactions of a transcription factor with the proximal sequence element of small nuclear RNA genes. Molecular & Cellular Biology, 12(7): 3247-3261.

Murphy, S. (1997). Differential in vivo activation of the class II and class III snRNA genes by the POU-specific domain of Oct-1. Nucleic Acids Research, 25(11): 2068-2076.

Murphy, M., Ahn, J., Walker, K. K., Hoffman, W. H., Evans, R. M., Levine, A. J., & George, D. L. (1999). Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. Genes & Development, 13(19): 2490-2501.

Muth, V., Nadaud, S., Grummt, I., & Voit, R. (2001). Acetylation of TAF(I)68, a subunit of TIF-IB/SL1, activates RNA polymerase I transcription. EMBO Journal, 20(6): 1353-1362.

Nagase, H. & Nakamura, Y. (1993). Mutations of the APC (adenomatous polyposis coli) gene. Human Mutation, 2(6): 425-434.

Nesbit, C. E., Tersak, J. M., & Prochownik, E. V. (1999). MYC oncogenes and human neoplastic disease. Oncogene, 18(19): 3004-3016.

Nguyen, V. T., Kiss, T., Michels, A. A., & Bensaude, O. (2001). 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. Nature, 414(6861): 322-325.

Nolte, R. T., Conlin, R. M., Harrison, S. C., & Brown, R. S. (1998). Differing roles for zinc fingers in DNA recognition: structure of a six-finger transcription factor IIIA complex. Proceedings of the National Academy of Sciences of the United States of America, 95(6): 2938-2943.

Notterman, D. A., Alon, U., Sierk, A. J., & Levine, A. J. (2001). Transcriptional gene expression profiles of colorectal adenoma, adenocarcinoma, and normal tissue examined by oligonucleotide arrays. Cancer Research, 61(7): 3124-3130.

Oettel, S., Härtel, F., Kober, I., Iben, S., & Seifart, K. H. (1997). Human transcritpion factors IIIC2, IIIC1 and a novel component IIIC0 fulfil different aspects of DNA binding to various pol III genes. Nucleic Acids Research, 25(12): 2440-2447.

O'Mahony, D. J., Xie, W., Smith, S. D., Singer, H. A., & Rothblum, L. I. (1992). Differential phosphorylation and localisation of the transcription factor UBF in vivo in response to serum deprivation. The Journal of Biological Chemistry, 267(1): 35-38.

O'Neill, V. A., Eden, F. C., Pratt, K., & Hatfield, D. L. (1985). A human opal suppressor tRNA gene and pseudogene. Journal of Biological Chemistry, 260(4): 2501-2508.

Orlandin, M., Semplici, F., Ferruzzi, R., Meggio, F., Pinna, L. A., & Oliviero, S. (1998). Proein kinase CK2alpha' is induced by serum as a delayed early gene and cooperates with Ha-ras in fibroblast transformation. The Journal of Biological Chemistry, 273(33): 21291-21297.

Paule, M. R. (1998). Transcription of Ribosomal RNA Genes by Eukaryotic RNA Polymerase I. Berlin: Springer-Verlag.

Paule, M. R. & White, R. J. (2000). Survey and summary: transcription by RNA polymerases I and III. Nucleic Acids Research, 28(6): 1283-1298.

Pelletier, G., Stefanovsky, V. Y., Faubladier, M., Hirschler-Laszkiewicz, I., Savard, J., Rothblum, L. I., Cote, J., & Moss, T. (2000). Competitive recruitment of CBP and Rb-HDAC regulates UBF acetylation and ribosomal transcription. Molecular Cell, 6(5): 1059-1066.

Perna, P. J., Harris, G. H., Iida, C. T., Kownin, P., Bugren, S., & Paule, M. R. (1992). The start site of the Acanthamoeba castellanii ribosomal RNA transcription unit. Gene Expression, 2(1): 71-78.

Perou, C. M., Jeffrey, S. S., van de Rijn, M., Rees, C. A., Eisen, M. B., Ross, D. T., Pergamenschikov, A., Williams, C. F., Zhu, S. X., Lee, J. C., Lashkari, D., Shalon, D., Brown, P. O., & Botstein, D. (1999). Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proceedings of the National Academy of Sciences of the United States of America, 96(16): 9212-9217.

Pertschuk, L. P., Feldman, J. G., Kim, D. S., Nayeri, K., Eisenberg, K. B., Carter, A. C., Thelmo, W. T., Rhong, Z. T., Benn, P., & Grossman, A. (1993). Steroid hormone receptor immunohistochemistry and amplification of c-myc protooncogene. Relationship to disease-free survival in breast cancer. Cancer, 71(1): 162-171.

Phelan, C. M., Lancaster, J. M., Tonin, P., Gumbs, C., Cochran, C., Carter, R., Ghadirian, P., Perret, C., Moslehi, R., Dion, F., Faucher, M. C., Dole, K., Karimi, S., Foulkes, W., Lounis, H., Warner, E., Goss, P., Anderson, D., Larsson, C., Narod, S. A., & Futreal, P. A. (1996). Mutation analysis of the BRCA2 gene in 49 site-specific breast cancer families. Nature Genetics, 13(1): 120-122.

Phelan, C. M., Rebbeck, T. R., Weber, B. L., Devilee, P., Ruttledge, M. H., Lynch, H. T., Lenoir, G. M., Stratton, M. R., Easton, D. F., Ponder, B. A., Cannon-Albright, L., Larsson, C., Goldgar, D. E., & Narod, S. A. (1996). Ovarian cancer risk in BRCA1 carriers is modified by the HRAS1 variable number of tandem repeat (VNTR) locus. Nature Genetics, 12(3): 309-311.

