A thesis entitled

Regulation of RNA polymerase III transcription during differentiation

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Dimitris Athineos

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University of Glasgow

Abstract

The differentiation of F9 embryonal carcinoma (EC) cells into parietal endoderm (PE)-like cells provides a tractable model for studying molecular events during early and inaccessible stages of murine development. It has been previously shown that PE formation is accompanied by a ~10-fold decrease in RNA polymerase (pol) III transcription. This down-regulation was attributed to a reduction in the activity of the basal pol III factor TFIIIB; however, the mechanism involved was not established. The work presented here was aimed at elucidating the precise changes that lead to a decrease in pol III transcriptional activity during F9 differentiation.

The protein levels of all three components of the TFIIIB complex, Brf1, Bdp1 and TBP, were found to decrease. In the case of Brf1 and Bdp1, this decrease did not reflect a change in their stability, as proteasome inhibition did not have an effect on protein abundance. On the other hand, a specific down-regulation of the mRNAs encoding Brf1 and Bdp1 was observed. Temporal analysis of F9 differentiation, however, revealed that the decline in TFIIIB levels follows that in pol III transcription in the same extracts. Furthermore, over-expression of Brf1 did not result in an increase in pol III transcription in PE cells. These data point to TFIIIB playing a secondary role in the down-regulation of pol III during differentiation.

TFIIIB activity, and consequently pol III transcription, can be regulated by a number of proteins. For example, c-Myc activates pol III transcription, while the retinoblastoma protein (pRb) is a repressor. Time course analysis revealed that c-Myc down-regulation and pRb up-regulation closely follows the decline in pol III transcription during differentiation. Furthermore, recombinant c-Myc efficiently restored transcription in PE extracts. These two proteins, therefore, provide likely

candidates for the primary regulatory mechanism of pol III transcription in response to differentiation, through their effect on TFIIIB.

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Abbreviations

ATP	Adenosine triphosphate
bp	Base pairs
Bdp1	B double-prime 1
Brf1, Brf2	TFIIB-related factor 1, 2
BSA	Bovine serum albumin
Cdk	Cyclin-dependent kinase
CK2	Casein kinase 2
CMV	Cytomegalovirus
CTP	Cytosine triphosphate
db-cAMP	Dibutyryl cyclic AMP
DEPC-H_2O	Diethylpyrocarbonate water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DSE	Distal sequence element
DTT	Dithiothreitol
EC	Embryonal carcinoma
EDTA	Ethylene diamine tetra-acetic acid
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated protein kinase
FBS	Foetal bovine serum
GTP	Guanine triphosphate
h	Human
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HECT	Homologous to E6-AP C-terminus

Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
HPRT	Hypoxanthine guanine phosphoribosyl transferase
IBMX	3-Isobutyl-1-methylxanthine
ICM	Inner cell mass
ICR	Internal control region
IVT	*In vitro* transcription
kD	Kilodalton
MAPK	Mitogen activated protein kinase
MEK	MAP/ERK kinase
mRNA	Messenger RNA
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PE	Parietal endoderm
PIC	Preinitiation complex
PMSF	Phenylmethylsulfonyl fluoride
Pol	RNA Polymerase
PSE	Proximal sequence element
PTF	Proximal sequence element binding transcription factor
PYS	Parietal yolk sac
RING	Really interesting new gene
rNTPs	Ribonucleotidetriphosphates
RA	Retinoic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SINEs	Short interspersed elements
SNAPc	snRNA activating protein complex
snRNA	Small nuclear RNA
SV40	Simian virus 40
TAF	TBP-associated factor
TBP	TATA-binding protein
TBS	Tris buffered saline
TEMED	N, N, N', N'-tetramethylethylenediamine
TF	Transcription factor
Tris	Tris (hydroxymethyl) methylamine
tRNA	Transfer RNA
ts	Temperature sensitive
TTP	Thymidine triphosphate
Tween 20	Polyoxyethylene sorbitan monolaurate
U	Units
UV	Ultraviolet light
UTP	Uridine triphosphate
V	Volts
VE	Visceral endoderm
y	Yeast

Declaration

All work presented within this thesis was performed entirely by myself and in no way forms part of any other thesis. The work was carried out during my time as a post-graduate student at the Division of Biochemistry and Molecular Biology, Faculty of Biomedical and Life Sciences, University of Glasgow, UK, from January 2002, while under the supervision of Professor Robert J. White.

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Dedicated to my mother

Αφιερωμενο στη μητερα μου

CHAPTER 1

INTRODUCTION

1.1 RNA POLYMERASE III

1.1.1 Eukaryotic RNA polymerases

Most eukaryotic cells contain four DNA-dependent RNA polymerases. Three of these are nuclear, while mitochondria contain their own unique RNA polymerase (Sentenac, 1985). Plants are an exception, with an extra nuclear RNA polymerase, IV, (Onodera *et al.*, 2005), in addition to the one found in chloroplasts. The nuclear RNA polymerases were originally identified through chromatography on DEAE Sephadex columns (Roeder and Rutter, 1969). They are classified according to various properties, including salt requirements, template preferences and differential sensitivity to α-amanitin, a toxin isolated from the poisonous *Amanita* mushrooms (Kedinger *et al.*, 1970; Sentenac, 1985). In mammals, RNA polymerase II (pol II) shows the highest sensitivity to the toxin, with a 50% reduction in its activity at only 25 ng/ml. RNA polymerase III (pol III) has an intermediate sensitivity (50% of activity at 20 μg/ml), while RNA polymerase I (pol I) is completely resistant (Schwartz *et al.*, 1974). The situation in *Saccharomyces cerevisiae* is different, with pol III being highly resistant, while pol I exhibits intermediate sensitivity. As in mammals, yeast pol II is very sensitive to α-amanitin (Schultz and Hall, 1976). Pols I and II are also inhibited by actinomycin D, with pol I being the more sensitive of the two enzymes, while tagetitoxin (a bacterial phytotoxin produced by *Pseudomonas*

syringae) inhibits pol III preferentially (Steinberg et al., 1990). More than any other property of the RNA polymerases, the sensitivity to these toxins has been used to determine which genes are transcribed by which polymerase. Accordingly, pol I-transcribed genes are termed class I, pol II-transcribed genes class II and pol III-transcribed genes class III.

The best characterised nuclear RNA polymerases are from *S. cerevisiae*, where the genes for all subunits have been cloned. Individual subunits are named after the enzyme they belong to (i.e. A for pol I, B for pol II and C for pol III) and their apparent size (in kDa). Pol III, with 17 subunits, appears to be the most complex of the nuclear RNA polymerases, compared to the 14 and 12 subunits of pols I and II, respectively. Of these, 5 subunits are shared between the three polymerases (ABC27, ABC23, ABC14.5, ABC10α and ABC10β), while two are shared with pol I (AC40 and AC19). The two largest subunits of pol III (C160 and C128) are homologous to the equivalent subunits of pols I and II, while C25 and C11 are homologous to two pol I subunits. The remaining six (C82, C53, C37, C34, C31 and C17) are unique to pol III. The functional core of pol III is made up of five subunits, C160, C128, AC40, AC19 and ABC23. Not surprisingly, these subunits are either shared between the polymerases, or are homologous, both to each other and to subunits from the solitary archaeal or bacterial RNA polymerase (Geiduschek and Kassavetis, 2001). The pol III-specific subunits appear to be important for recognition of the TFIIIC – TFIIIB – DNA preinitiation complex (see below). Recently, human pol III was purified, and its subunits identified by mass spectrometry (Hu *et al.*, 2002). Analysis revealed that the human enzyme contains orthologs of all the yeast pol III subunits.

Despite the shared and homologous subunits, pols I, II and III differ both in localisation and target genes. Pol I is responsible for the transcription of a single gene, whose product, the 45S pre-ribosomal RNA, is later processed into the 5.8S,

18S and 28S ribosomal RNA (rRNA) molecules. Pol I has, therefore, only one promoter structure to recognise. Nevertheless, pol I is believed to account for as much as 70% of the total nuclear transcription (in HeLa cells), occurring at distinct areas within the nucleus, called nucleoli. Specifically, the rRNA is synthesised in the fibrillar centres, while ribosome assembly takes place in the surrounding granular area (Shaw and Olsen, 1984).

Pol II is at the other extreme, being responsible for the transcription of all protein-coding genes, as well as most small nuclear RNA (snRNA) genes. In order for the cell to efficiently control the transcription of all these genes, pol II promoters are suitably diverse, both in their regulatory and core regions. Despite the large number of genes, pol II transcription accounts for 20% of HeLa total nuclear transcription. Actively transcribing pol II seems to be spatially separated at particular locations within the nucleoplasm, of which around 8,000 may exist in HeLa cells (Pombo *et al.*, 1999).

Pol III transcribes a much smaller set of genes than pol II. Its products are small, untranslated RNAs, including the 5S rRNA, transfer RNA (tRNA) and U6 snRNA (see below). Transcription of this set of genes by pol III provides the remaining 10% of nuclear transcription in HeLa cells. Consistent with the smaller set of target genes, promoters of class III genes do not exhibit the variety seen in class II genes, being divided into three main types (see below). As is the case for pol II, active pol III is concentrated at specific sites, which are separate from the pol II sites. Around 2,000 of these sites are thought to exist in the nucleus of a HeLa cell, each one containing approximately five active polymerases (Pombo *et al.*, 1999).

1.1.2 Class III genes

The genes transcribed by RNA polymerase III have the common characteristic of encoding small, untranslated RNAs that serve various essential functions in cells. For example, 5S rRNA and tRNA are required for protein synthesis, while U6 snRNA and H1 and MRP RNAs are involved in post-transcriptional processing. Interestingly, the adenoviral VA genes, thought to be important for diverting the translational machinery of an infected cell for the virus' own needs, are also transcribed by pol III. The majority of pol III templates in mammals, however, belong to the short interspersed repeat (SINE) family of genes, the function of which is not known.

- 5S rRNA

The 5S rRNA is the smallest of the ribosomal RNAs, at around 120 nucleotides, and the only one transcribed by pol III. After its synthesis, it becomes associated with the large subunit of the ribosome, although its exact role is still unknown.

5S rRNA genes are often, but not always, situated in repeated clusters. For example, in *S. cerevisiae*, the genes for each of the four rRNAs are located in a shared repeat, even though 5S rRNA is synthesised by a different polymerase than the other three (Nath and Bollon, 1977). It is worth noting that the expression of the rRNA genes in this repeat is not interdependent, as pol III inhibition does not affect pol I transcription (Gudenus *et al.*, 1988). In humans, there are about 200 to 300 copies of the 5S gene, many of which are located in clusters of tandem repeats, although some single copies could also exist (Sorensen and Frederiksen, 1991).

- tRNA

tRNA molecules are 70 – 90 nucleotides in length. Their primary function is to serve as adaptors between messenger RNA (mRNA) and amino acids, thus converting the genetic information encoded in the mRNA sequence into an amino acid sequence. This is made possible by the fact that each tRNA is linked to a particular amino acid, matching it to a specific codon in the message. Therefore, a tRNA that recognises the codon for leucine, and brings leucine to the growing polypeptide chain is termed $tRNA^{Leu}$, etc. Eukaryotic cells contain 50 – 100 distinct tRNA species (Sharp *et al.*, 1985), although relative amounts of different tRNAs vary considerably from one cell type to another, depending on codon usage (Garel, 1976). There are approximately 350 tRNA genes in *S. cerevisiae*, most of which are dispersed throughout the genome without clustering (Guthrie and Abelson, 1982). In contrast, irregular clustering seems to be the rule in higher eukaryotes. For example, a 46 kb region in chromosome 2 in *Drosophila melanogaster* contains 18 tRNA genes (Dingermann *et al.*, 1982). In humans, around 500 tRNA genes encode 60 – 90 tRNA isoacceptors (Hatlen and Attardi, 1971; IHGSC, 2001). Some of these genes are scattered individually throughout the genome, e.g. $tRNA^{Met}$ (Santos and Zasloff, 1981). Although some small scale clustering has been previously reported, analysis of the draft human genome sequence revealed considerable clustering on a genome-wide scale. Thus, more than 25% of all tRNA genes (around 140) are found in a 4 Mb region (~ 0.1% of the genome) of chromosome 6, with smaller clusters found on other chromosomes. In fact, more than half of the identified tRNA genes (280 out of 497) are located on either chromosome 1 or chromosome 6 (IHGSC, 2001).

- U6 snRNA

Part of the post-transcriptional processing of pre-mRNA to mature mRNA, involving the removal of introns, is carried out by the spliceosome (reviewed in Kramer, 1996). The spliceosomes, which are present in the nuclei of all eukaryotic cells at high numbers (10^6/cell), are RNA-protein complexes, part of a group of structurally related snRNPs (small nuclear ribonucleoproteins). In addition to the protein components, spliceosomes contain five snRNA species. Four of these are synthesised by pol II, while the fifth, U6, is synthesised by pol III (Kunkel *et al.*, 1986). Trypanosomes are an exception, since U2, in addition to U6, is made by pol III (Fantoni *et al.*, 1994). U6 is the smallest of the five snRNAs in the spliceosome, 106 nucleotides in length, but it is also the most conserved (Brow and Guthrie, 1988). In *S. cerevisiae*, U6 snRNA is encoded by an essential, single-copy gene (Brow and Guthrie, 1988). In humans, however, there appear to be more than 40 copies of the gene, dispersed throughout the genome, with many more pseudogenes and related sequences (IHGSC, 2001).

- 7SL RNA

7SL is a 300 nucleotide-long, pol III-transcribed RNA that forms the scaffold of the signal recognition particle. This RNP plays an important role in the co-translational insertion of nascent polypeptides into the endoplasmic reticulum (Walter and Blobel, 1982). There are three 7SL genes in humans, with more than 700 pseudogenes (IHGSC, 2001).

- 7SK RNA

7SK is an abundant RNA, 330 nucleotides in length, transcribed from a single copy gene in humans (Murphy et al., 1986). Despite its ubiquitous nature and high degree of conservation (Ullu *et al.*, 1982), the role of 7SK has remained elusive. Murphy *et al.* (1986) showed that it can interact with eight polypeptides, forming a 12S RNP. Recently, two reports demonstrated that 7SK can act as a negative regulator of pol II elongation, by inhibiting P-TEFb (Nguyen *et al.*, 2001; Yang *et al.*, 2001). P-TEFb is a heterodimer of Cdk9 and cyclins T1 or T2, which phosphorylates pol II at its C-terminal domain, leading to the formation of more processive elongation complexes (Price, 2000). 7SK seems to associate with P-TEFb, blocking its kinase activity.

- H1 and MRP RNAs

H1 is the 369 nucleotide-long RNA component of RNase P, an endoribonuclease that is involved in the post-transcriptional processing of the 5' terminus of tRNA (Bartkiewicz *et al.*, 1989; Lee and Engelke, 1989). H1 RNA has several blocks of sequence homology with MRP RNA (Gold *et al.*, 1989). MRP RNA is a component of the RNase MRP, another endoribonuclease involved in pre-rRNA processing (Morrissey and Tollervey, 1995; Schmitt and Clayton, 1993). Both H1 and MRP RNAs are transcribed from single-copy genes in humans (Baer *et al.*, 1990; Chang and Clayton, 1989).

- Products of unknown function (vault, Y, BC1 and BC200 RNAs)

Vaults are the largest known cytoplasmic RNPs, with a mass three times that of ribosomes (~ 13 MDa), but of unknown function (reviewed in Kickhoefer *et al.*, 1996; Scheffer *et al.*, 2000). Although proteins contribute to most of a vault's mass, it contains multiple copies of vault RNAs (vRNAs). vRNAs from different species vary in length (86 – 142 nucleotides), as well as gene copy number. For example, there is a single gene in rats (Kickhoefer *et al.*, 1993), while the human genome contains three distinct genes, encoding different, but very similar, vault RNAs (van Zon *et al.*, 2001).

BC1 and BC200 expression is very restricted, being confined to a specific subset of neurons in the central and peripheral nervous systems of rodents (for BC1) and primates (for BC200) (Tiedge *et al.*, 1993; Tiedge *et al.*, 1991). Even there, they can only be found in the somatic and dendritic domains of the neurons, as part of RNPs. A proposed role for these RNPs is in the transport and/or translation of dendritic mRNAs, although no conclusive evidence has been discovered yet (Tiedge *et al.*, 1993; Tiedge *et al.*, 1991). Despite the impressive similarity in expression patterns, suggesting a similar function, there is little sequence homology between BC1 and BC200 RNAs (Martignetti and Brosius, 1993). In fact, BC1 is thought to have evolved from a $tRNA^{Ala}$, while BC200 from an Alu or 7SL RNA (DeChiara and Brosius, 1987; Martignetti and Brosius, 1993).

Y RNAs are 69 to 112 nucleotides in length and are found in complexes with the Ro autoantigen (Wolin and Steitz, 1983). Although the function of these RNPs is not known, it has been suggested that they might play a role in the 5S rRNA quality control pathway (O'Brien and Wolin, 1994).

- SINEs

The most quantitatively important class of pol III-transcribed genes in higher organisms is the SINEs (short interspersed elements) (reviewed in Jelinek and Schmid, 1982). For example, *Xenopus laevis* contains 20,000 copies of the satellite 1 repetitive element, comprising approximately 1% of its total haploid genome (Lam and Carroll, 1983). The major SINE in primates is the Alu family (reviewed in Mighell *et al.*, 1997). In the haploid human genome, there are 500,000 to 1 million copies of Alu genes, representing 6 – 13% of total. The Alu consensus sequence is ~280 base pairs long, and consists of two similar, but distinct monomers, only the first of which contains a functional pol III promoter. Rodents have their own abundant SINE families. Rat genomes contain large numbers of the ID element (~ 135,000), while the most common ones found in mice belong to the B1 and B2 families (100,000 and 80,000 respectively) (Bennett *et al.*, 1984; Sapienza and St-Jacques, 1986).

The principal SINE families appear to have evolved from class III genes of known physiological role. Several SINES, e.g. B2 and ID, are thought to have arisen from tRNA genes (Daniels and Deininger, 1985). In contrast, the B1 and Alu families show sequence homology with the 7SL RNA (Ullu and Tschudi, 1984). Despite this fact, a clearly defined functional role has not been demonstrated for any SINE family (Howard and Sakamoto, 1990). Proposed functions include roles in regulating the expression of adjacent genes, splicing, translation, DNA replication, cell stress responses, regulation of growth and the turnover of specific mRNAs (White, 2002). Because SINEs are propagated by retrotransposition, in which the transcript is reverse transcribed into DNA and then inserted into a new genomic site, they can have significant impact on the structure and evolution of the genome, even though

the transcripts themselves might be bereft of function. The most obvious effect is the disruption of sequences at the site of integration, potentially leading to mutagenesis. On a larger scale, the presence of so many homologous sequences in the genome could lead to recombination events.

- Viral genes

Certain viruses contain genes that are transcribed by pol III. The best characterised example is the adenoviral genes VA1 and VA2. These ~ 160 bp genes are transcribed at high levels during the late stages of viral infection (Soderlund *et al.*, 1976; Weinmann *et al.*, 1974), where they have been shown to stimulate the translation of viral mRNAs (Thimmappaya *et al.*, 1982).

Another example is the Epstein-Barr virus (EBV). Like adenovirus, EBV contains two small genes, approximately 165 base pairs in length, called EBER1 and EBER2, that are transcribed by pol III (Rosa *et al.*, 1981). Although the exact mechanism is not yet clear, EBER RNAs have been shown to have transforming potential under certain circumstances (White, 2004b). This is the first example of an oncogenic RNA and provides evidence for the possibility that a pol III product can transform cells.