Pieler, T., Hamm, J., & Roeder, R. G. (1987). The 5S gene internal control region is composed of three distinct sequence elements, organized as two functional domains with variable spacing. Cell, 48(1): 91-100.

Pietilainen, T., Lipponen, P., Aaltomaa, S., Eskelinen, M., Kosma, V. M., & Syrjanen, K. (1995). Expression of c-myc proteins in breast cancer as related to established prognostic factors and survival. Anticancer Research, 15(3): 959-964.

Piras, G., Dittmer, J., Radonovich, M. F., & Brady, J. N. (1996). Human T-cell leukemia virus type I Tax protein transactivates RNA polymerase III promoter in vitro and in vivo. Journal of Biological Chemistry, 271(34): 20501-20506.

Pogue-Geile, K., Geiser, J. R., Shu, M., Miller, C., Wool, I. G., Meisler, A. I., & Pipas, J. M. (1991). Ribosomal protein genes are overexpressed in colorectal cancer: isolation of a cDNA clone encoding the human S3 ribosomal protein. Molecular & Cellular Biology, 11(8): 3842-3849.

Pombo, A., Jackson, D. A., Hollinshead, M., Wang, Z., Roeder, R. G., & Cook, P. R. (1999). Regional specialization in human nuclei: visualization of discrete sites of transcription by RNA polymerase III. EMBO Journal, 18(8): 2241-2253.

Ponz de Leon, M., Sassatelli, R., Benatti, P., & Roncucci, L. (1993). Identification of hereditary nonpolyposis colorectal cancer in the general population. The 6-year experience of a population-based registry. Cancer, 71(11): 3493-3501.

Powell, S. M., Zilz, N., Beazer-Barclay, Y., Bryan, T. M., Hamilton, S. R., Thibodeau, S. N., Vogelstein, B., & Kinzler, K. W. (1992). APC mutations occur early during colorectal tumorigenesis. Nature, 359(6392): 235-237.

Pretlow, T. P., Brasitus, T. A., Fulton, N. C., Cheyer, C., & Kaplan, E. L. (1993). K-ras mutations in putative preneoplastic lesions in human colon. Journal of the National Cancer Institute, 85(24): 2004-2007.

Pruss, D., Hayes, J. J., & Wolffe, A. P. (1995). Nucleosomal anatomy--where are the histones? Bioessays, 17(2): 161-170.

Radebaugh, C. A., Gong, X., Bartholomew, B., & Paule, M. R. (1997). Identification of previously unrecognized common elements in eukaryotic promoters. A ribosomal RNA gene initiator element for RNA polymerase I. Journal of Biological Chemistry, 272(6): 3141-3144.

Reddy, R., Henning, D., Das, G., Harless, M., & Wright, D. (1987). The capped U6 small nuclear RNA is transcribed by RNA polymerase III. Journal of Biological Chemistry, 262(1): 75-81.

Reeder, R. H. (1999). Regulation of RNA polymerase I transcription in yeast and vertebrates. Progress in Nucleic Acid Research & Molecular Biology, 62: 293-327.

Richmond, T. J., Finch, J. T., Rushton, B., Rhodes, D., & Klug, A. (1984). Structure of the nucleosome core particle at 7 A resolution. Nature, 311(5986): 532-537.

Rocco, J. W. & Sidransky, D. (2001). p16(MTS-1/CDKN2/INK4a) in cancer progression. Experimental Cell Research, 264: 42-55.

Roeder, R. G. & Rutter, W. J. (1969). Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. Nature, 224(216): 234-237.

Rogalsky, V., Todorov, G., & Moran, D. (1993). Translocation of retinoblastoma protein associated with tumor cell growth inhibition. Biochemical & Biophysical Research Communications, 192(3): 1139-1146.

Rogers, J. H. (1985). The origin and evolution of retroposons. International Review of Cytology, 93: 187-279.

Ronning, O. W., Lindmo, T., Pettersen, E. O., & Seglen, P. O. (1981). The role of protein accumulation in the cell cycle control of human NHIK 3025 cells. Journal of Cellular Physiology, 109(3): 411-418.

Ross, J. F., Liu, X., & Dynlacht, B. D. (1999). Mechanism of transcriptional repression of E2F by the retinoblastoma tumor suppressor protein. Molecular Cell, 3(2): 195-205.

Rothberg, P. G. (1987). The role of the oncogene c-myc in sporadic large bowel cancer and familial polyposis coli. Seminars in Surgical Oncology, 3(3): 152-158.

Roux-Dosseto, M., Romain, S., Dussault, N., Desideri, C., Piana, L., Bonnier, P., Tubiana, N., & Martin, P. M. (1992). c-myc gene amplification in selected node-negative breast cancer patients correlates with high rate of early relapse. European Journal of Cancer, 28A(10): 1600-1604.

Rozenberg, Y. Y. & Howard, B. D. (1994). Contrasting morphological changes in PC12 flat cells expressing two different forms of exogenous oncogenic ras. Experimental Cell Research, 211(1): 59-67.

Rubin, C. M., Houck, C. M., Deininger, P. L., Friedmann, T., & Schmid, C. W. (1980). Partial nucleotide sequence of the 300-nucleotide interspersed repeated human DNA sequences. Nature, 284(5754): 372-374.

Russanova, V. R., Driscoll, C. T., & Howard, B. H. (1995). Adenovirus type 2 preferentially stimulates polymerase III transcription of Alu elements by relieving repression: a potential role for chromatin. Molecular & Cellular Biology, 15(8): 4282-4290.

Rustgi, A. K., Dyson, N., & Bernards, R. (1991). Amino-terminal domains of c-myc and N-myc proteins mediate binding to the retinoblastoma gene product. Nature, 352(6335): 541-544.

Ryan, K. M. & Vousden, K. H. (1998). Characterization of structural p53 mutants which show selective defects in apoptosis but not cell cycle arrest. Molecular & Cellular Biology, 18(7): 3692-3698.

Sadowski, C. L., Henry, R. W., Lobo, S. M., & Hernandez, N. (1993). Targeting TBP to a non-TATA box cis-regulatory element: a TBP-containing complex activates transcription from snRNA promoters through the PSE. Genes & Development, 7(8): 1535-1548.

Saitoh, S., Cunningham, J., De Vries, E. M., McGovern, R. M., Schroeder, J. J., Hartmann, A., Blaszyk, H., Wold, L. E., Schaid, D., & Sommer, S. S. (1994). p53 gene mutations in breast cancers in midwestern US women: null as well as missense-type mutations are associated with poor prognosis. Oncogene, 9(10): 2869-2875.

Sawan, A., Randall, B., Angus, B., Wright, C., Henry, J. A., Ostrowski, J., Hennessy, C., Lennard, T. W., Corbett, I., & Horne, C. H. (1992). Retinoblastoma and p53 gene expression related to relapse and survival in human breast cancer: an immunohistochemical study. Journal of Pathology, 168(1): 23-28.

Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., & Howley, P. M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell, 63(6): 1129-1136.

Scheffner, M., Munger, K., Byrne, J. C., & Howley, P. M. (1991). The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proceedings of the National Academy of Sciences of the United States of America, 88(13): 5523-5527.

Scheffner, M., Huibregtse, J. M., Vierstra, R. D., & Howley, P. M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell, 75(3): 495-505.

Schlissel, M. S. & Brown, D. D. (1984). The transcriptional regulation of Xenopus 5s RNA genes in chromatin: the roles of active stable transcription complexes and histone H1. Cell, 37(3): 903-913.

Schmid, M., Haaf, T., & Grunert, D. (1984). 5-Azacytidine-induced undercondensations in human chromosomes. Human Genetics, 67(3): 257-263.

Schramm, L., Pendergrast, P. S., Sun, Y., & Hernandez, N. (2000). Different human TFIIIB activities direct RNA polymerase III transcription from TATA-containing and TATA-less promoters. Genes & Development, 14(20): 2650-2663.

Schramm, L. & Hernandez, N. (2002). Recruitment of RNA Polymerase III to its target promoters. Genes & Development, 16: 2593-2620.

Schuuring, E., Verhoeven, E., Mooi, W. J., & Michalides, R. J. (1992). Identification and cloning of two overexpressed genes, U21B31/PRAD1 and EMS1, within the amplified chromosome 11q13 region in human carcinomas. Oncogene, 7(2): 355-361.

Schwartz, L. B., Sklar, V. E. F., Jaehning, J. A., Weinmann, R., & Roeder, R. G. (1974). Isolation and partial characterisation of the multiple forms of deoxyribonucleic acid polymerase in the mouse myeloma, MOPC 135. The Journal of Biological Chemistry, 249(18): 5889-5897.

Schwarz, E., Freese, U. K., Gissmann, L., Mayer, W., Roggenbuck, B., Stremlau, A., & zur Hausen, H. (1985). Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature, 314(6006): 111-114.

Scott, M. R. D., Westphal, K. H., & Rigby, P. W. J. (1983). Activation of mouse genes in transformed cells. Cell, 34: 557-567.

Scott, P. H., Cairns, C. A., Sutcliffe, J. E., Alzuherri, H. M., McLees, A., Winter, A. G., & White, R. J. (2001). Regulation of RNA polymerase III transcription during cell cycle entry. Journal of Biological Chemistry, 276(2): 1005-1014.

Segil, N., Guermah, M., Hoffmann, A., Roeder, R. G., & Heintz, N. (1996). Mitotic regulation of TFIID: inhibition of activator-dependent transcription and changes in subcellular localization. Genes & Development, 10: 2389-2400.

Serova, O. M., Mazoyer, S., Puget, N., Dubois, V., Tonin, P., Shugart, Y. Y., Goldgar, D., Narod, S. A., Lynch, H. T., & Lenoir, G. M. (1997). Mutations in BRCA1 and BRCA2 in breast cancer families: are there more breast cancer-susceptibility genes? American Journal of Human Genetics, 60(3): 486-495.

Setzer, D. R. & Brown, D. D. (1985). Formation and stability of the 5 S RNA transcription complex. Journal of Biological Chemistry, 260(4): 2483-2492.

Sewing, A., Wiseman, B., Lloyd, A. C., & Land, H. (1997). High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1. Molecular & Cellular Biology, 17(9): 5588-5597.

Shan, B., Zhu, X., Chen, P. L., Durfee, T., Yang, Y., Sharp, D., & Lee, W. H. (1992). Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. Molecular & Cellular Biology, 12(12): 5620-5631.