1.1.3 Promoter structure of class III genes

Depending on their organisation, class III gene promoters have been divided into three main categories, termed type 1, type 2 and type 3 (Figure 1.1). Type 1 and 2 promoters, present in most class III genes, have the unusual characteristic of being intragenic, i.e. within the transcribed region of the gene. These discontinuous

structures, called internal control regions (ICRs), consist of essential sequence blocks separated by nonessential nucleotides. This feature sets them apart from the third promoter type. Type 3 promoters are devoid of intragenic elements, relying instead on sequences in the 5' flanking region, upstream of the transcription start site. In this respect, type 3 promoters resemble promoters of pol I- and pol II-transcribed genes.

1.1.3.1 Type 1 promoters

The 5S rRNA gene is the only example of a type 1 pol III promoter. The best characterised 5S promoter is that of *Xenopus* (Figure 1.1). Extensive studies have defined the minimum ICR between base pairs 50 and 97 downstream of the transcription start site (+50 to +97). This region consists of three separate elements: the A-block (+50 to +64), the intermediate element (IE, +67 to +72) and the C-block (+80 to +97) (Bogenhagen, 1985; Pieler *et al.*, 1985a; Pieler *et al.*, 1985b). The importance of these sequences is demonstrated by the fact that mutations in the A- or C-blocks of a major 5S gene in *Xenopus* oocytes abolish transcription (Keller *et al.*, 1990). Although the sequence of the bases between these elements is not important for transcription, the spacing is. For example, it has been shown that alterations in the spacing between the A-block and the intermediate element, from -3 to +10 base pairs, results in reduced transcription and inability to form a stable transcription complex (Pieler *et al.*, 1987). Studies have demonstrated that the region between the start site and the A-block is also important for expression of *Xenopus* 5S genes, especially in conditions suboptimal for transcription (Keller *et al.*, 1990). In optimal conditions, however, the minimal ICR (+50 to +97) is sufficient for accurate transcription (Pieler *et al.*, 1987).

The situation is slightly different in *S. cerevisiae*. *In vitro* transcription of the 5S rRNA gene requires only two promoter elements: the C-block, located at +81 to +94, as well as a region from -14 to +8, called sse (Challice and Segall, 1989). Surprisingly, the region from +9 to +80, which includes an A-block homology, is not absolutely required *in vitro*, although it contributes to the efficiency of transcription. *In vivo*, however, point mutations in the A-block, IE or C-block substantially reduce transcription (Lee *et al.*, 1995). The distance between the sse and C-block is also very important, with changes of more than a few base pairs resulting in a large reduction in 5S expression (Challice and Segall, 1989).

1.1.3.2 Type 2 promoters

This type of promoter is utilised by the majority of class III genes, including tRNA and 7SL, the viral VA and EBER genes, as well as SINE families, like Alu, B1 and B2. It consists of two essential and highly conserved regions of about 10 base pairs each, called A- and B-blocks, generally separated by 30 – 40 base pairs (Figure 1.1). It is worth noting that, in *Xenopus*, A-blocks from type 1 and type 2 promoters are related in sequence and interchangeable. This, however, is not the case in *Neurospora* (Ciliberto *et al.*, 1983; Shi and Tyler, 1991). These two regions have been shown to be essential promoter elements, while other internal and flanking sequences can have modulatory effects on transcription. Analysis of tRNA genes from various organisms, including *Xenopus*, *Drosophila*, nematode and yeast, revealed two highly conserved regions, corresponding to the A- and B-blocks, with consensus sequences TGGCNNAGTGG and GGTTCGANNCC, respectively (Galli *et al.*, 1981). The conservation of these sequences is such that they can also be found

in certain bacterial and chloroplast tRNA genes (Folk *et al.*, 1982; Gruissem *et al.*, 1982). Furthermore, chimeric genes with an A-block from one tRNA gene and a B-block from another have been shown to be active, demonstrating the functional compatibility of A- and B-blocks from different genes (Galli *et al.*, 1981). Part of the reason for this remarkable conservation might stem from the fact that the A- and B-blocks encode the D- and T-loops, respectively, which are required for tRNA function. Several studies have demonstrated the importance of these two elements in tRNA transcription. For example, point mutations in these sequences can have a substantial effect on transcription efficiency (Newman *et al.*, 1983; Reyes *et al.*, 1986; Traboni *et al.*, 1984).

As mentioned before, the flanking regions of type 2 promoters, although not necessary for transcription *in vitro*, may play a regulatory role *in vivo*. Indeed, the very limited homology observed in the flanking sequences between even related tRNA genes makes them good candidates for selective transcriptional control, which is necessary to the adaptation of tRNA genes to different codon and amino acid utilisation in different cell types. This is observed, for example, in members of the $tRNA^{Tyr}$ gene family in *X. laevis* (Gouilloud and Clarkson, 1986). 5' flanking sequences are likely to have some impact upon the transcription of most, if not all, tRNA genes, with numerous examples demonstrated in yeast, *Drosophila*, *Xenopus* and humans. Similarly, 3' flanking sequences have been shown to influence transcription efficiency (White, 2002).

The location of the A-block in type 2 promoters differs from that seen in type 1 promoters, the former being much closer to the start site. In *Xenopus* tRNA genes, for example, it is located between +10 and +20 bp, whereas the 5S A-block is situated at +50 bp (Galli *et al.*, 1981). The site at which initiation can occur seems to be primarily determined by the A-block, although the exact position is determined by

the local sequence, influenced by the upstream flanking region (White, 2002). In contrast to the A-block, the position of the B-block is quite variable, in part due to the presence of short introns in some tRNA genes. In yeast, for example, where 10% of tRNA genes contain introns, the separation between A- and B-blocks can be 27 to 93 base pairs (Geiduschek and Tocchini-Valentini, 1988). Generally, distances of 30 to 60 base pairs appear to be optimal for transcription, although separation up to 400 base pairs can be tolerated (Baker *et al.*, 1987; Dingermann *et al.*, 1983). Remarkably, both elements are recognised and bound by a single protein complex, TFIIIC (see below).

1.1.3.3 Type 3 promoters

The type 3 promoter was originally identified in mammalian U6 snRNA genes (Das et al., 1988; Kunkel and Pederson, 1988) and in the human 7SK gene (Murphy et al., 1986). They have also been found in human H1 and MRP RNA genes (Baer *et al.*, 1990; Topper and Clayton, 1990). This type of promoter is strikingly different from types 1 and 2, in that the essential elements are upstream of the transcription start site, within the 5' flanking region (Figure 1.1). In mice and humans, for example, this region is necessary and sufficient for expression of U6 snRNA (Das *et al.*, 1988). The core elements of type 3 promoters consist of a proximal sequence element (PSE), located between -70 and -50 bp and a TATA box, between -30 and -25 bp (Das *et al.*, 1988). The PSE found in U6 promoters is almost identical to the one from U2 snRNA genes, which are transcribed by pol II, and the two are interchangeable (Lobo and Hernandez, 1989). In fact, the PSE on its own constitutes the core promoter for pol II-transcribed U snRNAs. Point mutations in the TATA

box can reduce or abolish transcription by pol III (Kunkel and Pederson, 1989). Curiously, class II U snRNAs do not contain TATA boxes, and its deletion from the U6 promoter will switch transcription from pol III to pol II (Lobo and Hernandez, 1989). Also critical in U6 expression is the distance between PSE and the TATA box, with expression becoming severely compromised if the separation is altered (Goomer and Kunkel, 1992).

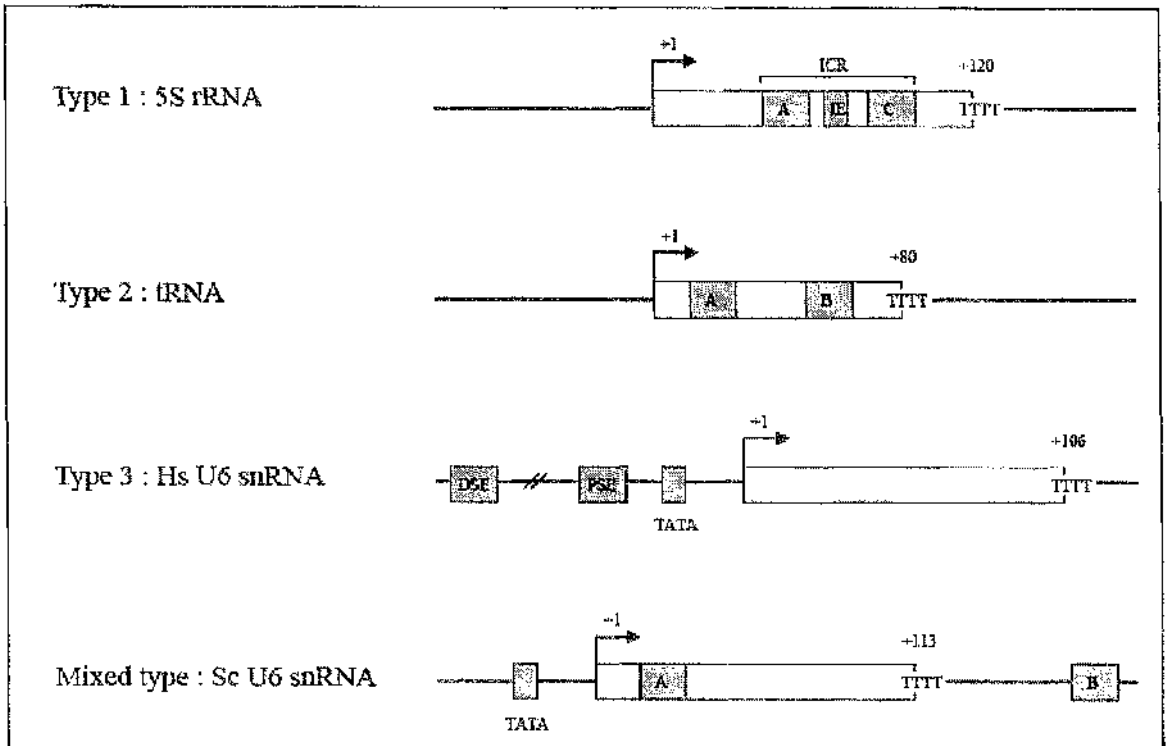


Figure 1.1 Different types of RNA polymerase III promoters

The type 1 promoter of the *X. laevis* 5S rRNA gene consists of an internal control region (ICR), which can be subdivided into the A-block (+50 to +64), the intermediate element (IE, +67 to +72) and the C-block (+80 to +97). Type 2 promoters, represented here by the *X. laevis* $tRNA^{Leu}$ promoter, consist of an A-block (+8 to +19 in this case) and a B-block (+52 to +62). A typical type 3 promoter is that found in the human U6 snRNA gene, with a distal sequence element (DSE) far upstream of the start site (-215 to -240) that enhances transcription and a core promoter composed of the proximal sequence element (PSE, -65 to -48) and a TATA box (-32 to -25). Finally, some promoters are a combination of features from the three main promoter types. The example presented here is the *S. cerevisiae* U6 snRNA, which contains a TATA box at a position similar to the human gene (-30 to -23), an A-block (+21 to +31) and a B-block outside the transcribed region (+234 to +244). The start site is represented by +1 and the termination site by TTTT.

A sequence located about 150 base pairs upstream of the PSE, called the distal sequence element (DSE), has been shown to enhance expression *in vivo*. This element can also be found in U1 and U2 snRNA genes (Kunkel and Pederson, 1988). The positioning of the transcription start site is determined by both the PSE and the TATA box, although neither plays a dominant role. The precise sequence, however, around the start site can also influence the position and level of transcription (Goomer and Kunkel, 1992).

1.1.3.4 Mixed promoters

Some pol III promoters contain a combination of the typical promoter elements described before, and, as such, cannot be readily classified. Figure 1.1 shows one example, namely the *S. cerevisiae* U6 snRNA promoter. It consists of three elements, one upstream and two downstream of the start site, all of which are required for efficient transcription *in vivo* (Brow and Guthrie, 1990). The TATA box and A-blocks are located at similar positions as the ones in the human U6 and tRNA promoters, respectively, while the B-block is unusually located approximately 120 base pairs downstream of the coding region.

Another gene that shows mixed characteristics in its promoter is the human 7SL gene. Although it possesses A- and B-blocks, their sequence is fairly degenerate (Ullu and Weiner, 1985). Significant expression requires both these internal, as well as external, sequences, which include an ATF-binding site and a putative TATA box (Bredow *et al.*, 1990; Howe and Shu, 1993). The upstream sequences of the human 7SL gene can be efficiently substituted by those of the 7SK gene, which has a type 3

promoter structure (Kleinert *et al.*, 1988). Yeast 7SL genes, on the other hand, conform to the typical type 2 promoter structure (Willis, 1993).

Similarly, the EBV EBER2, silkworm $tRNA^{Ala}$, *Xenopus* $tRNA^{Sec}$ and rat vault RNA genes, while containing A- and B-blocks that are homologous to those from type 2 promoters, also possess upstream sequence elements found in type 3 promoters (White, 2002).

1.1.4 Transcription factors required for pol III transcription

Pol III by itself shows no specificity towards particular DNA sequences, and will initiate transcription randomly (Cozzarelli *et al.*, 1983; Weil *et al.*, 1979). It requires the assistance of transcription factors, which recognise the appropriate subset of promoters and recruit the polymerase to the start site of the correct genes (Parker and Roeder, 1977).

Chromatographic separation of HeLa cell extracts over phosphocellulose revealed that two fractions, called PC-B (0.1 – 0.35 M KCl) and PC-C (0.35 0.6M KCl) were required for specific transcription of tRNA and VA genes (Segall *et al.*, 1980). The general factors included in these fractions were designated TFIIIB and TFIIIC, respectively. It was subsequently demonstrated that many other class III genes, e.g. vault RNA, B1 and B2, required these two fractions in combination, while either fraction alone did not support transcription (Carey *et al.*, 1986a; Kickhoefer *et al.*, 1993; Singh *et al.*, 1985). Transcription of 5S was shown to require, in addition to PC-B and PC-C, a third phosphocellulose fraction, PC-A (Engelke *et al.*, 1980; Segall *et al.*, 1980). This gene specific-factor was called TFIIIA.

Since these initial experiments, it has been made clear that the crude phosphocellulose fractions contain many factors that are involved in pol III transcription. The terms TFIIIA, TFIIIB and TFIIIC will from now on be used to indicate the protein complexes that constitute the basal transcription factors for class III genes. Additional factors that regulate this basal transcription machinery will be described in a later chapter.

1.1.4.1 TFIIIB

Although TFIIIB was purified from yeast in 1986 (Klekamp and Weil, 1986), it was not until a few years later that its importance in pol III transcription became truly apparent. Kassavetis *et al.* (1990) demonstrated that, once recruited to the promoter of a tRNA or 5S gene, TFIIIB would remain there, even under conditions that stripped away the initial recruiting factors or prohibited the formation of new complexes. Furthermore, TFIIIB could then support several rounds of accurately initiated transcription. This led to the suggestion that, at least in yeast, TFIIIB was sufficient to recruit pol III to the appropriate gene and direct several rounds of transcription, and that the function of the other factors, TFIIIC and/or TFIIIA, were to recruit TFIIIB to the promoter (Kassavetis *et al.*, 1990). The TFIIIB complex is composed of three polypeptides: TBP, Brf1/Brf2 and Bdp1.

- TBP

It was initially believed that the TATA-binding protein (TBP) was only involved in the transcription of class II genes with TATA boxes in their promoters. The suspicion that this might not be the case came from the observation that the class III gene encoding U6 snRNA contained a TATA box, while the other U snRNAs, transcribed by pol II, did not. It was then shown that, not only was this TATA box responsible for selecting pol III over pol II in yeast and human U6 snRNA genes (Lobo and Hernandez, 1989; Mattaj *et al.*, 1988), but also that transcription from the U6 promoter required TBP (Lobo *et al.*, 1991; Margottin *et al.*, 1991; Simmen *et al.*, 1991). TBP is also required by TATA-less class III genes. This was demonstrated by *in vitro* competition experiments, where TATA box sequences from class II promoters specifically competed for a human factor required by various TATA-less class III genes, including 5S, tRNA and VA (White *et al.*, 1992a; White *et al.*, 1992b). Furthermore, recombinant TBP restored the transcription of TATA-less class III genes in systems where TBP was depleted by fractionation or inactivated by heat treatment (White *et al.*, 1992a; White *et al.*, 1992b). It is now known that TBP is utilised by all three polymerases (Hernandez, 1993; Rigby, 1993). Its universal role has been demonstrated in yeast, where mutations in TBP affect transcription by all three polymerases (Cormack and Struhl, 1992; Schultz *et al.*, 1992).

TBP genes have been cloned from a wide variety of organisms, including humans, mice, frogs, flies, plants and yeast (Hernandez, 1993). Most organisms have only one TBP gene, although the genome of some plant species, like *Arabidopsis*, contain two genes that encode highly similar proteins (Gasch *et al.*, 1990). All TBP genes encode a small polypeptide, 27 – 38 kDa in size. It consists of an N-terminal region that is

variable in both size and sequence, and a very highly conserved C-terminus (Hernandez, 1993). The function of the N-terminal region is still being debated, with conflicting reports concerning its significance. It has been suggested that it can modulate the activity of the conserved C-terminal domain (Kuddus and Schmidt, 1993; Lescure *et al.*, 1994; Mittal and Hernandez, 1997). The C-terminal region contains two direct imperfect repeats (~ 40% identity) of 66 – 67 residues, flanking a short, basic region. Binding of TBP to a TATA box occurs through contact of these repeats with the minor groove of the DNA (Starr and Hawley, 1991; Yamamoto *et al.*, 1992), inducing substantial bending of the DNA (Horikoshi *et al.*, 1992). X-ray crystallographic analysis of one of the *Arabidopsis* TBP polypeptides revealed that the C-terminal domain folds into a highly symmetrical structure, resembling a saddle (Nikolov *et al.*, 1992). The concave underside, lined by β-sheets, is wide enough to accommodate DNA, whereas the convex topside, made up of α-helices, is accessible for interactions with other factors (Nikolov *et al.*, 1992).

Proteins that form complexes with TBP are called TBP-associated factors (TAFs). Fractionation of HeLa extracts has revealed that several different TBP – TAF complexes exist in cells, each one serving a different polymerase. In the case of pol I, the TBP-containing complex, SL-1 (also called TIF-IB), contains three TAFs (Comai *et al.*, 1992), while TFIID, supporting transcription by pol II, contains around 10 TAFs (Pugh and Tjian, 1992). As mentioned before, the pol III-specific factor, TFIIIB, is composed of TBP and two TAFs, Brf1 (or Brf2) and Bdp1.

- Characterisation of *S. cerevisiae* TFIIIB

S. cerevisiae TFIIIB can be separated, by cation exchange chromatography, into two activities, called B' and B'', both of which are required for transcription of tRNA in the presence of purified TFIIIC and pol III (Kassavetis *et al.*, 1991). Photocrosslinking experiments revealed the presence of two polypeptides in the TFIIIB fraction, 70 and 90 kDa, recruited to the tRNA promoter by interaction with TFIIIC (Bartholomew *et al.*, 1990; Bartholomew *et al.*, 1991). Significant progress in the characterisation of *S. cerevisiae* TFIIIB was made when three groups, independently, cloned the gene encoding the 70 kDa subunit, now referred to as Brf1 (TFIIB-related factor 1 – for a description of a universal nomenclature of TFIIIB components see (Willis, 2002). It was cloned both as a suppressor of a mutation in the A-block of a tRNA gene (Lopez-De-Leon *et al.*, 1992) and as a suppressor which, at high copy number, rescues certain temperature-sensitive mutations in TBP (Buratowski and Zhou, 1992; Colbert and Hahn, 1992). The latter data suggested that Brf1 can interact with TBP, and that TBP might be a part of the TFIIIB activity. This was shown to be the case for both yeast and mammalian TFIIIB activities by biochemical methods (Chiang *et al.*, 1993; Huet and Sentenac, 1992; Kassavetis *et al.*, 1992; Lobo *et al.*, 1992; Taggart *et al.*, 1992; White and Jackson, 1992).