Sharma, K. & Tollervey, D. (1999). Base pairing between U3 small nucleolar RNA and the 5' end of 18S rRNA is required for pre-rRNA processing. Molecular & Cellular Biology, 19(9): 6012-6019.

Sharp, S. J., Schaack, J., Cooley, L., Burke, D. J., & Soll, D. (1985). Structure and transcription of eukaryotic tRNA genes. CRC Critical Reviews in Biochemistry, 19(2): 107-144.

Shattuck-Eidens, D., Oliphant, A., McClure, M., McBride, C., Gupte, J., Rubano, T., Pruss, D., Tavtigian, S. V., Teng, D. H., Adey, N., Staebell, M., Gumpper, K., Lundstrom, R., Hulick, M., Kelly, M., Holmen, J., Lingenfelter, B., Manley, S., Fujimura, F., Luce, M., Ward, B., Cannon-Albright, L., Steele, L., Offit, K., Gilewski, T., Norton, L., Brown, K., Schulz, C., Hampel, H., Schluger, A., Giulotto, E., Zoli, W., Ravaioli, A., Nevanlinna, H., Pyrhonen, S., Rowley, P., Loader, S., Osborne, M. P., Daly, M., Tepler, I., Weinstein, P. I., Scalia, J. L., Michaelson, R., Scott, R. J., Radice, P., Pierotti, M. A., Garber, J. E., Isaacs, C., Peshkin, B., Lippman, M. E., Dosik, M., Caligo, M. A., Greenstein, R. M., Pilarski, R., Weber, B., Burgemeister, R., Frank, T. S., Skolnick, M. H., & Thomas, A. (1997). BRCA1 sequence analysis in women at high risk for susceptibility mutations. Risk factor analysis and implications for genetic testing. Jama, 278(15): 1242-1250.

Shibata, D., Schaeffer, J., Li, Z. H., Capella, G., & Perucho, M. (1993). Genetic heterogeneity of the c-K-ras locus in colorectal adenomas but not in adenocarcinomas. Journal of the National Cancer Institute, 85(13): 1058-1063.

Shirasawa, S., Furuse, M., Yokoyama, N., & Sasazuki, T. (1993). Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science, 260(5104): 85-88.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., & Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proceedings of the National Academy of Sciences of the United States of America, 96(10): 5522-5527.

Sierra, A., Castellsague, X., Escobedo, A., Moreno, A., Drudis, T., & Fabra, A. (1999). Synergistic cooperation between c-Myc and Bcl-2 in lymph node progression of T1 human breast carcinomas. Breast Cancer Research & Treatment, 54(1): 39-45.

Silverman, A. L., Park, J. G., Hamilton, S. R., Gazdar, A. F., Luk, G. D., & Baylin, S. B. (1989). Abnormal methylation of the calcitonin gene in human colonic neoplasms. Cancer Research, 49(13): 3468-3473.

Silvestrini, R., Benini, E., Daidone, M. G., Veneroni, S., Boracchi, P., Cappelletti, V., Di Fronzo, G., & Veronesi, U. (1993). p53 as an independent prognostic marker in lymph node-negative breast cancer patients. Journal of the National Cancer Institute, 85(12): 965-970.

Singer, M. F. (1982). SINEs and LINEs: highly repeated short and long interspersed sequences in mammalian genomes. Cell, 28(3): 433-434.

Singh, K., Carey, M. F., Saragosti, S., & Botchan, M. (1985). Expression of enhanced levels of small RNA polymerase III transcripts encoded by the B2 repeats in simian virus 40-transformed mouse cells. Nature, 314(11): 553-556.

Sinn, E., Wang, Z., Kovelman, R., & Roeder, R. G. (1995). Cloning and characterization of a TFIIIC2 subunit (TFIIIC beta) whose presence correlates with activation of RNA polymerase III-mediated transcription by adenovirus E1A expression and serum factors. Genes & Development, 9(6): 675-685.

Sionov, R. V. & Haupt, Y. (1999). The cellular response to p53: the decision between life and death. Oncogene, 18(45): 6145-6157.

Smirnov, D. A., Hou, S., & Ricciardi, R. P. (2000). Association of histone deacetylase with COUP-TF in tumorigenic Ad12-transformed cells and its potential role in shut-off of MHC class I transcription. Virology, 268(2): 319-328.

Smith, A. J., Stern, H. S., Penner, M., Hay, K., Mitri, A., Bapat, B. V., & Gallinger, S. (1994). Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons. Cancer Research, 54(21): 5527-5530.

Sobulo, O. M., Borrow, J., Tomek, R., Reshmi, S., Harden, A., Schlegelberger, B., Housman, D., Doggett, N. A., Rowley, J. D., & Zeleznik-Le, N. J. (1997). MLL is fused to CBP, a histone acetyltransferase, in therapy-related acute myeloid leukemia with a t(11;16)(q23;p13.3). Proceedings of the National Academy of Sciences of the United States of America, 94(16): 8732-8737.

Söderlund, H., Pettersson, U., Vennström, B., & Phillipson, L. (1976). A new species of virus-coded low molecular weight RNA from cells infected with adenovirus type 2. Cell, 7: 585-593.

Song, D. H., Sussman, D. J., & Seldin, D. C. (2000). Endogenous protein kinase CK2 participates in Wnt signalling in mammary epithelial cells. The Journal of Biological Chemistry, 275(31): 23790-23797.

Sørensen, P. D. & Frederiksen, S. (1991). Characterization of human 5S rRNA genes. Nucleic Acids Research, 19(15): 4147-4151.

Sparks, A. B., Morin, P. J., Vogelstein, B., & Kinzler, K. W. (1998). Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Research, 58(6): 1130-1134.

Srivastava, S., Zou, Z. Q., Pirollo, K., Blattner, W., & Chang, E. H. (1990). Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature, 348(6303): 747-749.

Stefanovsky, V. Y., Bazett-Jones, D. P., Pelletier, G., & Moss, T. (1996). The DNA supercoiling architecture induced by the transcription factor xUBF requires three of its five HMG-boxes. Nucleic Acids Research, 24(16): 3208-3215.

Stefanovsky, V. Y., Pelletier, G., Hannan, R., Gagnon-Kugler, T., Rothblum, L. I., & Moss, T. (2001). An immediate response of ribosomal transcription to growth factor stimulation in mammals is mediated by ERK phosphorylation of UBF. Molecular Cell, 8(5): 1063-1073.

Stein, T., Crighton, D., Boyle, J. M., Varley, J. M., & White, R. J. (2002a). RNA polymerase III transcription can be derepressed by oncogenes or mutations that compromise p53 function in tumours and Li-Fraumeni syndrome. Oncogene, 21(19): 2961-2970.

Stein, T., Crighton, D., Warnock, L. J., Milner, J., & White, R. J. (2002b). Several regions of p53 are involved in repression of RNA polymerase III transcription. Oncogene, 21(36): 5540-5547.

Stoppa-Lyonnet, D., Laurent-Puig, P., Essioux, L., Pages, S., Ithier, G., Ligot, L., Fourquet, A., Salmon, R. J., Clough, K. B., Pouillart, P., Bonaiti-Pellie, C., & Thomas, G. (1997). BRCA1 sequence variations in 160 individuals referred to a breast/ovarian family cancer clinic. Institut Curie Breast Cancer Group. American Journal of Human Genetics, 60(5): 1021-1030.

Storey, A., Thomas, M., Kalita, A., Harwood, C., Gardiol, D., Mantovani, F., Breuer, J., Leigh, I. M., Matlashewski, G., & Banks, L. (1998). Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. Nature, 393(6682): 229-234.

Stork, P., Loda, M., Bosari, S., Wiley, B., Poppenhusen, K., & Wolfe, H. (1991). Detection of K-ras mutations in pancreatic and hepatic neoplasms by non-isotopic mismatched polymerase chain reaction. Oncogene, 6(5): 857-862.

Stunkel, W., Kober, I., & Seifart, K. H. (1997). A nucleosome positioned in the distal promoter region activates transcription of the human U6 gene. Molecular & Cellular Biology, 17(8): 4397-4405.

Sutcliffe, J. E., Cairns, C. A., McLees, A., Allison, S. J., Tosh, K., & White, R. J. (1999). RNA polymerase III transcription factor IIIB is a target for repression by pocket proteins p107 and p130. Molecular & Cellular Biology, 19(6): 4255-4261.

Sutcliffe, J. E., Brown, T. R., Allison, S. J., Scott, P. H., & White, R. J. (2000). Retinoblastoma protein disrupts interactions required for RNA polymerase III transcription. Molecular & Cellular Biology, 20(24): 9192-9202.

Sutter, T., Doi, S., Carnevale, K. A., Arber, N., & Weinstein, I. B. (1997). Expression of cyclins D1 and E in human colon adenocarcinomas. Journal of Medicine, 28(5-6): 285-309.

Szabo, C. I. & King, M. C. (1997). Population genetics of BRCA1 and BRCA2. American Journal of Human Genetics, 60(5): 1013-1020.

Tavtigian, S. V., Simard, J., Rommens, J., Couch, F., Shattuck-Eidens, D., Neuhausen, S., Merajver, S., Thorlacius, S., Offit, K., Stoppa-Lyonnet, D., Belanger, C., Bell, R., Berry, S., Bogden, R., Chen, Q., Davis, T., Dumont, M., Frye, C., Hattier, T., Jammulapati, S., Janecki, T., Jiang, P., Kehrer, R., Leblanc, J. F., & Goldgar, D. E. (1996). The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds. Nature Genetics, 12(3): 333-337.

Taya, Y. (1997). RB kinases and RB-binding proteins: new points of view. Trends in Biochemical Sciences, 22(1): 14-17.

Teichmann, M., Wang, Z., & Roeder, R. G. (2000). A stable complex of a novel transcription factor IIB- related factor, human TFIIIB50, and associated proteins mediate selective transcription by RNA polymerase III of genes with upstream promoter elements. Proceedings of the National Academy of Sciences of the United States of America, 97(26): 14200-14205.

Tetsu, O. & McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature, 398(6726): 422-426.

Thimmappaya, B., Weinberger, C., Schneider, R. J., & Shenk, T. (1982). Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. Cell, 31(3 Pt 2): 543-551.

Thor, A. D., Moore, D. H., II, Edgerton, S. M., Kawasaki, E. S., Reihsaus, E., Lynch, H. T., Marcus, J. N., Schwartz, L., Chen, L. C., & Mayall, B. H. (1992). Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. Journal of the National Cancer Institute, 84(11): 845-855.

Topper, J. N. & Clayton, D. A. (1990). Characterization of human MRP/Th RNA and its nuclear gene: full length MRP/Th RNA is an active endoribonuclease when assembled as an RNP. Nucleic Acids Research, 18(4): 793-799.