The *BRF1* gene is essential for growth, and its importance for pol III transcription was demonstrated by several experiments. Disruption of the gene resulted in a rapid decrease in the *in vivo* transcription of tRNA, but not of class I and II genes (Buratowski and Zhou, 1992; Lopez-De-Leon *et al.*, 1992). Similarly, while extracts from *BRF1* mutants were defective for transcription by pol III (but wild-type for pol I and pol II), addition of recombinant ScBrf1 (*S. cerevisiae* Brf1) could reconstitute

the transcription of 5S and tRNA genes (Colbert and Hahn, 1992). Addition of recombinant ScBrf1 to wild-type extracts resulted in stimulation of pol III transcription, suggesting that Brf1 is normally a limiting factor *in vivo* (Colbert and Hahn, 1992; Lopez-De-Leon *et al.*, 1992). The cloning of the gene encoding the B'' subunit, now referred to as Bdp1 (B-double prime 1), completed the characterisation of *S. cerevisiae* TFIIIB (Ishiguro et al., 2002).

- Characterisation of human TFIIIB

Identification of the components of TFIIIB in mammals proved to be more complicated. Whereas all pol III promoters in *S. cerevisiae* require the same TFIIIB complex (Joazeiro *et al.*, 1994), the initial characterisation of mammalian TFIIIB showed that type 1 and 2 promoters use different components in the TFIIIB fraction than type 3. Transcription from type 1 and 2 promoters requires a complex consisting of TBP, Bdp1 and a homologue of yeast Brf1, HsBrf1 (*Homo sapiens* Brf1) (Mital *et al.*, 1996; Wang and Roeder, 1995). On the other hand, type 3 promoters were shown to utilise, in addition to TBP and Bdp1, a protein related to Brf1 in its N-terminus, but not its C-terminal region (Mital *et al.*, 1996; Schramm *et al.*, 2000; Teichmann *et al.*, 2000; Wang and Roeder, 1995), now referred to as Brf2 (Willis, 2002). *S. cerevisiae* Brf1 has, therefore, at least two homologues in human cells, HsBrf1 and HsBrf2. From now on, this specialised complex will be referred to as Brf2-TFIIIB.

In addition to Brf2, mammalian cells also contain alternatively spliced variants of Brf1 (McCulloch *et al.*, 2000). One of these, Brf1_v2 has been implicated in human U6 transcription, even though the full-length protein, Brf1, is not involved (McCulloch *et al.*, 2000).

Using a combination of database searches for sequences similar to the SANT domain of yeast Bdp1 (see below) and library screening, Schramm *et al.* (2000) isolated and cloned Bdp1 from human cells. As is the case with Brf1, a number of human Bdp1 splice variants have been identified (Kelter *et al.*, 2000; Schramm *et al.*, 2000). Which of these alternatively spliced forms of Bdp1 is involved in pol III transcription is still unclear (Schramm and Hernandez, 2002).

- Structural characteristics of Brf1/Brf2 and Bdp1

Human and *S. cerevisiae* Brf1, as well as HsBrf2, contain two distinct domains in the N-terminal half of the polypeptide, both of which are conserved between Brf1 (and Brf2) and TFIIB (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-De-Leon *et al.*, 1992). One consists of a zinc-binding domain at the extreme N-terminus, while the 'core domain', occupying most of the remaining N-terminal half, is made up of two, 76 residues-long imperfect repeats. Although the C-terminal half, which is absent in TFIIB, is significantly less conserved, it contains, in yeast, three well-conserved regions, designated homology regions H1, H2 and H3 (Khoo *et al.*, 1994). H2 and H3 are also conserved in HsBrf1 (Andrau *et al.*, 1999; Mital *et al.*, 1996). Mutagenesis experiments have shown that Brf1 contains two distinct TBP-binding domains, which interact with opposite faces of the TBP-DNA complex. As a result, Brf1 is able to bind stably to TBP in the absence of DNA, unlike TFIIB (Kassavetis *et al.*, 1991; Wang and Roeder, 1995). The C-terminal H2 region comprises the high-affinity TBP-binding surface of Brf1, interacting primarily with the convex surface and side of the first TBP pseudo-repeat (Colbert *et al.*, 1998; Kassavetis *et al.*, 1998b). The second TBP-binding site is thought to lie in the core domain in the N-

terminal half. The importance of the TBP-binding site in the C-terminus is demonstrated by the fact that the N-terminal half of Brf1 does not bind stably to a TBP-DNA complex (Kassavetis *et al.*, 1998b). HsBrf2, which lacks an H2 domain at its C-terminus, is able to bind the TBP-DNA complex through the N-terminal direct repeat region, although the interaction is weak (Cabart and Murphy, 2001; Schramm *et al.*, 2000).

Yeast Bdp1 contains a domain related to a Myb repeat, found in the SWI-SNF and ADA complexes, the transcriptional co-repressor N-Cor and yeast TFIIIB Bdp1, and thus referred to as the SANT domain (Aasland *et al.*, 1996). This domain is absolutely required for TFIIIC-dependent (but not TFIIIC-independent) pol III transcription (Kumar *et al.*, 1997). A region just upstream of the SANT domain is required for transcription from linear, but not supercoiled, templates (Kassavetis *et al.*, 1998a). Although human Bdp1 is much larger than its yeast counterpart, the two proteins share three regions of homology: the SANT domain, which is 43% identical, and the regions immediately upstream and downstream of it, with 21% and 17% identity, respectively (Schramm *et al.*, 2000). The larger size of human Bdp1 comes from its C-terminal extension, which contains 19 imperfect repeats of 26 – 28 amino acids, which include potential phosphorylation sites. ScBdp1 has been shown to contact ScBrf1 and TBP, as well as the DNA itself (Colbert *et al.*, 1998; Shah *et al.*, 1999). This binding to DNA has been postulated to contribute to the stability of the TFIIIB-DNA complex (Grove *et al.*, 1999).

1.1.4.2 TFIIIC

Like TFIIIB, TFIIIC was first purified from yeast, where it is also referred to as the τ factor. *S. cerevisiae* TFIIIC consists of six subunits, τ138, τ131, τ98, τ91, τ60 and τ55, all of which have been cloned and shown to be essential for cell viability (Bartholomew *et al.*, 1990; Braun *et al.*, 1992; Swanson *et al.*, 1991). Limited protease treatment revealed that *S. cerevisiae* TFIIIC consists of two domains, τ_A and τ_B, separated by a flexible linker (Marzouki *et al.*, 1986). These data are supported by direct visualisation of TFIIIC – tDNA complexes using scanning transmission electron microscopy (STEM), where TFIIIC appears as either two tightly packed or clearly separated globular domains, depending on the separation of the A- and B-blocks (Schultz *et al.*, 1989). Although the τ_B domain binds strongly to the B-block and appears to be the major determinant for binding, τ_A, which binds weakly to the A-block, is also required for transcription (Figure 1.2).

In humans, the TFIIIC fraction was originally resolved into two activities, TFIIIC1 and TFIIIC2 (Dean and Berk, 1987; Yoshinaga *et al.*, 1987). Although both seem to be required for 5S, tRNA and VA1 gene transcription, TFIIIC1 was found to be sufficient for the transcription of the 7SK and U6 genes (both of which have type 3 promoters) (Oettel *et al.*, 1997; Yoon *et al.*, 1995; Yoshinaga *et al.*, 1987). The situation became more complicated with the description of two other TFIIIC1 activities, TFIIIC1-like and TFIIIC1', with different chromatographic but similar functional properties (Oettel *et al.*, 1997; Wang and Roeder, 1996). A recent report, however, suggests that HsBdp1 is an essential component of the TFIIIC1 activities, with different HsBdp1 alternatively spliced variants being components of the TFIIIC1 and TFIIIC1-like activities (Wieser *et al.*, 2004).

The TFIIIC2 fraction was found to contain an activity that corresponds, at least in part, with yeast TFIIIC and resides in a complex of five polypeptides of 220, 110, 102, 90 and 63 kDa (Kovelman and Roeder, 1992; Yoshinaga *et al.*, 1989). This complex is called TFIIIC2 or TFIIIC2a, and is the complex that is actively involved in pol III transcription. The TFIIIC2 fraction also contains an inactive complex, designated TFIIIC2b (Kovelman and Roeder, 1992). TFIIIC2b, which represents approximately 10 – 20% of total TFIIIC in growing HeLa cells, lacks the TFIIIC110 subunit, but seems to contain a 77 kDa subunit that is absent from TFIIIC2a (Kovelman and Roeder, 1992; Yoshinaga *et al.*, 1989). From now on, TFIIIC2a will be referred to as TFIIIC (factor, as opposed to activity).

All five subunits have been cloned. TFIIIC220, TFIIIC110 and TFIIIC90 show no significant homology to any of the yeast subunits, whereas TFIIIC63 and TFIIIC102 display weak homology to τ95 (22%) and τ131 (31%), respectively (Hsieh *et al.*, 1999c). TFIIIC220 is functionally similar to the yeast τ138, as it is responsible for recognising the B-block of the promoter (L'Etoile *et al.*, 1994; Lagna *et al.*, 1994), although the two proteins share no sequence homology. Similarly, TFIIIC90 seems to be functionally equivalent to the τ60 subunit of yeast TFIIIC, as they interact with the same set of proteins (Hsieh *et al.*, 1999b). TFIIIC90, however, has an added intrinsic HAT (histone acetyltransferase) activity, for both free and nucleosomal H3 (Hsieh *et al.*, 1999b). TFIIIC110 also has an intrinsic HAT activity, and can acetylate free and nucleosomal histones H3 and H4, as well as nucleosomal histone H2B (Kundu *et al.*, 1999).

1.1.4.3 Additional general factors

An additional general factor, TFIIIE, has been described in yeast (Dieci *et al.*, 1993). It appears to be required for the efficient transcription of 5S rRNA and tRNA genes using partially purified factors, and can stimulate transcription of tRNA and U6 genes in a reconstituted system using recombinant TFIIIB and highly purified TFIIIC and pol III (Dieci *et al.*, 1993; Ruth *et al.*, 1996). Nevertheless, a defined role for TFIIIE in pol III transcription has not yet been identified. Possibilities that have been suggested include stimulation of TFIIIB recruitment, stabilisation of the transcription complex and catalysis of the conformational rearrangements in TFIIIB (Ruth *et al.*, 1996).

An activity required for transcription of tRNA and 5S genes has also been identified in silkworm, termed TFIIID (Ottonello *et al.*, 1987). A similar TFIIID activity has not been demonstrated in other systems, and it has been suggested that silkworm TFIIIC and TFIIID represent a subdivision of the components associated with TFIIIC in other organisms (Sprague, 1992; Young *et al.*, 1991).

1.1.4.4 Gene-specific factors

- TFIIIA

Transcription of the 5S rRNA gene requires, in addition to the general factors TFIIIB and TFIIIC, the presence of a gene-specific factor, TFIIIA (Engelke et al., 1980). TFIIIA was the first eukaryotic transcription factor to be purified and also the first to have its gene cloned (Engelke et al., 1980; Ginsberg et al., 1984). Surprisingly, it was

not first purified from yeast, like TFIIIB and TFIIIC, but rather from early *X. laevis* oocytes, due to its considerable abundance, as a complex with 5S rRNA (Engelke et al., 1980). It has since been purified from human cells (Moorefield and Roeder, 1994), *S. cerevisiae* (Wang and Weil, 1989) and silkworm (Smith et al., 1995).

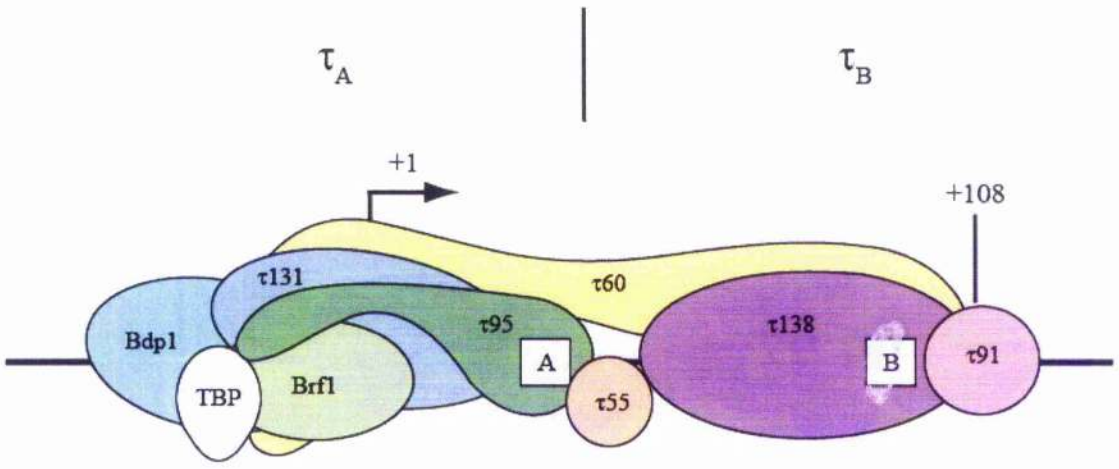


Figure 1.2 TFIIIC and TFIIIB interactions with a tRNA promoter

The position of the various *S. cerevisiae* TFIIIB and TFIIIC subunits on a SUP4 tRNA gene, as determined by cross-linking and protein-protein association experiments. The start site (+1), end of the gene (+108) and A- and B-blocks are indicated.

X. laevis TFIIIA is 344 amino acids long and, apart from 90 N-terminal and 10 C-terminal residues, is composed of nine tandem 27-amino acid repeats, which form zinc fingers (Miller et al., 1985). These are essential for the DNA-binding activity of TFIIIA (Hanas et al., 1983), and are a common feature in DNA-binding proteins. Using another zinc-finger protein, it was demonstrated that the α-helix contained in each finger makes contacts with the major groove of the DNA (Pavletich and Pabo,

1991). The DNA-binding domain of TFIIIA can be separated into three regions. Zinc fingers 1 – 3 bind to the C-block of the 5S promoter and are thought to contribute most of the binding energy of TFIIIA. Fingers 7 – 9 make contacts with the A-block, while finger 5 recognises the IE. The other two zinc fingers, 4 and 6, provide the appropriate spacing between the three regions (Clemens et al., 1992; Hayes and Clemens, 1992; Liao et al., 1992). Although TFIIIA proteins from other organisms also contain nine zinc fingers (e.g. Archambault et al., 1992; Moorefield and Roeder, 1994), it seems to have evolved quite rapidly, with considerable divergence observed even among frog species. For example, the *X. laevis* protein shows only 84% identity to the *X. borealis* TFIIIA and 63% to *Rana catesbeiana* (Gaskins and Hanas, 1990). This may be explained, in part, by the fact that the only essential function of TFIIIA in *S. cerevisiae* is in 5S rRNA transcription (Camier et al., 1995).

In *Xenopus*, at least, TFIIIA has additional functions in both the storage and transport of 5S rRNA (Tafuri and Wolffe, 1993). Binding of TFIIIA to 5S leads to the formation of the 7S RNP, which can then be exported from the nucleus (Guddat et al., 1990). This can lead to depletion of TFIIIA from the nucleus, providing a negative feedback loop for regulating the production of 5S rRNA.

- SNAPc

Transcription from the type 3 promoters of U6 snRNA and 7SK does not require TFIIIA or TFIIIC, since they are devoid of the internal promoter elements that the two factors recognise (Schramm and Hernandez, 2002). The gene-external PSE of type 3 promoters is instead recognised by another transcription factor, independently identified as SNAPc or PTF (Murphy et al., 1992; Sadowski et al., 1993; Yoon et al.,

1995). SNAPc/PTF is comprised of five subunits of 190, 50, 45, 43 and 19 kDa (Schramm and Hernandez, 2002). The two largest, SNAP190 and SNAP50 are responsible for contacts with the DNA, whereas the remaining subunits cannot bind DNA independently (Henry et al., 1995; Yoon et al., 1995).

The importance of SNAPc for U6 and 7SK transcription is demonstrated by the fact that depletion of endogenous SNAPc specifically represses transcription from the U6 and 7SK promoters, whereas it does not affect VA1 transcription (Sadowski et al., 1996; Yoon and Roeder, 1996). Interestingly, U1 and U2 transcription was also inhibited, and addition of highly purified SNAPc restored transcription of all four genes, suggesting that the same factor, SNAPc, is involved in pol II and pol III transcription of U snRNAs.

1.1.5 Assembly of the pre-initiation complex

Efficient and accurate transcription requires the assembly of transcription factors at the relevant gene promoter, in an ordered and stepwise manner, which will allow the recruitment of the polymerase and its placement over the correct start site. The pathway of transcription complex assembly for RNA polymerase III is summarised in Figure 1.3.

In type 2 promoters, TFIIIC recognises and binds the A- and B-blocks of the promoter (Lassar et al., 1983). This interaction allows the recruitment of TFIIIB and pol III (Bieker et al., 1985; Setzer and Brown, 1985). Transcription from type 1 promoters requires prior binding of TFIIIA, which recognises all three elements of the ICR (Engelke et al., 1980; Sakonju et al., 1981). This TFIIIA-DNA complex allows binding of TFIIIC (Lassar et al., 1983). TFIIIA can be, therefore, viewed as a

specificity factor, which changes the promoter-recognition properties of TFIIIC and targets it to the 5S promoter. Following binding of TFIIIC, the recruitment pathway is similar to that in type 2 promoters. In type 3 promoters, SNAPc binds to the PSE,

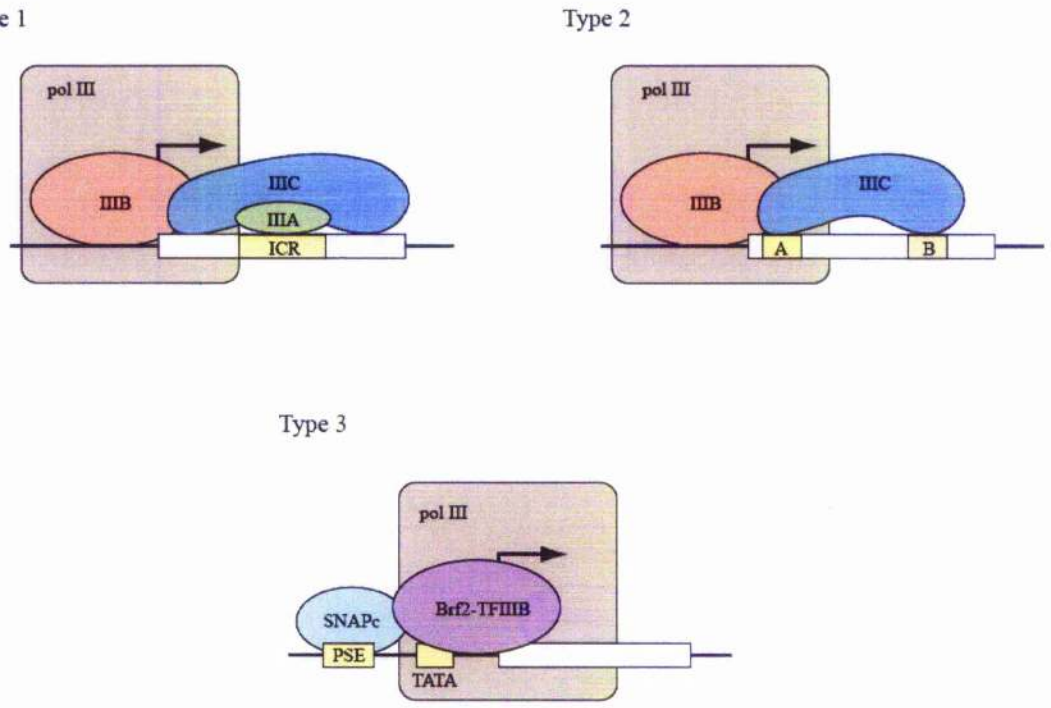


Figure 1.3 Different pathways for recruitment of TFIIIB and RNA polymerase III

Pre-initiation complexes assembled on the three promoter types. In type 1 promoters, TFIIIA recognises and binds the ICR, allowing the recruitment of TFIIIC. This is followed by recruitment of TFIIIB and then pol III. The A- and B-blocks of type 2 promoters are recognised by TFIIIC, which then recruits TFIIIB and then pol III. In type 3 promoters, SNAPc and the Brf2-TFIIIB complex bind the PSE and TATA box, respectively. This is followed by the recruitment of pol III.

while the TATA box is recognised by the TBP component of the Brf2-TFIIIB complex (Schramm et al., 2000; Teichmann et al., 2000; Yoon et al., 1995). Binding of SNAPc and Brf2-TFIIIB to the promoter leads to the recruitment of pol III (Chong

et al., 2001), presumably through protein-protein interactions, although this has not yet been demonstrated.

Once assembled, class III transcription complexes are extremely stable, and their components remain associated after initiation, supporting multiple rounds of transcription (Bogenhagen et al., 1982; Lassar et al., 1983). Subsequent rounds proceed 5- to 10-fold more rapidly than the initial round of transcription (Dieci and Sentenac, 1996).

1.1.6 Regulation of pol III transcription

The previous chapters described the components of the basal pol III transcription machinery, and how they all come together to allow expression of class III genes. In order for the cell to modulate the output of pol III to suit its requirements at any given time, as well as in response to various stimuli, a host of additional factors is employed. Some of these will be described below.

1.1.6.1 Activities that reduce pol III transcription

- Dr1

Dr1 is a 19 kDa phosphoprotein that was isolated from HeLa cells due to its ability to repress pol II transcription (Inostroza *et al.*, 1992). Although it can function independently, a 28 kDa co-repressor called DRAP1 can bind to Dr1 and enhance its ability to inhibit transcription (Mermelstein *et al.*, 1996). In the case of pol II, this is

accomplished by binding to TBP and disrupting its interaction with TFIIB and/or TFIIA (Inostroza *et al.*, 1992; Yeung *et al.*, 1994).

Dr1 can also inhibit transcription by pol III, both *in vitro* and *in vivo*. Highly purified or recombinant Dr1 can repress the expression of class III genes when added to crude extracts or fractionated factors (White *et al.*, 1994), while its overexpression in yeast has been shown to repress tRNA transcription (Kim *et al.*, 1997). This repression can be relieved either by raising the levels of TBP or by adding TFIIIB, but not TFIIIC, fractions (White *et al.*, 1994). Similarly to the pol II system, Dr1 binding to TBP prevents its interaction with Brf1, by competing for overlapping binding sites (White *et al.*, 1994). Interestingly, Dr1 does not affect pol I transcription, either *in vitro* or *in vivo* (Kim *et al.*, 1997; White *et al.*, 1994). It has, therefore, the potential to tip the balance of transcription in the favour of pol I, when, for example, rRNA production is lagging behind that of 5S rRNA.

- pRb/p107/p130

pRb is a 105 kDa nuclear phosphoprotein, encoded by the retinoblastoma susceptibility gene *RB*. The *RB* gene was isolated because of its association with retinoblastoma, a childhood retinal malignancy, although it has also been found mutated in other human tumours (Friend *et al.*, 1986; Whyte, 1995). *RB* was one of the first tumour suppressor genes to be identified, and has been extensively studied. It plays an important role both in cell growth (increase in cell size) and proliferation (increase in cell number), as the ability of cells to undergo growth- or proliferation-arrest is severely compromised if pRb is inactivated (Weinberg, 1995; Whyte, 1995).

One of the most studied functions of pRb is its involvement in the regulation of the cell cycle (Zheng and Lee, 2001), especially the G1-to-S phase transition. In the absence of mitogenic stimuli, pRb is critical for arresting the cell cycle at the restriction (R) point, two-thirds through the G1 phase. Under favourable conditions, however, pRb is inactivated through hyperphosphorylation by cyclin D- and cyclin E-dependent kinases, allowing the cells to pass the R point, committing to DNA synthesis and division (Zheng and Lee, 2001). pRb has also been implicated with cell cycle regulation beyond the G1 phase, regulation of genome integrity and differentiation (Zheng and Lee, 2001).

pRb can be functionally divided into three regions: the N- and C-termini, between amino acids 1 – 378 and 768 – 869 (of the human protein), respectively, and the pocket domain (Welch and Wang, 1993; Welch and Wang, 1995). The pocket domain can be further divided into the A and B subdomains, separated by an inessential spacer. The structural integrity of the pocket domain is required for interaction with most of pRb targets, and is frequently found mutated in cancers (Chen *et al.*, 1995; Weinberg, 1995).

While pRb has been shown to repress pol II-transcribed genes, either through interactions with E2F (Stevaux and Dyson, 2002) or by recruiting histone deacetylases (HDACs) to promoters (Brehm and Kouzarides, 1999), it does so in a limited way: the overall level of pol II transcription is not affected in primary fibroblasts from *RB1* mice, compared to equivalent cells from wild-type mice (White *et al.*, 1996). In the same system, however, pol III transcriptional activity is enhanced 5-fold (Larminie *et al.*, 1997; White *et al.*, 1996). Furthermore, overexpression of pRb in transfected cells, as well as addition of recombinant pRb to a reconstituted system with partially purified factors, resulted in the repression of various class III genes, including 5S rRNA, tRNA and U6 snRNA (White *et al.*,

1996). Thus, pRb was shown to be a general repressor of pol III transcription, a function requiring the A/B pocket domain, as well as its C-terminal region (White *et al.*, 1996).

pRb-mediated repression of class III genes is accomplished via distinct mechanisms, owing to the different transcription factor requirements between type 1/2 and type 3 promoters. Binding of pRb to TFIIIB has been shown to disrupt its interaction to TFIIIC, and thus block its recruitment to type 1 and 2 promoters (Chu *et al.*, 1997; Larminie *et al.*, 1997; Sutcliffe *et al.*, 2000). Type 3 promoters, on the other hand, exemplified by U6 snRNA, do not require TFIIIC for the recruitment of TFIIIB, and indeed use a different TFIIIB complex, i.e. Brf2-TFIIIB. In this case, pRb has been shown to be recruited to the promoter itself, though protein-protein interactions with SNAPc and Brf2-TFIIIB (Hirsch *et al.*, 2004). The molecular mechanisms that underlie this repression, however, are still not clear.

pRb can also inhibit pol I transcription, by binding the upstream binding factor (UBF) and preventing it from recruiting SL-1 and the stimulatory co-factor CBP (Hannan *et al.*, 2000; Pelletier *et al.*, 2000). General repression of both pol I and pol III transcription by pRb provides the cell with a single mechanism that can efficiently block the expression of many genes involved in protein synthesis, thus affecting cell growth. Separately, through its E2F-related regulation of many cell cycle-related genes, pRb can also influence cell proliferation.

pRb has two relatives, p107 and p130, with which it shares 30 – 35% amino acid identity. This homology mostly lies in the A/B pocket region, hence the name 'pocket proteins' that is often used for the three proteins collectively. p107 and p130 are more closely related to each other, however, than to pRb, with ~ 50% amino acid identity and highly conserved regions extending throughout the protein. This includes the spacer sequence between the A/B subdomains of the pocket region

(Mulligan and Jacks, 1998). In contrast to pRb, the spacer is essential to the function of p107 and p130, as it contains the binding site for cyclin A/Cdk2 and cyclin E/Cdk2 complexes (Mulligan and Jacks, 1998).

Although there is some functional overlap between the pocket proteins, there are also some clear differences (reviewed in Classon and Dyson, 2001). For example, like pRb, p107 and p130 can each inhibit cell growth and proliferation if overexpressed in tumour cells, an effect associated with cell cycle arrest at the G1 phase. On the other hand, no human tumours have been shown to contain mutations in the *p107* gene, while only a few contain mutations of the *p130* gene, suggesting that they might not share the tumour suppressing function of pRb. Similar to pRb, p107 and p130 can repress pol III transcription, at least from type 1 and 2 promoters (Sutcliffe *et al.*, 1999). Overexpression of p107 and p130 in transfected cells, and recombinant proteins *in vitro*, can repress the expression of 5S rRNA and VA1, while pol III transcription is enhanced in *p107 -/- p130-/-* primary fibroblasts. This repression is achieved in a manner similar to pRb, i.e. by binding to TFIIIB and preventing its association with, and therefore its recruitment by, TFIIIC (Sutcliffe *et al.*, 1999).

- p53

p53 is another tumour suppressor, activation of which, in response to several stimuli, can lead to cell growth and proliferation arrest and apoptosis (Vogelstein *et al.*, 2000). p53, unlike pRb, is not an essential cell cycle regulator, as has been revealed by studies on *p53-/-* mice, which develop normally (Donehower *et al.*, 1992). These mice, however, display a strong predisposition to cancer, with 74% of them

developing tumours within 6 months of birth. This demonstrates that p53 provides an important checkpoint against tumourigenesis.

Although many biochemical functions have been ascribed to p53, the best characterised is its ability to bind to specific sequences in DNA and activate the expression of adjacent genes (el-Deiry, 1998). DNA binding is mediated by the central core domain (residues 100 – 300), while the acidic N-terminal domain (residues 1 – 43) mediates transcriptional activation through interactions with TBP and several TAFs in TFIID (Farmer *et al.*, 1996). p53 can also repress certain class II genes without p53-binding sites, by recruiting HDACs (Murphy *et al.*, 1999), e.g. c-*fos* (Kley *et al.*, 1992) and cyclin A (Yamamoto *et al.*, 1994).

p53 can also repress pol III transcription (Cairns and White, 1998; Chesnokov *et al.*, 1996). The best demonstration of this came from *p53*$^{-/-}$ primary fibroblasts, where tRNA and 5S rRNA expression is significantly elevated, compared to cells from wild-type mice (Cairns and White, 1998). p53-mediated pol III repression is not an indirect response to cell cycle changes, as certain mutations in p53 that block its transcriptional repression function do not affect its ability to arrest cells at G1 (Stein *et al.*, 2002a; Stein *et al.*, 2002b). Furthermore, repression of the transcription of various pol III templates can be demonstrated *in vitro*, using recombinant p53 (Cairns and White, 1998; Chesnokov *et al.*, 1996; Stein *et al.*, 2002b).

Although class III genes do not contain p53-binding sites, the DNA-binding domain of p53 is critical for the repression of pol III transcription, with regions both in the N- and C-termini contributing to this function (Stein *et al.*, 2002b). Instead, repression is achieved by protein-protein interactions with TFIIIB (Cairns and White, 1998). It is now understood that p53, through binding to the TBP component of TFIIIB, prevents the interactions between TFIIIC and TFIIIB that lead to recruitment of the latter to most class III promoters (Crighton *et al.*, 2003). Indeed, p53 induction leads to

reduced recruitment of TFIIIB to tRNA promoters *in vivo* (Crighton *et al.*, 2003). For type 3 promoters, where TFIIIC is not required for pre-initiation complex assembly, the ability of p53 to prevent the association of TFIIIB with pol III might come into play, although the exact molecular mechanism is still not clear.

As is the case with pRb, p53 can also inhibit pol I transcription (Zhai and Comai, 2000). Since the major products of pol I and pol III transcription, rRNA and tRNA, are essential for the translational capacity of the cells, their repression by p53 might contribute to its growth-arresting function.

1.1.6.2 Activities that stimulate pol III transcription

- Oct-1 and Staf

In addition to the PSE, type 3 promoters have an upstream regulatory element called DSE (Schramm and Hernandez, 2002). The DSE contains octamer motifs that are recognised by Oct-1, a member of the POU domain family of transcription factors (Rosenfeld, 1991). Oct-1, and the related factor Oct-2, can stimulate transcription from type 3 promoters by stabilising the binding of SNAPc to the PSE (Mittal *et al.*, 1996; Murphy *et al.*, 1992).

Staf is another factor that can enhance transcription from class III genes with a type 3 promoter. It is a 65 kDa polypeptide, containing seven tandemly repeated zinc fingers (Schuster *et al.*, 1995), through which it has been shown to bind to the DSE element of several vertebrate U snRNA genes (Schaub *et al.*, 1997). The majority of DSEs (~ 70%) contain an Oct-1 binding site within the Staf site, suggesting that the two proteins may act cooperatively to activate transcription (Myslinski *et al.*, 1993; Schaub *et al.*, 1997).

- CK2

CK2, formerly known as casein kinase 2, is a highly-conserved serine/threonine kinase, distributed ubiquitously in eukaryotes (reviewed in Litchfield, 2003). It is typically found in tetrameric complexes, consisting of two catalytic (α and/or α') and two regulatory β subunits. Within the cell, CK2 has been detected in the nucleus and the cytoplasm, as well as associated with specific structures and organelles, including the plasma membrane, endoplasmic reticulum and ribosomes. Unsurprisingly, CK2 has been shown to participate in the phosphorylation and regulation of a broad array of cellular targets, and thus is implicated in a large number of cellular processes (Litchfield, 2003). For example, candidate cellular CK2 substrates include proto-oncogene products, like c-Myc and c-Jun, the tumour suppressors p53 and BRCA1 and transcriptional regulators, such as Max (Litchfield, 2003).

The involvement of CK2 in the regulation of pol III transcription was first demonstrated in *S. cerevisiae*, using a strain carrying a temperature-sensitive mutation in the α' catalytic subunit of CK2 (Hockman and Schultz, 1996). Using these cells, it was shown that, at the non-permissive temperature, pol III transcription was down-regulated. It was later shown that CK2 activates pol III transcription by phosphorylating the TBP component of TFIIIB, enhancing its recruitment to the promoters of class III genes (Ghavidel et al., 1999; Ghavidel and Schultz, 1997; Ghavidel and Schultz, 2001).

The situation seems to be quite similar in mammalian cells, where CK2 has been shown to have a potent effect on pol III transcription (Johnston *et al.*, 2002). CK2 binds and phosphorylates TFIIIB, facilitating its recruitment by TFIIIC. In human

cells, however, the CK2 target for phosphorylation appears to be Brf1, as opposed to TBP, although all three components of TFIIIB, Brf1, Bdp1 and TBP, can be phosphorylated *in vitro* (Johnston *et al.*, 2002). In the case of type 3 promoters, which do not require Brf1, Hu *et al.* (2003) showed, using a well defined U6 transcription system, that CK2 phosphorylation of the Brf2-TFIIIB complex was inhibitory. On the other hand, treatment of the highly purified pol III complex resulted in activation of transcription, suggesting that, at least in this system, CK2 can have both positive and negative regulatory effects. Further work suggests that the CK2 target in Brf2-TFIIIB is Bdp1, and that its phosphorylation – and inactivation – occurs in a cell cycle-specific manner (Hu *et al.*, 2004). It is worth noting that CK2 has been shown to also stimulate rRNA synthesis by pol I, both in mammals and yeast (Belenguer *et al.*, 1989; Ghavidel and Schultz, 2001; Voit *et al.*, 1992). This provides the cell with yet another opportunity to coordinate the output of pols I and III, perhaps helping to ensure that the supply of rRNA and tRNA is appropriate for the physiological status of the cell.

- ERK

Mitogen-activated protein kinase (MAPK) pathways are signalling systems that translate various extracellular stimuli, e.g. growth factors, to appropriate intracellular responses, culminating in the alteration of expression of a particular set of genes. They exist in all eukaryotes, and control fundamental processes, such as growth and proliferation, differentiation, survival and apoptosis. The basic arrangement consists of a G-protein upstream of a core module of three kinases: a MAPK kinase kinase (MAPKKK or MAP3K), that phosphorylates and activates a MAPK kinase

(MAPKK or MAP2K), which in turn activates MAPK. One of these modules, the ERK (extracellular-signal regulated kinase) pathway, is involved in growth control in all its facets, including cell proliferation, transformation, differentiation and apoptosis and is employed by a wide variety of growth and differentiation factors (reviewed in Kolch, 2000; Robinson and Cobb, 1997). It features Ras as the G-protein, Raf as MAP3K, MEK (MAPK/ERK kinase) as MAP2K and ERK as MAPK. Amongst the many growth-promoting activities of the ERK pathway is its ability to link growth factor signalling to ribosome biogenesis in mammalian cells (Whitmarsh and Davis, 2000).

ERK has been shown to phosphorylate, and thus activate, UBF, increasing the synthesis of rRNA by pol I (Stefanovsky *et al.*, 2001). In order for this to be physiologically relevant, 5S rRNA synthesis must increase concomitantly. This has indeed proven to be the case, as serum induction of quiescent fibroblasts leads to an ERK-dependent activation of pol III transcription (Felton-Edkins *et al.*, 2003a). Importantly, the effects were shown to be independent of pRb or the cell cycle. Instead, ERK was shown to bind and phosphorylate Brf1, both *in vitro* and *in vivo*. This phosphorylation seems to promote binding of TFIIIB to TFIIIC and pol III, enhancing its recruitment to most pol III promoters.

- c-Myc

c-*myc* belongs to a small family of proto-oncogenes, the other members of which are N- and L-*myc*, and has been shown to be involved in the regulation of the cell cycle, cell growth and apoptosis, among others (Grandori *et al.*, 2000). c-Myc is a transcription factor containing a basic helix-loop-helix-leucine zipper (bHLH-LZ)

domain at its C-terminus, which mediates DNA binding and dimerisation, and a transcription activation domain at its N-terminus. Heterodimerisation of c-Myc with Max, which also contains a bHLH-LZ domain, allows it to recognise and bind the hexameric DNA sequence CACGTG (belonging to the larger class of sequences known as E-boxes, CANNTG), resulting in the activation of transcription from promoters that carry such an element (Murre *et al.*, 1989). This transcription activation function is mediated, at least in part, by the recruitment of a histone acetyltransferase (McMahon *et al.*, 2000). c-Myc has also been shown to repress transcription of certain target genes (Li *et al.*, 1994).

c-Myc is thought to promote cell cycle progression by activating the genes encoding cyclin D2 and Cdk4, while repressing $p21^{Cip1}$, a Cdk inhibitor (Bouchard *et al.*, 2001; Coller *et al.*, 2000; Hermeking *et al.*, 2000). In conjunction with the indirect activation of another Cdk inhibitor, $p27^{Kip1}$, by cyclin D2, the net effect of c-Myc would be the prolonged activation of cyclin E/Cdk2 and inhibition (phosphorylation) of pRb by Cdk2 and Cdk4 (Coller *et al.*, 2000). In addition to its proliferative role, c-Myc seems to be crucially involved in cell growth regulation. In some cell types, these two functions can be separated, with growth being induced in the absence of increased proliferation (e.g. Beier *et al.*, 2000; e.g. Schuhmacher *et al.*, 1999). On the other hand, loss of c-Myc results in reduced protein synthesis and growth (Johnston *et al.*, 1999; Mateyak *et al.*, 1997). Growth-related genes that are directly induced by c-Myc include several ribosomal proteins, translation factors, nucleolar proteins and rRNA-processing factors (Coller *et al.*, 2000; Guo *et al.*, 2000; Rosenwald, 1996; Schlosser *et al.*, 2003).

c-Myc has been shown to activate pol I transcription, both indirectly, by inducing UBF, a pol I-specific transcription factor (Poortinga *et al.*, 2004) and directly, by binding to SL-1 and promoting its recruitment to promoters (Grandori *et al.*, 2005).

c-Myc's repertoire has been recently expanded to include pol III, as it was shown to potently activate tRNA and 5S rRNA transcription (Gomez-Roman *et al.*, 2003). Surprisingly, c-Myc was found at the promoter of these genes, even if they do not contain E-boxes. It was observed, instead, that interactions between TFIIIB and the N-terminal transactivation domain of c-Myc were responsible for its recruitment (Gomez-Roman *et al.*, 2003).