Tremethick, D., Zucker, K., & Worcel, A. (1990). The transcription complex of the 5 S RNA gene, but not transcription factor IIIA alone, prevents nucleosomal repression of transcription. Journal of Biological Chemistry, 265(9): 5014-5023.

Tse, C., Fletcher, T. M., & Hansen, J. C. (1998). Enhanced transcription factor access to arrays of histone H3/H4 tetramer-DNA complexes *in vitro*: Implications for replication and transcription. Proceedings of the National Academy of Sciences of the United States of America, 95: 12169-12173.

Tuan, J. C., Zhai, W., & Comai, L. (1999). Recruitment of TATA-binding protein-TAFI complex SL1 to the human ribosomal DNA promoter is mediated by the carboxy-terminal activation domain of upstream binding factor (UBF) and is regulated by UBF phosphorylation. Molecular & Cellular Biology, 19(4): 2872-2879.

Ullu, E. & Tschudi, C. (1984). Alu sequences are processed 7SL RNA genes. Nature, 312(5990): 171-172.

Ura, K., Kurumizaka, H., Dimitrov, S. I., Almouzni, G., & Wolffe, A. P. (1997). Histone acetylation: influence on transcription, nucleosome mobility and positioning, and linker histone-dependent transcriptional repression. EMBO Journal, 16(8): 2096.

Vallett, S. M., Brudnak, M., Pellegrini, M., & Weber, H. W. (1993). In vivo regulation of rRNA transcription occurs rapidly in nondividing and dividing Drosophila cells in response to a phorbol ester and serum. Molecular & Cellular Biology, 13(2): 928-933.

Van Lint, C., Emiliani, S., & Verdin, E. (1996). The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Expression, 5(4-5): 245-253.

Varley, J. M., Evans, D. G., & Birch, J. M. (1997). Li-Fraumeni syndrome--a molecular and clinical review. British Journal of Cancer, 76(1): 1-14.

Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., & Bos, J. L. (1988). Genetic alterations during colorectal-tumor development. New England Journal of Medicine, 319(9): 525-532.

Vogelstein, B., Lane, D., & Levine, A. J. (2000). Surfing the p53 network. Nature, 408(6810): 307-310.

Vogelstein, B. & Kinzler, K. W. (2002). Colorectal Tumors. In B. Vogelstein & K. W. Kinzler (Eds.), The Genetic Basis of Human Cancer, 2nd ed.: Chpt 34. USA: The McGraw-Hill Companies Inc.

Voit, R., Schnapp, S., Kuhn, A., Rosenbauer, H., Hirschmann, P., Stunnenberg, H. G., & Grummt, I. (1992). The nucleolar transcription factor mUBF is phosphorylated by caesin kinase II in the C-terminal hyperacidic tail which is essentail for transactivation. EMBO Journal, 11(6): 2211-2218.

Voit, R., Kuhn, A., Sander, E. E., & Grummt, I. (1995). Activation of mammalian ribosomal gene transcription requires phosphorylation of the nucleolar transcription factor UBF. Nucleic Acids Research, 23(14): 2593-2599.

Voit, R., Schafer, K., & Grummt, I. (1997). Mechanism of repression of RNA polymerase I transcription by the retinoblastoma protein. Molecular & Cellular Biology, 17(8): 4230-4237.

Voit, R., Hoffmann, M., & Grummt, I. (1999). Phosphorylation by G1-specific cdk-cyclin complexes activates the nucleolar transcription factor UBF. EMBO Journal, 18(7): 1891-1899.

Voit, R. & Grummt, I. (2001). Phosphorylation of UBF at serine 388 is required for interaction with RNA polymerase I and activation of rDNA transcription. Proceedings of the National Academy of Sciences of the United States of America, 96(24): 13631-13636.

Volarević, S., Stewart, M. J., Ledermann, B., Zilberman, F., Terracciano, L., Montini, E., Grompe, M., Kozma, S. C., & Thomas, G. (2000). Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. Science, 288: 2045-2047.

Walter, P. & Blobel, G. (1982). Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. Nature, 299(5885): 691-698.

Waltzer, L. & Bienz, M. (1998). *Drosophila* CBP represses the transcription factor TCF to antagonize Wingless signalling. Nature, 395: 521-525.

Wang, H. D., Yuh, C. H., Dang, C. V., & Johnson, D. L. (1995). The hepatitis B virus X protein increases the cellular level of TATA-binding protein, which mediates transactivation of RNA polymerase III genes. Molecular & Cellular Biology, 15(12): 6720-6728.

Wang, Z. & Roeder, R. G. (1995). Structure and function of a human transcription factor TFIIIB subunit that is evolutionarily conserved and contains both TFIIB- and high-mobility-group protein 2-related domains. Proceedings of the National Academy of Sciences of the United States of America, 92(15): 7026-7030.

Wang, Z. & Roeder, R. G. (1996). TFIIIC1 acts through a downstream region to stabilize TFIIIC2 binding to RNA polymerase III promoters. Molecular & Cellular Biology, 16(12): 6841-6850.

Wang, H. D., Trivedi, A., & Johnson, D. L. (1997). Hepatitis B virus X protein induces RNA polymerase III-dependent gene transcription and increases cellular TATA-binding protein by activating the Ras signaling pathway. Molecular & Cellular Biology, 17(12): 6838-6846.

Wang, Z. & Roeder, R. G. (1997). Three human RNA polymerase III-specific subunits form a subcomplex with a selective function in specific transcription initiation. Genes & Development, 11(10): 1315-1326.