1.1.7 Physiological regulation of pol III transcription

Previously, the role of individual proteins in the regulation of pol III transcription was described. In living cells, however, these factors do not act independently, but are part of networks that modulate cell behaviour in response to particular stimuli. The impact of two of these processes, cell cycle and differentiation, on pol III activity will be described further.

1.1.7.1 Cell cycle

In cycling cells, the transcriptional activity of pol III varies during the different stages of the cell cycle. It is low during early G1, increasing gradually as cells enter S phase. Pol III activity remains high, about 2- to 3-fold compared to G1, throughout S and G2, decreasing again as cells enter mitosis (see below) (White *et al.*, 1995a). TFIIIB activity was shown to follow the same pattern, suggesting that it is an important target for the cell cycle regulation of pol III transcription. TFIIIC binding activity, on the other hand, was unaffected throughout the cell cycle (White *et al.*,

1995a). Detailed time course analysis has revealed that the significant rise in TFIIIB and pol III activity during late G1 coincides with pRb inactivation through phosphorylation by cyclin D- and E-dependent kinases, near the restriction (R) point (Johnson *et al.*, 1974; Mauck and Green, 1974). The involvement of pRb is also supported by the fact that pRb binding to TFIIIB is substantially diminished after the cells have passed the R point (Scott *et al.*, 2001). pRb remains inactive through the rest of the cell cycle, until after mitosis, when its dephosphorylation renders it capable once again to bind TFIIIB at early G1 (Mittnacht, 1998).

Pol III activity is also reduced when cells exit the cell cycle, presumably reflecting a reduced requirement for protein synthesis (Johnson *et al.*, 1974; Mauck and Green, 1974). In this quiescent state, G0, TFIIIB activity was shown to be reduced, and in fact limiting for pol III transcription, whereas TFIIIC activity, which is sometimes limiting in cycling cells, was unaffected (Scott *et al.*, 2001). Similarly to cycling cells, pRb appears to be involved in the repression of TFIIIB in G0, where it is present in its hypophosphorylated, active form (Grana *et al.*, 1998). pRb was indeed shown to bind to TFIIIB in growth-arrested cells, an interaction that diminished following mitogenic stimulation – and thus entry into the cell cycle. Furthermore, *RB1*$^{-/-}$ cells exhibited a reduced ability to down-regulate pol III transcription following exit from the cell cycle (Scott *et al.*, 2001). The fact that some decrease in pol III transcription is still observed in these cells suggests that other mechanisms might also be involved. p107 and p130, related pRb and both able to repress pol III transcription through binding to TFIIIB (Sutcliffe *et al.*, 1999), are attractive candidates. Between these two, however, p130 is more likely to be relevant, since it is highly expressed in quiescent cells and in an active form (Smith *et al.*, 1996).

In mitosis, all nuclear transcription is repressed (reviewed in Gottesfeld and Forbes, 1997). Repression of pol III transcription has been shown to be a result of

phosphorylation and inactivation of TFIIIB. In *Xenopus*, this seems to involve cdc2 and one or more additional kinases (Gottesfeld *et al.*, 1994; Leresche *et al.*, 1996). HeLa cells, although possessing a more complicated cell cycle than early *Xenopus* embryos, appear to employ a similar mechanism. Thus, TFIIIB was found to be specifically inactivated during mitosis, attributed to the inactivation of one or more of its TAF components (White *et al.*, 1995b). Further work demonstrated that the Brf1 subunit of TFIIIB is specifically phosphorylated during mitosis, leading to a reduction in TFIIIB and pol III occupancy of 5S rRNA and tRNA genes (Fairley *et al.*, 2003). Interestingly, Bdp1 and pol III are preferentially released from promoters, with Brf1 and TBP showing only a slight (~ 20%) reduction. TFIIIC occupancy remains undiminished during mitosis (Fairley *et al.*, 2003). Accordingly, addition of recombinant Brf1 or Bdp1, but not TBP, relieves mitotic repression of pol III transcription (Fairley *et al.*, 2003; White *et al.*, 1995b).

1.1.7.2 Differentiation

In situ hybridisation experiments in early mouse embryos revealed an interesting pattern of expression of the B2 gene (Vasseur *et al.*, 1985; for a brief description of the first stages of mouse embryogenesis, see Chapter 1.2). B2 transcripts were present in oocytes and increased in abundance following fertilisation. By day 4, B2 expression could be detected in the inner cell mass (ICM), but was down-regulated in the trophectoderm. At 7.5 days post-coitum, B2 RNA was present in the embryonic ectoderm and mesoderm, but was undetectable in the embryonic and extraembryonic endoderm. These experiments, therefore, revealed that pol III transcription was tightly linked to the developmental stages of the early mouse embryo.

A murine cell line, F9, has been widely used to study early mouse embryogenesis, because it can be induced to mimic particular differentiation events (described in detail in Chapter 1.2). Using this system, it has been shown that differentiation results in a decrease in the abundance of class III transcripts, as a consequence of reduced pol III activity. For example, B2, VA1 and tRNA synthesis is reduced (White *et al.*, 1989). Importantly, it was shown that differentiated cells exhibit reduced TFIIIB, but not TFIIIC, activity (White *et al.*, 1989). This observation was supported in a later report, using more purified fractions (Alzuherri and White, 1998). It was also shown that the protein levels of at least two components of TFIIIB, Brf1 and TBP, decreased following differentiation, whereas two TFIIIC subunits were unaffected (Alzuherri and White, 1998). Since, however, Brf1 is not required for transcription from type 3 promoters, some additional mechanism should be involved.

Part of that answer may lie in the observation that the third component of TFIIIB, Bdp1, is also down-regulated following differentiation (Weser *et al.*, 2004). Interestingly, this group identified Bdp1 as an essential component of the previously uncharacterised TFIIIC1 activity (-ies), which they had earlier suggested was limiting in F9 PE cells (Meissner *et al.*, 2002).

1.1.8 Deregulation of pol III transcription by transformation

A wide variety of transformed cell types have been shown to express abnormally high levels of pol III products, including lines transformed by DNA or RNA tumour viruses and chemical carcinogens (e.g. Carey et al., 1986b; Gottesfeld et al., 1996; Kramerov et al., 1990; Larminie et al., 1999; Scott et al., 1983). Elevated pol III

activity has also been observed in tumours in situ, compared to adjacent healthy cells, thus validating the observations made in cell lines (Chen et al., 1997a; Chen et al., 1997b; Winter et al., 2000). Increased pol III activity, therefore, seems to correlate well with the transformed state. Less understood, in many cases, is the mechanism by which pol III deregulation occurs. Some possible mechanisms will be discussed below.

pRb is an important cell cycle regulator. The most common way that cancer cells use to overcome its restraining effect is through its constitutive hyperphosphorylation. This is achieved either by activating the cyclin-dependent kinases responsible for phosphorylating pRb and/or by inactivating the inhibitors of these kinases. For example, 30 – 40% of primary breast tumours overexpress cyclin D1 (Bates and Peters, 1995), while many glioblastomas and some gliomas have amplifications of the cdk4 gene (Weinberg, 1995). Furthermore, pancreatic, oesophageal and bladder carcinomas frequently contain deletions or mutations in the gene for p16, a cyclin D-dependent kinase inhibitor (Rocco and Sidransky, 2001; Sherr, 2001). The overall effect of these changes is the constitutive hyperphosphorylation of pRb, rendering it unable to bind its targets, one of which is TFIIIB (Scott et al., 2001). Accordingly, pol III transcription can be activated by overexpression of cyclin D or Cdk4, or by depletion of p16 (Scott et al., 2001).

Many tumours carry mutations in pRb itself. The majority (98%) of pRb mutations found in tumours include the extended pocket domain (residues 393 – 892) (Harbour, 1998). This region has been shown to be necessary and sufficient for the interaction between pRb and TFIIIB, and mutations within it can prevent pRb from repressing pol III transcription (Felton-Edkins et al., 2003b; Larminie et al., 1997).

Another important regulator of pol III transcription is p53, mutations of which in tumours have been well documented (Hollstein et al., 1994; Hollstein et al., 1991).

Most of these map to its central domain, which is essential for TFIIIB repression (Stein et al., 2002b). Pol III transcription, therefore, may be derepressed in cancers with such mutations. In some tumours, p53 function can also be lost due to viral or cellular oncogenes. For example, Hdm2, the human homologue of Mdm2 (an E3 ligase that targets p53 for degradation), is overexpressed in certain cancer types, including osteosarcomas and soft-tissue tumours (Momand et al., 1998). Stein,et al. (2002b) demonstrated that Hdm2 can stimulate pol III transcription by relieving TFIIIB from p53-mediated repression.

In addition to the factors that repress pol III transcription, several proteins or pathways lead to its activation. Thus, CK2, c-Myc and the ERK MAPK cascade all result in the up-regulation of pol III transcription. Elevated activity of all of these factors has been demonstrated in human cancers. The most striking example is ERK. 20% of all human tumours, including 90% of pancreatic cancers, have abnormal activity of Ras, the upstream kinase of the ERK pathway. ERK itself is induced in approximately 30% of tumours (Downward, 2003). Up-regulation of these activating factors presents another potential mechanism for deregulating pol III transcription in transformed and tumour cell types.

The most obvious way for tumours to increase pol III transcription, although not as common, is by increasing the levels of the pol III-specific transcription factors. This has been shown to be the case with both TFIIIC and TFIIIB. Studies have shown that TFIIIC is overexpressed in SV40- and polyomavirus-transformed cell lines (Felton-Edkins and White, 2002; Larminie et al., 1999; White et al., 1990), as well as in ovarian carcinomas (Winter et al., 2000). In cell culture models, SV40 and polyomavirus stimulates the expression of the Bdp1 subunit of TFIIIB, while hepatitis B virus causes induction of TBP (Felton-Edkins and White, 2002; Wang et

al., 1997). Importantly, the Brf1 subunit of TFIIIB is sometimes overexpressed in breast and cervical carcinomas (Daly et al., 2005).

1.1.9 Concluding remarks

TFIIIB is an essential, pol III-specific transcription factor, whose role is to recruit the polymerase to the appropriate promoters. Although its own recruitment is dependent on other factors, namely TFIIIC or SNAPc, under particular conditions in yeast, TFIIIB by itself is sufficient to recruit pol III and to initiate accurate transcription. The central role of TFIIIB is underlined by the fact that all but a few regulators that exert an influence on pol III transcription act through this multisubunit complex (Figure 1.4). In addition to the regulators mentioned previously, several viral proteins also affect pol III transcription through TFIIIB, directly or indirectly (White, 2004a). Thus, TFIIIB stands at the crossroads of many regulatory pathways, and its activity determines to a large extent the levels of pol III transcription.

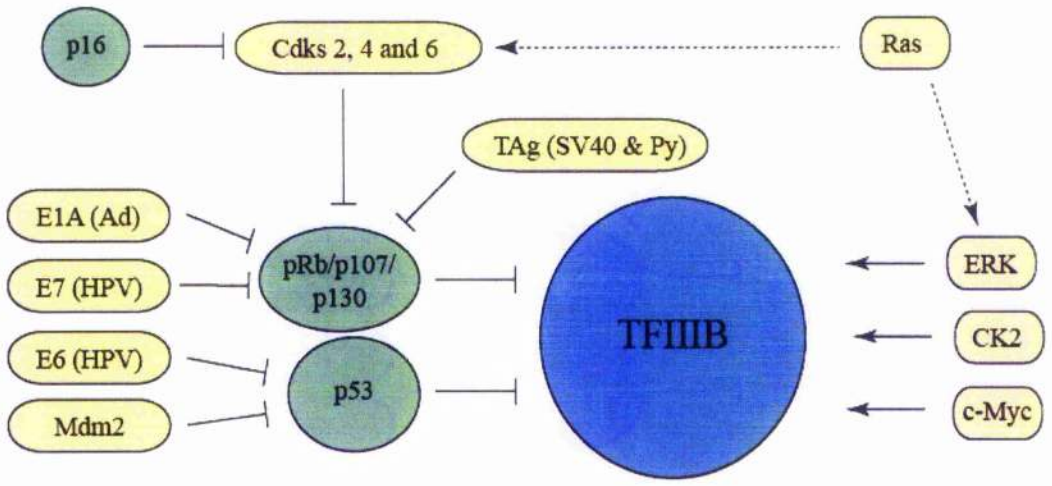


Figure 1.4 Antagonistic influences targeting TFIIIB

Repressors (green) and enhancers (yellow) of TFIIIB activity. The pocket proteins and p53 directly repress TFIIIB. This restraint is removed by the adenoviral E1A product, the large T antigens of SV40 and polyomavirus, the E6 and E7 products of high-risk human papillomaviruses, as well as the cellular oncogene Mdm2 and the cyclin D- and E-dependent kinases. p16 helps to restore repression of TFIIIB, by inhibiting pRb phosphorylation by the Cdks. On the other hand, TFIIIB is directly activated by the kinases ERK and CK2 and the cellular oncogene c-Myc. Another oncogene, Ras, can indirectly stimulate TFIIIB function both by activating ERK and cyclin D production.

1.2 The F9 cell system

1.2.1 Early mouse embryogenesis

Following fertilisation, the mouse egg, encapsulated in a transparent cell coat called the zona pellucida, starts dividing. Within 2 ½ days, it reaches an 8-cell stage called the morula. Further divisions lead to the first differentiation event in mouse embryogenesis, the formation of the blastocyst at day 4. This is comprised of an outer, spherical layer of cells, the trophectoderm, and a cluster of cells inside it, located at one of the poles, called the inner cell mass (ICM). The trophectoderm will eventually form the placenta, while the ICM will give the whole of the embryo proper, through further growth and differentiation events. The first stages of this process include the formation of the primitive endoderm, a layer of cells on the periphery of the ICM (~ day 4 ½), which will then give rise to visceral and parietal endoderm by days 5 to 6 (Grover and Adamson, 1986 and references therein). Part of the regulation that will determine cell type is governed by interactions with neighbouring cells. For example, if presumptive visceral endoderm cells come into contact with trophectoderm-derived, extraembryonic ectoderm, they will differentiate into parietal endoderm (Hogan and Tilly, 1981). The fate of the cells is also temporally controlled, as visceral endoderm cells from the 6th day of gestation can give rise to both visceral and parietal endoderm, whereas cells from the 7th day can only generate visceral endoderm (Gardner, 1982).

1.2.2 Teratomas

Clearly, the regulation of differentiation during early mouse embryogenesis is very complicated. Studying these mechanisms is made more difficult by the small amount of material one can work with and the heterogeneity of the starting populations. Both of these problems were overcome with the observation that the stem cells of teratomas, embryonal carcinoma (EC) cells, are very similar to the ICM of the blastocyst-stage mouse embryo. In addition, EC cells have the ability to differentiate into cell types similar to those that result from ICM differentiation (thoroughly discussed in Silver *et al.*, 1983). Teratomas are tumours that nearly always occur in the gonads, composed of a disorganised mass of cells at various stages of maturation (Stevens and Hummel, 1957). They can form spontaneously, but can also be experimentally induced by a number of methods, including grafting pre- and postimplantation embryos to an extrauterine site, such as the testis (Stevens, 1970) or kidney (Damjanov *et al.*, 1971). When all the EC cells in a teratoma become differentiated, the tumours are benign. If, however, the tumours consist of a mixture of EC and differentiated cells or undifferentiated EC cells entirely, they are malignant and often referred to as teratocarcinomas. Single EC cells from teratomas have been shown to take part in normal embryo development when injected into blastocysts (Brinster, 1974; Mintz and Illmensee, 1975; Papaioannou *et al.*, 1975), suggesting that they still retain their ability to differentiate into various cell types. Although EC cells from teratocarcinomas can be cultured *in vitro*, it was observed that, because of their pluripotency, the differentiation was chaotic and unpredictable, making it difficult to study. For example, some cell lines give rise to numerous differentiated derivatives when cultured as dense monolayers (Nicolas *et al.*, 1976).

However, selection and adaptation of EC cells to tissue culture has lead to the establishment of several lines with a more limited differentiation potential (summarised in Silver *et al.*, 1983). The cell line that has been most widely used for *in vitro* studies of differentiation is the F9 cell line.

1.2.3 The F9 cell line

The F9 cells were derived from the transplantable, experimentally induced OTT6050 teratocarcinoma (Bernstine *et al.*, 1973), established by implanting a six-day-old embryo in the testis of a strain 129 mouse (Stevens, 1970). One of the advantages of this cell line is that the karyotype has remained fairly stable over the years (Alonso *et al.*, 1991). They have been used as a model to study a variety of mechanisms, such as the involvement of retinoic acid in gene expression and differentiation and the changes that occur when the ICM differentiates into the two types of extraembryonic endoderm, visceral and parietal, in the mouse embryo. However, because EC cells lose their tumourigenicity when they differentiate (Strickland and Sawey, 1980), they have also been used as a reverse model for transformation, especially in regard to proto-oncogenes.

1.2.3.1 Induction of differentiation

F9 EC cells show very little spontaneous differentiation, both *in vivo* and *in vitro* (Sherman and Miller, 1978). However, treatment with a variety of agents results in their differentiation into endoderm-like cells, depending on the culture conditions

and inducing agent. Treatment of monolayer or suspension cultures of EC cells with retinoic acid (RA) and dibutyryl cyclic AMP (db-cAMP – a more stable analogue of cyclic AMP) results in their differentiation into parietal endoderm-like (PE) cells (Grover and Adamson, 1986; Strickland and Mahdavi, 1978; Strickland *et al.*, 1980). The effects of db-cAMP on EC cells can be potentiated by the cyclic phosphodiesterase inhibitor IBMX, which further increases the half-life, and therefore the levels, of cAMP in the cells, or by other cAMP-elevating chemicals. Differentiation of F9 EC cells to PE has also been achieved using N'-N'-dimethylacetamide and 5-bromodeoxyuridine (Moore *et al.*, 1986), the fluorescent dye Hoechst 33342 (Steuer *et al.*, 1990), as well as certain ginsenosides together with db-cAMP (Lee *et al.*, 1996b). On the other hand, treatment of small aggregates (30 – 50) of EC cells in suspension with RA results in the formation of visceral endoderm-like cells (Hogan *et al.*, 1981). It has been shown, however, that differentiation of F9 cells to visceral or parietal endoderm involves an initially common biochemical pathway and that the two differentiation pathways are reversible for the first day (Grover and Adamson, 1986). These data support the hypothesis (Strickland, 1981) that RA converts EC cells to primitive endoderm-like cells, which, depending on external influences, form either visceral or parietal endoderm. Therefore, differentiation of the F9 cell line closely parallels events in early embryogenesis, where the ICM forms parietal and visceral endoderm, via an intermediate primitive endoderm stage (Figure 1.5). It has been suggested that, since differentiation of F9 cells normally leads to endoderm-like cells, this is a line of primitive endoderm cells, rather than true EC cells. However, there have been reports of F9 cells differentiating into derivatives of all three germ layers – ectoderm, mesoderm and endoderm – depending on culture conditions (Kellermann et al., 1987; Koopman and Cotton,

1986; Koopman and Cotton, 1987), suggesting that F9 EC cells are indeed pluripotent.