Wang, H. D., Trivedi, A., & Johnson, D. L. (1998). Regulation of RNA polymerase I-dependent promoters by the hepatitis B virus X protein via activated Ras and TATA-binding protein. Molecular & Cellular Biology, 18(12): 7086-7094.

Wang, Z. & Roeder, R. G. (1998). DNA topoisomerase I and PC4 can interact with human TFIIIC to promote both accurate termination and transcription reinitiation by RNA polymerase III. Molecular Cell, 1(5): 749-757.

Wang, C., Fu, M., Mani, S., Wadler, S., Senderowicz, A. M., & Pestell, R. G. (2001). Histone acetylation and the cell-cycle in cancer. Frontiers in Bioscience, 6: D610-629.

Wang, H. L., Wang, J., Xiao, S. Y., Haydon, R., Stoiber, D., He, T. C., Bissonnette, M., & Hart, J. (2002). Elevated protein expression of cyclin D1 and fra-1 but decreased expression of c-myc in human colorectal adenocarcinomas overexpressing beta-catenin. International Journal of Cancer, 101: 301-310.

Watson, P. H., Safneck, J. R., Le, K., Dubik, D., & Shiu, R. P. (1993). Relationship of c-myc amplification to progression of breast cancer from in situ to invasive tumor and lymph node metastasis. Journal of the National Cancer Institute, 85(11): 902-907.

Watson, P. H., Singh, R., & Hole, A. K. (1996). Influence of c-myc on the progression of human breast cancer. Current Topics in Microbiology & Immunology, 213(Pt 2): 267-283.

Weber, H. W., Vallett, S., Neilson, L., Grotke, M., Chao, Y., Brudnak, M., Juan, A. S., & Pellegrini, M. (1991). Serum, insulin and phorbol esters stimulate rRNA and tRNA gene expression in both dividing and nondividing Drosophila cells. Molecular & Cellular Biochemistry, 104(1-2): 201-207.

Weinberg, R. A. (1991). Tumor suppressor genes. Science, 254(5035): 1138-1146.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. Cell, 81(3): 323-330.

Weiner, A. M., Deininger, P. L., & Efstratiadis, A. (1986). Nonviral retroposons: genes, pseudogenes, and transposable elements generated by the reverse flow of genetic information. Annual Review of Biochemistry, 55: 631-661.

Weinmann, R., Raskas, H. J., & Roeder, R. G. (1974). Role of DNA-dependent RNA polymerases II and III in transcription of the adenovirus genome late in productive infection. Proceedings of the National Academy of Sciences of the United States of America, 71(9): 3426-3439.

Weisbrod, S. (1982). Active chromatin. Nature, 297(5864): 289-295.

Welcsh, P. L., Owens, K. N., & King, M. C. (2000). Insights into the functions of BRCA1 and BRCA2. Trends in Genetics, 16(2): 69-74.

Werner, M., Chaussivert, N., Willis, I. M., & Sentenac, A. (1993). Interaction between a complex of RNA polymerase III subunits and the 70-kDa component of transcription factor IIIB. Journal of Biological Chemistry, 268(28): 20721-20724.

Werness, B. A., Levine, A. J., & Howley, P. M. (1990). Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science, 248(4951): 76-79.

White, R. J., Stott, D., & Rigby, P. W. (1990). Regulation of RNA polymerase III transcription in response to Simian virus 40 transformation. EMBO Journal, 9(11): 3713-3721.

White, R. J., Gottlieb, T. M., Downes, C. S., & Jackson, S. P. (1995a). Cell cycle regulation of RNA polymerase III transcription. Molecular & Cellular Biology, 15(12): 6653-6662.

White, R. J., Gottlieb, T. M., Downes, C. S., & Jackson, S. P. (1995b). Mitotic regulation of a TATA-binding-protein-containing complex. Molecular & Cellular Biology, 15(4): 1983-1992.

White, R. J., Trouche, D., Martin, K., Jackson, S. P., & Kouzarides, T. (1996). Repression of RNA polymerase III transcription by the retinoblastoma protein. Nature, 382(6586): 88-90.

White, R. J. (1998). RNA Polymerase III Transcription. (Second Edition ed.). Berlin: Springer-Verlag.

White, R. J. (2001). Gene Transcription, Mechanisms and Control. Oxford: Blackwell Science.

Whyte, P. (1995). The retinoblastoma protein and its relatives. Seminars in Cancer Biology, 6(2): 83-90.

Willert, K., Brink, M., Wodarz, A., Varmus, H., & Nusse, R. (1997). Caesin kinase 2 associates with and phosphorylates Dishevelled. EMBO Journal, 16: 3089-3096.

Willis, I. M. (1993). RNA polymerase III. Genes, factors and transcriptional specificity. Eur J Biochem, 212: 1-11.

Winston, J. T., Coats, S. R., Wang, Y. Z., & Pledger, W. J. (1996). Regulation of the cell cycle machinery by oncogenic ras. Oncogene, 12(1): 127-134.

Winter, A. G., Sourvinos, G., Allison, S. J., Tosh, K., Scott, P. H., Spandidos, D. A., & White, R. J. (2000). RNA polymerase III transcription factor TFIIIC2 is overexpressed in ovarian tumors. Proceedings of the National Academy of Sciences of the United States of America, 97(23): 12619-12624.

Wolffe, A. P. (1990). New approaches to chromatin function. New Biologist, 2(3): 211-218.