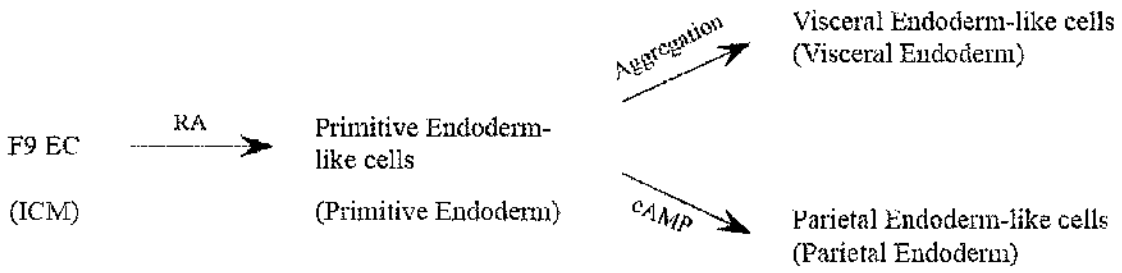


Figure 1.5 The development of extraembryonic endoderm in the F9 embryonal carcinoma (EC) cell system and in the mouse embryo

Treatment of monolayer cultures of F9 EC cells with retinoic acid (RA) leads to the formation of primitive endoderm-like cells. If allowed to aggregate, these cells will form visceral endoderm. On the other hand, if the intracellular cAMP is elevated, they form parietal endoderm (PE). In red are the corresponding stages in the mouse embryo, which the F9 cells mimic during their differentiation. The inner cell mass (ICM) gives rise to the primitive endoderm, which will then form the visceral and parietal endoderm.

It is worth noting that EC cells become committed to differentiate within hours of addition of the inducing reagents. Dong *et al.* (1990) showed that treatment with RA for 3 hours is enough to induce differentiation to visceral endoderm. Similarly, an 8-hour treatment with RA and db-cAMP is enough to commit cells to the PE differentiation pathway (Levine *et al.*, 1984).

Since I have exclusively used PE cells for my experiments, the remainder of this chapter will focus on the events following induction of differentiation of EC cells along this pathway alone.

1.2.3.2 Differentiation-induced changes

Differentiation of F9 cells is accompanied by a large number of changes, both morphologic and biochemical. EC cells normally grow as tightly packed colonies. Treatment with low doses (0.1 μM) of retinoic acid causes them, within 24 hours, to move apart from one another and the colonies to become less compact. Following that, the cells assume a flat morphology, with perinuclear granules (Strickland and Mahdavi, 1978). In the presence of both RA and db-cAMP, the cells become more rounded and often develop neural-like processes that can extend many cell diameters (Kuff and Fewell, 1980; Strickland *et al.*, 1980).

Another dramatic change that takes place following induction of differentiation towards parietal endoderm concerns the rate of proliferation. F9 EC cells have a high proliferation rate, with a doubling time of ~ 12 hours in the exponentially growing phase. The cell cycle is characterised by a very short G1 phase (less than 0.5 hours) and an S phase of about 10 hours, both of which can also be seen in the preimplantation mouse embryo (Rosenstraus *et al.*, 1982). Induction of differentiation by RA alone causes a lengthening of the G1 phase to approximately 2.5 hours and of the S phase to 12.5 hours. In both cases, the G2+M phase was around 2 hours. This increase in G1 and S phases leads to an overall increase in the doubling time of the cells, to about 17 hours (Rosenstraus *et al.*, 1982), which correlates with the onset of morphological changes and decrease in cell growth (Linder *et al.*, 1981).

In order for the cells to achieve the widespread changes mentioned before, it is obvious that major changes in gene expression must occur. Some of the first to be investigated in the F9 system were proteins that had been shown to be important in

the first stages of the mouse embryo. For example, parietal endoderm cells produce a thick basement membrane, known as Reichert's membrane, which they lay down over the cells of the trophoblast, the major components of which are laminin, entactin, type IV collagen and heparan sulphate proteoglycan (Smith and Strickland, 1981). It has been shown that differentiation of F9 EC cells to PE causes increased synthesis of all four of these (Carlin *et al.*, 1983; Strickland *et al.*, 1980; Zhang *et al.*, 1998), as well as plasminogen activator (Strickland and Mahdavi, 1978). Because of the similarities between the embryonic and F9-derived parietal endoderm in regard to these proteins, they have been used as differentiation markers, i.e. proteins the expression of which signifies production of parietal endoderm-like cells (e.g. Strickland *et al.*, 1980).

Over the years, a large number of genes whose expression is altered during differentiation have been identified. For example, alkaline phosphatase, Rex-1 and SSEA 1 expression have been shown to be repressed in PE cells, while Era-1, retinol-binding protein and transthyretin expression is induced (summarised in Alonso *et al.*, 1991). Using cDNA microarrays, Harris and Childs (2002) were able to examine the gene expression pattern of differentiating F9 cells on a larger scale. These researchers observed that induction of gene expression exhibits a biphasic pattern, with an initial peak at around 8 hours following addition of RA and db-cAMP and then a second peak around day 2, resembling the path of differentiation from EC to primitive endoderm and then to parietal endoderm (Figure 1.5). On the other hand, repressed genes were a slowly rising population, probably reflecting the effect of differentiation on global gene expression.

The regulation of some individual genes and proteins has attracted more attention, because of their involvement in cell growth and proliferation, differentiation and apoptosis. These will be described in more detail here.

- Myc and Max

Reduction in c-*myc* expression, in response to differentiation of F9 EC cells to PE, was first observed by Campisi *et al.* (1984). They showed that there is a sharp decline of c-*myc* mRNA within 3 days after induction of differentiation by RA and db-cAMP. It was later shown that reduction of c-*myc* mRNA occurs very early following induction, with a significant decrease observed after 6 hours, while minute amounts could be observed at 24 hours (Dony *et al.*, 1985). It was also observed that it was the half-life of the c-*myc* mRNA, rather than the expression of the c-*myc* gene, that was reduced following induction of differentiation, revealing a post-transcriptional method of regulation (Dony *et al.*, 1985). Further supporting evidence came from the work of Dean *et al.* (1986), who also showed that the decrease in c-*myc* mRNA occurred very early after addition of inducing reagents (between 8 and 12 hours), regulated at the post-transcriptional level. Significantly, c-*myc* mRNA levels were linked with proliferation, rather than differentiation, suggesting that c-*myc* is involved in the growth arrest observed in PE cells (Dean *et al.*, 1986). Investigation of the c-*myc* gene itself revealed no gross deletions or insertions within the gene, and no amplification or difference in copy number was observed, comparing F9 EC DNA with PYS2 (a parietal endoderm cell line) or mouse liver DNA (Griep and DeLuca, 1986).

Experiments using different cell lines suggested that c-*myc* down-regulation was necessary for differentiation (Coppola and Cole, 1986; Lachman and Skoultchi, 1984; Prochownik and Kukowska, 1986; Westin *et al.*, 1982). Griep and Westphal (1988) proposed a similar relationship between c-*myc* and differentiation in F9 cells, by showing that artificially decreasing c-*myc* levels, using antisense *myc* sequences,

induces differentiation. However, Schulz and Gais (1989), employing the reverse method of overexpressing *myc* in F9 cells, showed that differentiation itself is not affected, but rather c-*myc* is involved in the regulation of growth and proliferation, supporting the hypothesis proposed by Dean *et al.* (1986). To further complicate matters, a report from Nishikura *et al.* (1990) showed that enforced changes in c-*myc* mRNA levels, by using sense or antisense *myc* sequences, had no overall effect on proliferation or differentiation of F9 cells. The authors suggested that c-*myc*, although important to growth and differentiation, is not by itself sufficient to induce the changes observed after treatment with RA and db-cAMP, and that the cells in their experiments compensated for the loss or overexpression of *myc* by other mechanisms, with c-*fos* being a possibility. They also stated that, although appearing normal, their c-*myc* sense and antisense clones were pushed near the limit of their ability to compensate. These data overall suggest that c-*myc* plays an important role in the differentiation of F9 EC cells, given that it is regulated very soon following induction of differentiation, and that, at least in some systems, has direct effects on growth, proliferation and differentiation.

Another member of the myc family of proto-oncogenes, N-*myc*, which shares ~40% homology with c-*myc*, has also been shown to be strongly down-regulated in differentiating F9 cells (Sejersen *et al.*, 1986). As with c-*myc*, this reduction is the result of post-transcriptional regulation, but its importance for F9 differentiation is not clearly understood, since it occurs much later than c-*myc* (Sumegi *et al.*, 1986).

Heterodimerisation with the Max protein is thought to be necessary for the function of myc family members as transcriptional activators or repressors, at least for class II genes (reviewed in Grandori *et al.*, 2000). Max is essential for embryonic development, as *max* $^{-/-}$ mice die soon after implantation, exhibiting a generalised developmental arrest of both embryonic and extraembryonic tissues (including

visceral and parietal endoderm). The timing of the lethality in *max* $^{-/-}$ embryos coincides with depletion of maternal Max stores (Shen-Li *et al.*, 2000). *Max* expression is maintained throughout early embryogenesis, which is also true for differentiating F9 EC cells to parietal endoderm (Larsson *et al.*, 1997; Shen-Li *et al.*, 2000). In the latter case, however, Max protein levels have been shown to decline (Larsson *et al.*, 1997).

- p53

In addition to its role as a tumour suppressor, p53 is known to be involved in the regulation of the cell cycle and differentiation (Almog and Rotter, 1997; Gottlieb and Oren, 1996). The observation, therefore, that *p53* null mice exhibited a normal phenotype was somewhat puzzling, suggesting its dispensability for normal development (Donehower *et al.*, 1992). The precise function of p53 in early embryogenesis is not known. In the F9 cell system, p53 seems to respond to differentiation in two ways, one concerning its activity and the other its levels. Although EC cells contain wild type p53 protein (Pennica *et al.*, 1984), it has been shown that it is transcriptionally inactive in unstressed cells, becoming activated in response to differentiation or DNA damage (Lutzker and Levine, 1996; Mayo and Berberich, 1996). This fact might account for the lack of selection for *p53* gene mutations in testicular teratocarcinomas, since none has been reported in this type of tumour. Furthermore, in the case of DNA damage, the F9 cells undergo p53-mediated apoptosis, which may explain the good response of human testicular tumours to chemotherapy (Lutzker and Levine, 1996 and references therein).

Linzer and Levine (1979) first observed that F9 EC cells contain high levels of p53 protein when compared to normal cells – with no adverse effects on cell viability and growth. Differentiation of these cells to parietal endoderm, by treatment with RA and db-cAMP, is followed by a marked decrease in p53 levels, at both mRNA and protein level (Oren *et al.*, 1982; Reich *et al.*, 1983) – even though its transcriptional activity increases (see above). The amount of translatable *p53* mRNA decreased following differentiation by about 20-fold, with a concomitant decrease in protein levels, while the half-life of p53 protein remained relatively unchanged (Oren *et al.*, 1982; Reich *et al.*, 1983). It is worth noting that p53 half-life in F9 EC cells (~ 3 hours) was found to be considerably longer than that of the p53 protein from the non-transformed mouse cell line BALB/c 3T3 (~ 30 min) (Reich *et al.*, 1983). In support of a post-transcriptional mode of regulation, Dony *et al.* (1985) showed that, while the rate of transcription of the *p53* gene was relatively constant through differentiation, the amount of *p53* mRNA sharply declined 2-3 days after induction. Because of the kinetics of p53 down-regulation, coinciding with morphological and cell cycle changes in differentiating F9 cells, the authors suggested that it might be a secondary event, resulting from changes in the cell cycle. Using a slightly different system, Chandrasekaran *et al.* (1982) demonstrated the decrease of p53 protein levels between F9 EC and PYS-2 (a parietal endoderm cell line) cells, relating it to the onset of differentiation, rather than to changes in cell growth. In this report, however, decreased protein stability was suggested as a reason for the decline in p53 levels. Although it appears contradictory to another report, which suggests that protein stability is not affected in response to differentiation (Oren *et al.*, 1982), the difference between the chemically-induced F9 PE cells and the PYS-2 cell line could possibly account for the discrepancy. In contrast to these reports Kosaka *et al.* (1992) demonstrated that, following a rapid and transient decrease, *p53* mRNA levels

increase in differentiated F9 cells. These researchers, however, used temperature-inducible F9 cells, where shifting the culture temperature to 39°C results in differentiation of particular F9 EC mutants to either primitive or parietal endoderm-like cells. Treatment of these cells with RA leads to a decrease in p53 protein levels. Therefore, the authors suggested that reduced amounts of p53 are not necessarily associated with F9 EC differentiation, but may be associated only with RA treatment. Similar observations were made using another differentiation inducer, sodium butyrate (Kosaka *et al.*, 1991). Overall, it seems that regulation of p53 levels is linked to the differentiation of F9 EC cells to PE, rather than to proliferation, with a question mark as to the necessity of p53 down-regulation for differentiation to occur.

- pRb/p107/p130

The product of the retinoblastoma gene, pRb, appears to be essential for mouse development, as loss of both alleles of the *Rb* gene results in the death of the embryo, between 13 – 15 days of gestation. Various abnormalities were observed in *Rb*$^{-/-}$ embryos, especially in the haematopoietic and nervous system, as well as increased apoptosis (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Expression of the *Rb* gene could be detected at very low levels in early embryos (day 9.5 of gestation), subsequently rising and peaking at day 14.5 (Bernards *et al.*, 1989). Induction of *Rb* expression has also been observed in a number of cell types in response to differentiation, such as erythroleukaemia and pre-B cells and myoblasts (Coppola *et al.*, 1990). No such direct evidence exists for F9 cells, but in another embryonal carcinoma cell line, P19, RA-induced differentiation to neuroectodermal cells

(neurons and glia) is accompanied by a marked induction of *Rb* expression, with a subsequent accumulation of Rb mRNA and protein (Slack *et al.*, 1993).

Some indirect evidence concerning the expression of *Rb* in F9 cells before and after differentiation does exist. F9 EC cells have been shown to possess an E1A-like activity, which is down-regulated following differentiation (Imperiale *et al.*, 1984). E1A is an adenoviral protein that is necessary for the expression of another viral gene, *E2A* (Nevins, 1982). One way that E1A achieves this is by sequestering pRb, thus releasing the E2F transcription factor, which then activates *E2A* expression (Bandara and La Thangue, 1991; Raychaudhuri *et al.*, 1991; Zamanian and La Thangue, 1992). One explanation for the presence of an E1A-like activity in F9 EC cells might be that these cells contain very low amounts of pRb. Differentiation-induced up-regulation of *Rb* expression could then result in the disappearance of this E1A-like activity. Consistent with this hypothesis is the very short G1 phase of EC cells, where pRb is though to exert its negative influence on the cell cycle (reviewed in Zheng and Lee, 2001), and a lengthened G1 phase in differentiated F9 cells (Rosenstraus *et al.*, 1982).

Regardless of the actual levels of pRb in F9 EC and PE cells, there is no doubt that pRb function is regulated during differentiation, at least as part of E2F regulation. Going back to the E1A-like activity, two proteins or protein complexes were shown to be required for the efficient transcription of *E2A* in the absence of the E1A protein: ECRE-3, originally called TF68, and E2F, also called DRTF1 (for differentiation-regulated transcription factor 1), the activities of which are strongly down-regulated during differentiation of F9 cells (Imperiale *et al.*, 1984; La Thangue and Rigby, 1987; La Thangue *et al.*, 1990; Murray *et al.*, 1991). Using DNA binding assays, pRb was shown to be able to interact with E2F, to form pRb-E2F-DNA complexes, and that this interaction increased in F9 PE cells (Bandara and La

Thangue, 1991; Partridge and La Thangue, 1991). Furthermore, the interaction between pRb and E2F has been shown to repress E2F-dependent transcription (Hiebert *et al.*, 1992; Zamanian and La Thangue, 1992). Interestingly, ectopic expression of pRb in F9 EC cells does not have an effect on E2F-dependent transcription, as one would expect (Zamanian and La Thangue, 1993). Although this might argue against the hypothesis that the very short G1 phase is due to low levels of pRb, there could be an additional mechanism in EC cells to ensure that pRb is kept inactive. For example, cyclin D1, a known down-regulator of pRb, has been shown to exist at high levels in F9 EC cells, which decline during differentiation to parietal endoderm (Li *et al.*, 1999). Finally, expression of the E1A protein in F9 EC cells does not lead to activation of E2F-mediated transcription (Boeuf *et al.*, 1990). Since E1A activates E2F by sequestering pRb, this could be another indication of the low levels of pRb in F9 EC cells.

The other two members of the retinoblastoma family, p107 and p130, are not individually essential for mouse embryo development, as *p107*$^{-/-}$ or *p130*$^{-/-}$ mice develop normally, indicating that the two proteins are able to substitute for each other functionally (Cobrinik *et al.*, 1996; Lee *et al.*, 1996a). Double knockouts, however, die perinatally, showing deregulated limb development (Cobrinik *et al.*, 1996), suggesting that p107 and p130 perform growth-regulatory functions that cannot be substituted for by pRb. As is the case with pRb, no direct evidence concerning the expression of p107 and p130 in F9 EC and PE cells exists, although it has been shown that the ability of p107 to repress E2F-mediated transcription increases following differentiation (Zamanian and La Thangue, 1993). Ectopic expression of p107 in F9 EC cells does not lead to repression of *E2A* transcription (Zamanian and La Thangue, 1993), suggesting that, even at increased levels, p107 cannot override the cellular mechanisms that regulate *E2A* expression in F9 EC cells.

As mentioned before, F9 EC cells have a very short G1 phase, which is increased following differentiation (Rosenstraus *et al.*, 1982). Progression through the G1 checkpoint is controlled by the D- and E-type cyclins, complexed with CDK4/6 and CDK2, respectively (reviewed in Boonstra, 2003). In the F9 system, mRNA and protein levels of cyclins D1, D3 and E, as well as protein levels of CDK6 and activity of CDK4, have been shown to decline during differentiation to parietal endoderm (Faria *et al.*, 1998; Li *et al.*, 2004; Li *et al.*, 1999). Furthermore, the CDK inhibitor $p27^{Kip}$ is up-regulated in PE cells (Li *et al.*, 2004; Li *et al.*, 1999). $p27^{Kip1}$ is further activated by being released from cyclin D/CDK complexes, due to the reduction in cyclin D levels, and by reduced phosphorylation, due to the falling levels of cyclin E (Li *et al.*, 2004). Surprising, cyclin D2 mRNA and protein levels increase following differentiation (Li *et al.*, 2004; Li *et al.*, 1999). However, in addition to its role in promoting cell cycle progression in some cell systems, it has been suggested that cyclin D2 may function as a negative regulator of cell growth (Meyyappan *et al.*, 1998), which might also be the case in differentiated F9 cells.

- Ras/ERK pathway

The Ras family of proteins is involved in the transduction of extracellular signals that play an important role in cell growth, proliferation and differentiation. Activation of Ras ultimately leads to the activation of ERK, among other proteins (reviewed in Katz and McCormick, 1997). The *ras* family contains three genes, K-, H- and N-*ras*, which are differentially expressed in mice, both in adult tissues and during development (Leon *et al.*, 1987). Not all three genes are necessary for embryonic development, however, as N- and H-*ras* knockout mice are born and grow normally

(Crespo and Leon, 2000; Umanoff *et al.*, 1995). K-*ras* knockout mice, on the other hand, die after 12.5 days of gestation, suggesting that the product of this gene is essential for development (Johnson *et al.*, 1997; Koera *et al.*, 1997).

In differentiating F9 cells, H- and K-*ras* expression is not appreciably affected (Lockett and Sleigh, 1987). Ras activity, however, increases when F9 EC cells are treated with retinoic acid (Verheijen *et al.*, 1999), which when used by itself induces the formation of primitive endoderm-like cells (Grover and Adamson, 1986). Furthermore, transient expression of oncogenic H-Ras was shown to induce differentiation to primitive endoderm, a response mediated by ERK (Verheijen *et al.*, 1999). Further differentiation to parietal endoderm, by treatment with parathyroid hormone (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993), is accompanied by reduction in the activity of endogenous Ras. This is a necessary event, as ectopic expression of oncogenic Ras blocked parietal endoderm differentiation, but by itself was not sufficient for differentiation to occur. As before, the effects of oncogenic Ras were shown to be mediated by ERK (Verheijen *et al.*, 1999).

1.2.4 Virus infection

F9 cells, and other embryonal carcinoma cell lines, have the interesting property of being resistant to infection by a number of viruses, which resembles the situation during the very early stages of embryogenesis. Two well documented examples are two DNA viruses from the papovaviridae family, Simian Virus 40 (SV40) and the murine polyomavirus (PyV). Swartzendruber and Lehman (1975) showed that, following infection of embryonal carcinoma cells by these two viruses, no viral antigens could be detected. Differentiated derivatives of these cells, on the other

hand, were found to be susceptible to viral infection. Further experiments showed that the enhancer sequences of both SV40 and PyV function poorly in F9 EC cells, but increase in activity as cells differentiate (Linney and Donerly, 1983; Sleigh and Lockett, 1985). An additional level of repression was demonstrated by Segal *et al.* (Segal *et al.*, 1979), who showed that the small amount of SV40 transcripts that were produced in EC cells were not processed into mature mRNA. RA-induced differentiation alleviated this block and allowed normal splicing of SV40 mRNA to occur (Segal and Khoury, 1979).

Another well studied example is that of the Moloney Murine leukaemia virus (MoMuLV), a C-type retrovirus. As with SV40 and PyV, the enhancer region of MoMuLV has been shown to function poorly in EC cells, with a subsequent increase in activity following differentiation (Linney *et al.*, 1984). It was observed that viral DNA becomes methylated in EC cells, but only after a considerable lag period following integration (Gautsch and Wilson, 1983; Stewart *et al.*, 1982). This methylation seems to provide an additional regulatory mechanism, since integrated viral genomes were not expressed in differentiated cells, even though enhancer activity was up-regulated. In contrast, production of viral proteins in differentiated cells was observed from newly-infecting genomes, or from integrated genomes following 5-bromodeoxyuridine treatment (Niwa *et al.*, 1983).

Other viruses that have been shown to be differentially regulated in embryonal carcinoma cells and their differentiated derivatives include the minute virus of mice (MVM), the mouse mammary tumour virus and the murine cytomegalovirus, whereas many other viruses, including vaccinia, influenza, vesicular stomatitis virus (VSV), and herpes simplex type 1 (HSV-1) replicate equally well in all cell types (reviewed in Kelly and Condamine, 1982).

1.3 The Ubiquitin – Proteasome System

1.3.1 Introduction

When protein homeostasis was being studied, the major focus for a long time was on gene expression and protein synthesis, with protein degradation relegated to an unregulated, non-specific mechanism. The discovery of the ubiquitin-proteasome pathway helped transform the image of protein degradation to that of a highly regulated and complex process, which serves as the major proteolytic pathway in cells. Because of its wide range of substrates, some of which are presented below, the ubiquitin-proteasome pathway has been implicated in a large number of cellular processes. Cell cycle progression, cell growth and proliferation, differentiation and development, response to environmental stimuli and stresses, the immune and inflammatory responses and apoptosis are all, to some extent, regulated by targeted proteolysis of key components (reviewed in Glickman and Ciechanover, 2002). This pathway is responsible for the degradation of abnormal or misfolded proteins, while non-proteolytic functions have also been described (for reviews, see Aguilar and Wendland, 2003; Hicke, 2001). It is now believed that the majority of protein breakdown in mammalian cells occurs via the ubiquitin-proteasome pathway, while the rest is carried out by the lysosomes (Figure 1.6).

1.3.2 Ubiquitin-mediated degradation

The main function of the ubiquitin-proteasome pathway is the regulated degradation of proteins, in response to various exogenous and endogenous signals. This process involves two discrete and successive steps. First, the protein to be degraded is tagged with multiple ubiquitin molecules, in the form of ubiquitin chains. This tag is subsequently recognised by the proteasome, which degrades the target protein and recycles the ubiquitin. Below, a brief description of the components involved in this processes is given, together with some details of their interactions.

1.3.2.1 Ubiquitin

Ubiquitin is a highly conserved, 76-residue polypeptide. It was first isolated in thymus, but was later found in all tissues and organisms (Goldstein *et al.*, 1975). This ubiquitous presence is, in fact, where ubiquitin got its name. Ubiquitin is essential for proteasome-mediated degradation (with a few exceptions), as it provides the recognition signal for the proteasome. Ubiquitin is conjugated to the substrate protein through its C-terminal glycine residue (G76), which forms an isopeptide bond with a lysine residue on the substrate. Since ubiquitin contains lysine residues, it is able to conjugate to itself, forming polymeric, branched structures, which can take the form of chains (Hershko and Heller, 1985). In fact, degradation of proteins requires, in most cases, the presence of chains of 4 or more ubiquitin moieties, linked to each other at a specific lysine residue (K48) (Chau *et al.*, 1989; Thrower *et al.*, 2000). The importance of K48-linked chains was demonstrated in *Saccharomyces cerevisiae*, where a lysine-to-arginine mutation was shown to be lethal (Finley *et al.*, 1994).

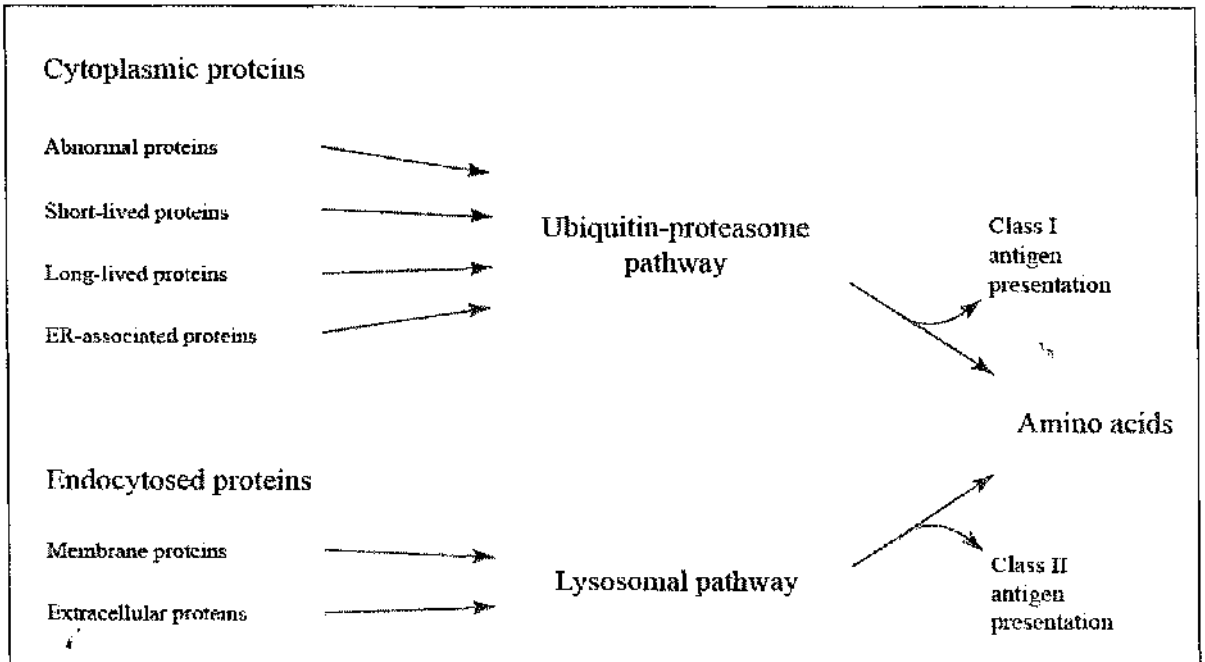


Figure 1.6 Pathways of protein breakdown in mammalian cells

In mammalian cells, most proteins are degraded by the ubiquitin-proteasome pathway. Some of the products from this pathway are used for class I antigen presentation. The lysosomal-endosomal pathway is utilised for the breakdown of extracellular and membrane proteins. This pathway provides peptides for class II antigen presentation.

1.3.2.2 Conjugating enzymes

In order for a substrate to be tagged with a polyubiquitin chain, the sequential action of three classes of conjugating enzymes is needed (Figure 1.7). The E1 enzyme activates ubiquitin and transfers it to an E2 protein. An E3 ubiquitin ligase, which imparts substrate specificity, then catalyses the conjugation of ubiquitin to the target protein.

- E1

Only one ubiquitin-activating enzyme, E1, has been found in most organisms, including humans and yeast, the deletion of which has been shown to be lethal in *S. cerevisiae* (McGrath *et al.*, 1991; Zacksenhaus and Sheinin, 1990). The chemistry of the E1 – ubiquitin reaction is well characterised (Haas and Rose, 1982) and involves the formation of a high energy thiol ester between the enzyme and ubiquitin, in an ATP-dependent manner. This activated ubiquitin is then transferred to one of several E2 enzymes (Figure 1.7). The high efficiency of the E1 enzyme allows the production of sufficient activated ubiquitin for all the downstream conjugation reactions (Pickart, 2001).

- E2

S. cerevisiae contains 11 E2s (also called ubiquitin-conjugating enzymes, ubiquitin-carrier proteins or Ubcs), while many more have been identified in higher organisms (Pickart, 2001). All E2s share a conserved core domain of about 150 amino acids. This domain contains the active site cysteine residue that will accept the ubiquitin from E1, forming another high energy thiol ester bond. Interestingly, the E2 active site lacks any obvious residues that can catalyse this transfer, or the transfer of ubiquitin to the substrate lysine. This has led to the suggestion that the E1 active site contributes to/catalyses the transfer from E1 to E2. Similarly, the E3 enzyme, in addition to bringing the substrate in proximity to the activated ubiquitin (see below), assists in the catalysis of the transfer (Pickart, 2001).

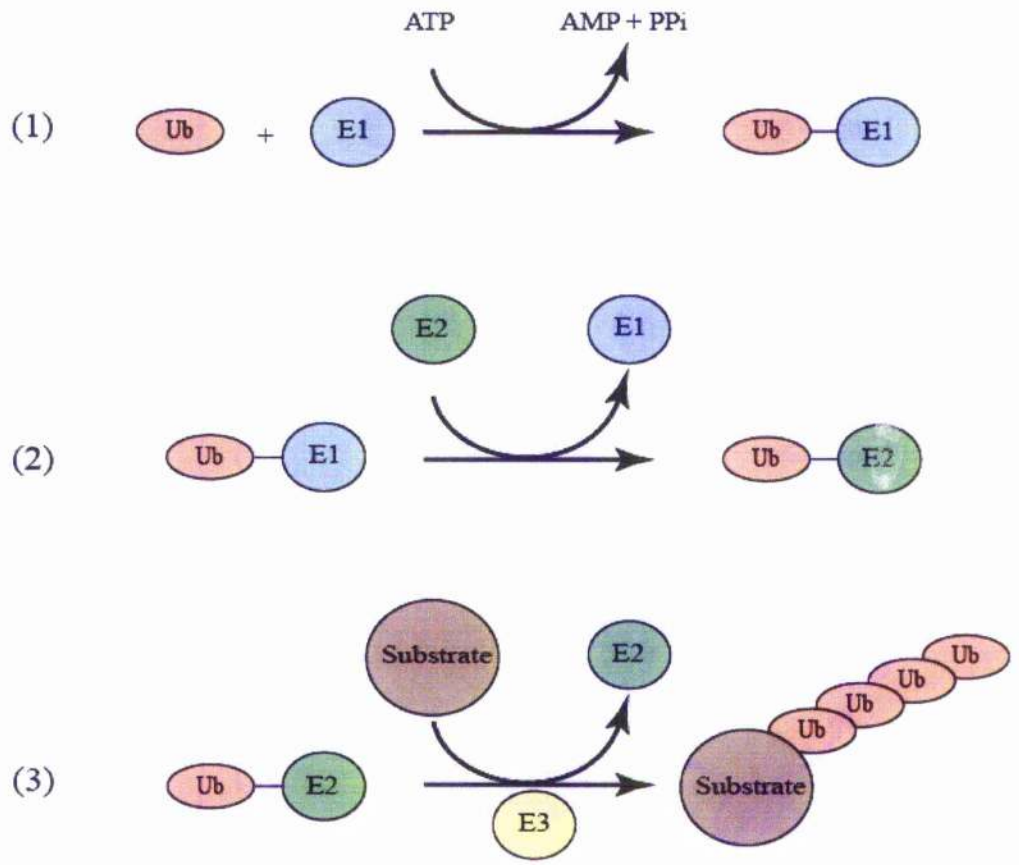


Figure 1.7 The ubiquitin conjugation pathway

Ubiquitin is activated by the ubiquitin-activating enzyme E1, in an ATP-dependent reaction (step 1). The activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme, E2 (step 2). Step 3 provides a simplified version of the final conjugation reaction. An E3 ubiquitin ligase enzyme specifically recognises the substrate, brings it into proximity with the ubiquitin carrying-E2 and catalyses the transfer of ubiquitin to the substrate. HECT family E3s accept the ubiquitin from E2, whereas in the case of RING E3s, the ubiquitin is transferred directly from E2 to the substrate.

As is the case with E1, which can – indeed, has to – interact with multiple E2s, so can E2 proteins interact with different E3s. Thus, the same E2 can play a role in several different processes, depending on the associated E3. Because of this, some E2s have overlapping functions, whereas others have more specific roles. For example, in *S. cerevisiae*, Ubc3/Cdc34 is essential for the transition from G1 to S

phase, while Ubc2/Rad6 is required for degradation of 'N-end-rule' substrates, but also DNA repair (Hershko and Ciechanover, 1998).

- E3

The E3 ubiquitin ligases comprise the largest and most varied family of enzymes in ubiquitin conjugation. The primary role of these enzymes is to bring the substrate protein in close proximity to ubiquitin-carrying E2 and to catalyse the transfer of ubiquitin. The E3 family is divided into two major groups, the HECT and RING E3s.

The HECT (homologous to E6-AP carboxyl terminal) E3s, as the name implies, contain a HECT domain of about 350 amino acids, which mediates E2 binding. This domain contains a strictly conserved cysteine residue, which acts as a site for a thiol ester formation with ubiquitin. In this class of E3s, therefore, the ubiquitin is transferred from E2 to E3 prior to conjugation to the substrate lysine. Substrate specificity is imparted by the unique N-terminus of each family member.

The best characterised example of a HECT E3, and the prototype member of the family, is E6-AP. In papillomavirus (HPV) – infected cells, the viral E6 protein forms a complex with cellular E6-AP (E6-associated protein) and specifically targets the tumour suppressor p53 for proteasomal degradation (Scheffner *et al.*, 1993). Cellular targets of E6-AP in uninfected cells include some Src family tyrosine kinases (Harris *et al.*, 1999; Oda *et al.*, 1999), the nucleotide excision repair factor HHR23A (Kumar *et al.*, 1999) and Mcm7, a subunit of the replication licensing factor (Kuhne and Banks, 1998).

The RING E3s take their name from the RING (really interesting new gene) finger domain. The characteristic feature of this domain is a series of histidine and cysteine

residues which coordinate two zinc ions. Database searches have revealed hundreds of proteins that contain this domain, implicated in a wide variety of cellular functions (reviewed in Borden, 2000; Deshaies, 1999; Joazeiro and Weissman, 2000). Because of the structure of the RING finger and its catalytic inactivity, it has been proposed that these domains function as molecular scaffolds, rather than chemical catalysts (Borden, 2000). Every known E3 without a HECT domain has a RING finger domain or a RING finger protein as its constituent. In contrast to the HECT E3s, however, ubiquitin is not transferred to RING E3s. Rather, it is transferred directly from the E2 enzyme to the substrate protein, although the E3 is still necessary for this process.

RING E3s can be further subdivided into single-subunit and multisubunit enzymes. In the single-subunit E3s, the substrate recognition and RING finger domains are obviously in the same polypeptide, although they are structurally distinct. The RING finger and regions flanking it facilitate E2 binding and transfer of ubiquitin to the target protein (Pickart, 2001). A well-known example of this type of E3 is Mdm2, which is responsible for the targeting of p53 for degradation (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997 and see below).

The multisubunit RING E3s are protein complexes containing a small RING finger protein. As was the case with the single-subunit enzymes, the RING finger protein, usually in conjunction with another protein in the complex, recruits the appropriate ubiquitin-charged E2. Substrate recognition is delegated to an altogether different protein. So far, three types of multisubunit RING E3s have been described. Two of these, the SCF (Skp1/Cullin/F-box protein) and VCB (pVHL/Elongin B/Elongin C) complexes are similar in organisation (reviewed in Tyers and Jorgensen, 2000). Both contain the Rbx1 RING finger protein, which, together with a cullin protein (Cul1 and Cul2, respectively) recruit the appropriate E2. Substrate specificity is imparted

by the F-box proteins in SCF, or pVHL, the product of the Von Hippel – Lindau tumour suppressor gene, in VCB E3s. The third type of multisubunit E3, the APC (anaphase promoting complex), has a different structural organisation, with Apc11 playing the role of the RING finger protein (Page and Hieter, 1999). Targets of the APC include mitotic cyclins, some anaphase inhibitors and spindle-associated proteins, as well as non-mitotic proteins (reviewed in Harper *et al.*, 2002; Page and Hieter, 1999).

1.3.2.3 The proteasome

The 26S proteasome is a very large (about 2.5 MDa), multicatalytic protease that degrades multiubiquitinated proteins into peptides 3 to 22 residues long (Kisselev *et al.*, 1999). The importance of the proteasome for the cell is highlighted by the fact that its subunits are highly conserved among all eukaryotes, and, in yeast, only three are not lethal when deleted (reviewed in Voges *et al.*, 1999). Proteasomes are present in the nucleus and the cytoplasm of all eukaryotic cells (Wojcik and DeMartino, 2003).

The proteasome is composed of two functional subcomplexes, the 20S catalytic particle and the 19S regulatory particle (Figure 1.8). The 20S particle has a barrel-like structure, consisting of four rings, with 7 different subunits each, stacked on top of each other. The two inner rings, termed β, contain the 6 catalytic sites of the proteasome, positioned on the inner surface of the barrel-like structure. The combination of these catalytic sites allows the proteasome to cleave proteins and peptides between any two amino acids (reviewed in Orlowski and Wilk, 2000). The two outer rings, termed α, are thought to regulate the access to the catalytic core, as

well as the interactions with the 19S particles, which cap both sides of the 20S complex (Figure 1.8).