Wolffe, A. P. & Pruss, D. (1996). Deviant nucleosomes: the functional specialization of chromatin. Trends in Genetics, 12(2): 58-62.

Wooster, R., Neuhausen, S., Mangion, J., Quirk, Y., Ford, D., Collins, N., Nguyen, K., Seal, S., Tran, T., Averill, D., Fields, P., Marshall, G., Narod, S., Lenoir, G., Lynch, H., Feunteun, J., Devilee, P., Cornelisse, C., Menko, F., Daly, P., Ormiston, W., McManus, R., Pye, C., Lewis, C., Cannon-Albright, L., Peto, J., Ponder, B., Skolnick, M., Easton, D., Golgar, D., & Stratton, M. (1994). Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. Science, 365(5181): 2088-2090.

Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., & Micklem, G. (1995). Identification of the breast cancer susceptibility gene BRCA2. Nature, 378(6559): 789-792.

Yamashita, Y., Shimada, M., Harimoto, N., Rikimaru, T., Shirabe, K., Tanaka, S., & Sugimachi, K. (2003). Histone deacetylase inhibitor trichostatin A induces cell-cycle arrest/apoptosis and hepatocyte differentiation in human hepatoma cells. International Journal of Cancer, 103: 572-576.

Yang, Z., Zhu, Q., Luo, K., & Zhou, Q. (2001). The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. Nature, 414(6861): 317-322.

Ying, C., Gregg, D. W., & Gorski, J. (1996). Estrogen-induced changes in rRNA accumulation and RNA polymerase I activity in the rat pituitary: correlation with pituitary tumor susceptibility. Molecular & Cellular Endocrinology, 118: 207-213.

Yoon, J. B., Murphy, S., Bai, L., Wang, Z., & Roeder, R. G. (1995). Proximal sequence element-binding transcription factor (PTF) is a multisubunit complex required for transcription of both RNA polymerase II- and RNA polymerase III-dependent small nuclear RNA genes. Molecular & Cellular Biology, 15(4): 2019-2027.

Yoshida, M., Kijima, M., Akita, M., & Beppu, T. (1990). Potent and specific inhibition of mammalian histone deactylase both in vivo and in vitro by trichostatin A. Journal of Biological Chemistry, 265(28): 17174-17179.

Yoshida, M., Furumai, R., Nishiyama, M., Komatsu, Y., Nishino, N., & Horinouchi, S. (2001). Histone deacetylase as a new target for cancer chemotherapy. Cancer Chemotherapy Pharmacology, 48(S1): S20-S26.

Yoshida, M., Horinouchi, S., & Beppu, T. (1995). Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. Bioessays, 17(5): 423-430.

Yoshinaga, S., Dean, N., Han, M., & Berk, A. J. (1986). Adenovirus stimulation of transcription by RNA polymerase III: evidence for an E1A-dependent increase in transcription factor IIIC concentration. EMBO Journal, 5(2): 343-354.

Yoshinaga, S. K., Boulanger, P. A., & Berk, A. J. (1987). Resolution of human transcription factor TFIIIC into two functional components. Proceedings of the National Academy of Sciences of the United States of America, 84(11): 3585-3589.

Yoshinaga, S. K., L'Etoile, N. D., & Berk, A. J. (1989). Purification and characterization of transcription factor IIIC2. Journal of Biological Chemistry, 264(18): 10726-10731.

Zhai, W., Tuan, J. A., & Comai, L. (1997). SV40 large T antigen binds to the TBP-TAF(I) complex SL1 and coactivates ribosomal RNA transcription. Genes & Development, 11(1605-17).

Zhai, W. & Comai, L. (1999). A kinase activity associated with simian virus 40 large T antigen phosphorylates upstream binding factor (UBF) and promotes formation of a stable initiation complex between UBF and SL1. Molecular & Cellular Biology, 19(4): 2791-2802.

Zhai, W. & Comai, L. (2000). Repression of RNA polymerase I transcription by the tumor suppressor p53. Molecular & Cellular Biology, 20(16): 5930-5938.

Zhang, L., Zhou, W., Velculescu, V. E., Kern, S. E., Hruban, R. H., Hamilton, S. R., Vogelstein, B., & Kinzler, K. W. (1997). Gene expression profiles in normal and cancer cells. Science, 276(5316): 1268-1272.

Zhang, W. & Bieker, J. J. (1998). Acetylation and modulation of erythroid Kruppel-like factor (EKLF) activity by interaction with histone acetyltransferases. Proceedings of the National Academy of Sciences of the United States of America, 95(17): 9855-9860.

Zhao, J., Yuan, X., Frodin, M., & Grummt, I. (2003). ERK-dependent phosphorylation of the transcription initiation factor TIF-IA is required for RNA polymerase I transcription and cell growth. Molecular Cell, 11(2): 405-413.

Zieve, G., Benecke, B. J., & Penman, S. (1977). Synthesis of two classes of small RNA species in vivo and in vitro. Biochemistry, 16(20): 4520-4525.

Zomerdijk, J. C. & Tjian, R. (1998). Initiation of transcription on human rRNA genes. In M. R. Paule (Ed.), Transcription of Ribosomal RNA Genes by Eukaryotic RNA Polymerase I.: Chpt 10. Berlin: Springer-Verlag.

zur Hausen, H. (1996). Papillomavirus infections--a major cause of human cancers. Biochimica et Biophysica Acta, 1288(2): F55-78.

zur Hausen, H. (2000). Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. Journal of the National Cancer Institute, 92(9): 690-698.