The 19S regulatory particle is responsible for recognising and binding the multiubiquitin chain, which it subsequently removes from the substrate. The untagged protein is then unfolded and translocated into the catalytic core of the proteasome (Voges *et al.*, 1999). The 19S complex is made up of at least 16 subunits, divided into the base and the lid (Figure 1.8). The base consists of 6 different AAA ATPases, which exhibit chaperone-like activity and may contribute to the unfolding of the target protein, and two non-ATPase regulatory proteins. Because of its interaction with the α ring of the 20S particle, the base might also play a role in the translocation of the target protein. The lid component of the 19S complex consists of at least 8 subunits, most of which are thought to be non-ATPase regulatory proteins. The exact function of all the subunits is still unclear, although one, Rpn11/S3, has been shown to be able to remove multiubiquitin chains from substrates (Verma *et al.*, 2002; Yao and Cohen, 2002). Deubiquitination of proteins is essential for their subsequent degradation.

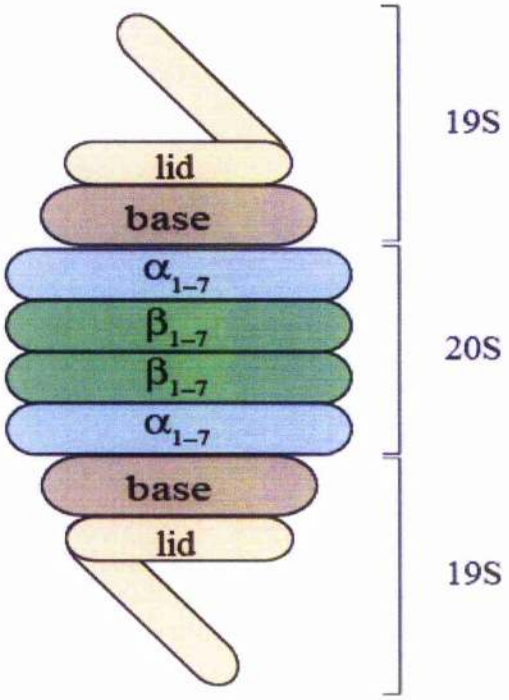


Figure 1.8 The 26S proteasome

A schematic representation of the proteasome and its subunits. It is composed of the 20S catalytic particle and two 19S regulatory particle, one at each end. The 20S complex forms a barrel-shaped structure, made up of four rings, two α and two β, each consisting of 7 non-identical subunits, stacked on top of each other. The 19S complex is responsible for the recognition of tagged proteins and their preparation for degradation by the 20S core. It consists of two subcomplexes, the base and the lid.

1.3.3 Degradation signals

Even though the enzymatic cascade responsible for the conjugation of ubiquitin to a substrate protein and its ultimate degradation by the proteasome is fairly well characterised, the recognition signals required for marking proteins for proteolysis are only now beginning to be clarified. These degradation signals appear to be

sequence or structural elements, mediating the interaction between the substrate and the appropriate E2/E3 complex.

The first of these elements to be identified was the destruction box of the mitotic cyclins, A and B (Glotzer *et al.*, 1991), and many other substrates of the APC (Deshaies, 1999; Koepp *et al.*, 1999; Page and Hieter, 1999). The minimal motif is 9 amino acids long and has the consensus sequence of R-A/T-A-L-G-X-I/V-G/T-N, with the arginine (R) and leucine (L) residues being absolutely conserved (Yamano *et al.*, 1996). Interestingly, the destruction box is neither ubiquitinated nor phosphorylated (see below), so it may serve as a binding site for the ligase subunit of the APC.

Recently, phosphorylation of the target protein has emerged as an important regulatory mechanism for the degradation of many proteins. Indeed, phosphorylation of numerous substrates seems to be necessary for recognition by the ubiquitin conjugation apparatus. For example, the Cdk inhibitor Sic1p and the STAT1 transcription factor are both targeted for degradation following phosphorylation (Kim and Maniatis, 1996; Schneider *et al.*, 1996). Rechsteiner and Rogers (1996) proposed that the PEST motif, a region rich in proline, glutamate, serine and threonine residues found in many short-lived proteins, might serve as a degradation signal. It has since been pointed out that PEST elements contain numerous S/TP sites, which are minimum phosphorylation sites for Cdks and other kinases (Yaglom *et al.*, 1995). Indeed, in several, but not all, cases, phosphorylation of residues within the PEST element are necessary for degradation, e.g. the yeast G1 cyclins Cln2 and Cln3, the mammalian G1 cyclins D1 and E, IκBα and β-catenin (Hershko and Ciechanover, 1998). In contrast, phosphorylation in some cases actually prevents degradation, presumably by masking the degradation signal. This type of regulation has been

demonstrated for c-Fos and c-Jun, following phosphorylation by MAP kinases (Musti *et al.*, 1997; Okazaki and Sagata, 1995).

Another type of degradation signal, which has been demonstrated for the yeast mating type transcription factor α2, is a hydrophobic surface on an amphipathic helix, recognised by a Ubc6/Ubc7-containing complex (Johnson *et al.*, 1998).

A well-characterised degradation signal, but with so far only one possible physiological candidate, is the so-called 'N-end rule' (Varshavsky, 1996). Using artificial substrates, it was demonstrated that ubiquitination and degradation was determined by the nature of the N-terminal amino acids. Rao *et al.* (2001) showed that a 33 kDa fragment produced during chromatid sister separation is degraded by the N-end rule pathway, an event that is essential for chromosome stability.

1.3.5 Cellular targets of the ubiquitin-proteasome pathway

A large number of proteins have been shown to be targeted to the proteasome for degradation. In recent years, in addition, non-degradation roles of the ubiquitin-proteasome pathway have become apparent. As a result, the ubiquitin-proteasome pathway is involved in the regulation of many cellular processes. A few examples are displayed in Table 1.1, along with particular cellular targets.

Table 1.1 Targets of the ubiquitin-proteasome pathway

Process	*Target(s)*	*References*
Cell cycle	e.g. cyclin A, B, D1, E, Cdk inhibitors	(Reed, 2003)
Transcription	e.g. E2F-1, Fos, Jun, Myc, p53, Hif1α, β-catenin, IκBs	(Hershko and Ciechanover, 1998; Muratani and Tansey, 2003)
Signal transduction	membrane receptors, e.g. growth hormone receptor, platelet-derived growth factor receptor	(Hicke, 1999)
Antigen processing	antigenic proteins	(Sijts et al., 2001)

1.3.6 Proteasome inhibitors

The identification of substrates of the ubiquitin-proteasome pathway, as well as of cellular processes that it is involved in, has been greatly assisted by the development of pharmacological inhibitors of the proteasome. These compounds can readily enter cells and, reversibly or irreversibly, block the proteolytic functions of the proteasome.

The best characterised and most widely used class of proteasome inhibitors is the peptide aldehydes. Compounds like MG115, MG132, ALLN and PSI are substrate analogues, primarily blocking the chymotryptic-like activity of the proteasome, which is thought to be rate limiting (Lee and Goldberg, 1998). Inhibition by peptide aldehydes can be reversed simply by their removal from the growth medium. Although they are very potent, the do not significantly affect cell viability and growth for 10 to 20 hours (Lee and Goldberg, 1998). One drawback, however, is that they are not entirely specific to the proteasome, as they have been shown to inhibit certain lysosomal proteases and the calpains.

Similar in structure, but chemically different, the peptide boronates are the newest and most potent proteasome inhibitors. They act by binding irreversibly to the active sites of proteasomes, without affecting the function of other cellular proteases (Adams *et al.*, 1998).

Lactocystin (or lactacystin), chemically and structurally different from the peptide inhibitors, is a naturally-occurring proteasome inhibitor, isolated from actinomycetes (Omura *et al.*, 1991). It irreversibly blocks proteasome function by covalently binding to β subunits of the 20S particle, resulting in the inhibition of the chymotryptic- and tryptic-like activities (Craiu *et al.*, 1997; Fenteany *et al.*, 1995). Lactocystin, which converts into its active form β-lactone in aqueous solutions, can also inhibit cathepsin A (Ostrowska *et al.*, 1997).

1.4 Objectives

The specific down-regulation of TFIIIB activity during the differentiation of F9 embryonal carcinoma cells to parietal endoderm-like cells has been demonstrated previously (Alzuherri and White, 1998; White *et al.*, 1989). The aim of this study was to elucidate the precise mechanism by which this down-regulation occurs. Since differentiation of these cells results in the loss of their transformed state, the mechanism by which pol III transcription is down-regulated could provide insights into the deregulation observed in transformed cells and tumours.

CHAPTER 2

Materials and Methods

2.1 Cell culture

Cell culture was performed in a class II hood, using aseptic technique and sterile equipment and reagents. The F9 cell line was routinely cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% foetal bovine serum (FBS), 1 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma) in a humidified atmosphere containing 5% CO_2 at 37°C. From here on, this will be referred to as growth medium. The stable cell lines derived from the F9 cells were cultured in growth medium which contained 500 µg/ml G418 sulphate (PAA laboratories) and referred to as selection medium. Differentiation of F9 EC cells was induced by addition of 0.1 µM retinoic acid (RA), 1 mM dibutyryl cyclic AMP (db-cAMP) and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) (all from Sigma) to growth medium, or selection medium for the stably-transfected clones, and will be referred to as differentiation medium from here on. Cells were kept in differentiation medium for 6-7 days, with the medium changed every 2 days.

F9 EC cells were passaged when subconfluent; approximately every 2 to 3 days. After media was aspirated from the flask, 2 ml of buffered trypsin-EDTA (0.05% trypsin, 0.02% EDTA, Sigma) were added to the cells and then aspirated immediately. A further 2 ml were added and left for approximately 2 minutes at 37°C. Following trypsinisation, fresh media was immediately added to the

dissociated cells in order to neutralise the trypsin. Appropriate volume of this cell suspension was then used.

Cryo-freezing was used for storage of all cell lines. Cells were trypsinised as described above and, following pelleting by centrifugation, they were resuspended in a solution of 80% DMEM, 20% FBS and 10% dimethylsulphoxide (DMSO, Sigma). Cells were aliquoted into cryo-tubes and frozen in stages, by initially being placed at –80°C overnight and subsequently being transferred to liquid nitrogen storage. Thawing of cells was performed rapidly by placing cryo-tubes in a waterbath at 37°C until just thawed. Cells were then mixed with fresh media, centrifuged and the supernatant aspirated off to ensure removal of DMSO prior to resuspension in growth medium.

2.2 Optimisation of transfection using β-galactosidase assays

In order to optimise the transfection process, varying amounts of CMV-β gal plasmid were transfected into cells using varying volumes of either Superfect (Qiagen) or Lipofectamine (Invitrogen). At the time of transfection, cells were ~80% confluent in a 6cm dish. After following the transfection protocol recommended by each manufacturer the transfected cells were washed twice in PBS, re-grown in DMEM/FBS and harvested in 400 µl 1xRLB (Promega; 25 nM Tris-H_3PO_4, pH 7.8, 2 mM 1,2-diaminocyclohexane tetraacetic acid, 2 mM dithiothrietol, 10% glycerol and 1% Triton X-100), microcentrifuged at 10,000 rpm for 5 minutes to remove cell debris and the lysates analysed for reporter gene activity.

For the β-galactosidase assays, 100 µl of cell lysate were incubated with 100 µl of 2x β– gal reagent (200 nM sodium phosphate buffer, pH 7.2, 2 nM magnesium chloride, 100 mM β-mercaptoethanol, 1.3 µg/ml ONPG) and incubated at 37°C until a yellow colour developed. Reactions were stopped by the addition of 600µl of 1M sodium carbonate and the activity was measured in a spectrophotometer (Pharmacia Biotech Ultraspec 1000) at 420 nm.

2.3 Stable transfection

F9 EC cells were plated out at 10^6 cells/10 cm dish 24 hours prior to transfection, resulting in a confluency of ~70 – 80% at the time of transfection. Two 10 cm dishes were transfected with 12 µg of pcDNA3.1-HA-Hs*BRF1* construct, while a further two 10 cm dishes were transfected with 12 µg of the empty pcDNA3.1-HA vector (Invitrogen). Mastermixes for each set of 10 cm dishes were made up comprising the appropriate plasmid DNA and DMEM. Then, for each transfection, 24 µl of Superfect reagent was added, mixed gently and incubated at room temperature for 10 minutes to allow DNA-Superfect complexes to form. While complexes were forming, cells were washed once with 2 ml of PBS. For each transfection, 2.4 ml of growth medium was added to the tube containing the complexes. This was mixed gently and the diluted complex solution overlaid onto the rinsed cells. Cells were incubated with the complexes for 3 hours at 37°C in a CO_2 incubator. Following incubation, the media was aspirated off and the cells washed twice with PBS. Fresh growth medium was then added and the cells were allowed to grow for a further 24 hours before adding the selection agent G418 sulphate, at a concentration of

500 μg/ml, to the culture medium. Fresh selection medium was replaced every four days. After about five days cells began to die, but were split if they reached confluency before massive cell death began. After two to four weeks, healthy G418-resistant colonies were isolated and transferred to individual plates or wells. Potential clones were then screened using HA-hBrf1 specific assays to identify the clones with the highest levels of HA-hBrf1. This was achieved by Western blot using an antibody against the HA tag.

2.4 Preparation of whole-cell extracts

All extracts were prepared from cells grown in 10 cm tissue culture dishes to facilitate scraping and were harvested at approximately 80% confluency or after 6-7 days in differentiation medium. Preparation was performed on ice as rapidly as possible, and all solutions and tubes were kept ice-cold to maintain cell activity. Two different methods were employed. In the first, cells were washed twice with 5ml of PBS before being scraped with a plastic spatula into 5 ml of ice-cold PBS. Cells were collected in 50 ml Falcon tubes and pelleted by slow centrifugation at 1500 rpm for 5 minutes at 4°C. A small volume of fresh ice-cold PBS was used to resuspend the cell pellets and allow the cells to be transferred to eppendorf tubes. These were then microcentrifuged at 2000 rpm for 2 minutes at 4°C to re-pellet the cells and the PBS removed. The volumes of the cell pellets were then estimated by comparing them with pre-measured volumes of water. Microextraction requires pellets to be between 50 – 150 μl, giving approximately 0.5 – 3 x 10^7 cells; larger pellets were subdivided. An equal volume of freshly made pre-cooled microextraction buffer (20 mM Hepes pH 7.8, 450 mM NaCl, 25% glycerol, 50 mM NaF, 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) was added to the cells and, following resuspension, the cells were immediately snap-frozen on dry ice. Cells were then placed in a 30°C waterbath until just thawed before being immediately returned to dry-ice. This freeze-thaw procedure was performed a total of 3 times to ensure optimal cell lysis, with cells then being microcentrifuged at 13,000 rpm for 7 minutes at 4°C after the third thaw. The supernatant was carefully transferred into a fresh tube, ensuring that the cell debris was left behind, and then promptly aliquoted and snap frozen. These whole-cell extracts were then stored at –70°C. This method of protein extraction was used for most experiments.

For experiments examining levels of phosphorylated proteins, an alternative extraction protocol was followed. Extracts prepared in this way are termed cell lysates. Cells grown on 10 cm dishes were washed twice with PBS. After the second wash, care was taken to remove all the PBS from the dishes. Subsequently, the cells were scraped into 250 μl of lysis buffer (20 mM HEPES pH 7.8, 150 mM NaCl, 25% glycerol, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM DTT, 0.2 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 0.5 mM PMSF, 40 μg/ml bestatin, 1 μg/ml trypsin inhibitor, 0.7 μg/ml pepstatin, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin), transferred to eppendorf tubes and incubated on ice for 10 minutes. After the incubation was completed, the cells were passed through a 26-gauge needle three times and centrifuged at 4°C for 10 minutes. The supernatant, containing the soluble proteins, was finally transferred to another eppendorf tube and snap frozen. The cell lysates were then stored at -70°C.

2.5 Measuring protein concentration

The protein concentration of samples was determined using the Bradford assay. Bradford reagent (Biorad) was diluted in distilled water (1-in-4 dilution). 1 ml of the diluted reagent was added to 1 µl of protein extract and mixed. The absorbance of the sample was then measured in a UV spectrophotometer at 595 nm. To estimate the protein concentration of the sample, a standard curve was constructed, with a concentration range of 0 to 12 mg/ml, using a 10 mg/ml stock solution of bovine serum albumin (BSA). If the sample being measured fell outside the linear range of the standard set, appropriate dilutions were made and the samples re-measured.

2.6 Separation of proteins by polyacrylamide gel electrophoresis

Proteins were resolved on denaturing polyacrylamide gels according to molecular weight by electrophoresis. Typically, 7.8% polyacrylamide resolving minigels (375 mM Tris pH 8.8, 0.1% SDS) were used with a stacking layer comprised of 4% polyacrylamide gel (125 mM Tris pH 6.8, 0.1% SDS) based on the discontinuous buffer system described by Laemmli (1970). Samples were boiled for 2 minutes in 1x protein sample buffer (62.5 mM Tris pH 6.8, 0.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.125% bromophenol blue) prior to loading. Electrophoresis was performed in 1 x SDS running buffer (0.1% SDS, 76.8 mM glycine, 10 mM Tris, pH 8.3) at a voltage of 150-200 V. The progress of the electrophoresis was monitored

using Rainbow molecular weight markers (Amersham Biosciences) or Prosieve colour protein markers (Cambrex).

2.7 Western blotting

Electrophoretic transfer of proteins resolved by SDS-PAGE to nitrocellulose (BioRad) or PVDF (Amersham Biosciences) membranes was achieved using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system. Transfer was carried out in 1 x transfer buffer (76.8 mM glycine, 10 mM Tris pH 8.3, 20% methanol) at 50 V for 1 hour. Correct transfer and equal loading was ensured by washing the membrane in Ponceau S solution (0.1% Ponceau S, 5% acetic acid; Sigma) for 5 min and then rinsing it with distilled water until bands were clearly visible on the membrane. After washing the membrane clean of dye, it was blocked in milk buffer [wash buffer (32.5 mM Tris, 150 mM NaCl, 0.2% Tween-20), plus 5% skimmed milk powder (Marvel)] for 1 hour at room temperature. Membranes were incubated with primary antibodies (typically a 1:1000 dilution in milk buffer) for 2 hours at room temperature or overnight at 4°C. Excess primary antibody was removed by washing the blot 3 times for 3 minutes in wash buffer before incubating for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000 dilution in milk buffer; DAKO). To ensure removal of excess secondary antibody, the blot was sequentially washed in batches of wash buffer, 3 times for 3 minutes, followed by 2 washes for 15 minutes. After one further 5 minute wash using 1 x TBS (2.5 mM Tris-HCl pH 7.6, 15 mM NaCl), the blot was developed using the enhanced chemiluminescence method (ECL and ECL+; Amersham Biosciences) as directed by the manufacturer.

2.8 Antibodies

Table 2.1 Antibodies

Antibody	*Source*
anti-actin (C-11)	Santa Cruz
anti-Bdp1 (2663)	(Schramm et al., 2000)
anti-Brf1 (128)	(Cairns and White, 1998)
anti-Brf1 (CSH145)	Generously donated by Nouria Hernandez
anti-c-Fos (4)	Santa Cruz
anti-CK2 α & α' (H-286)	Santa Cruz
anti-c-Myc (N-262)	Santa Cruz
anti-cyclin D1 (72-13G)	Santa Cruz
anti-HA (F-7)	Santa Cruz
anti-laminin B1 (A-1)	Santa Cruz
anti-Mad1 (C-19)	Santa Cruz
anti-Mad2 (G-16)	Santa Cruz
anti-Mad3 (H-206)	Santa Cruz
anti-Mad4 (N-19)	Santa Cruz
anti-Max (C-17)	Santa Cruz
anti-p44/42 MAPK (9102)	Cell Signalling Technology
anti-p53 (1C12)	Cell Signalling Technology
anti-p107 (C-18)	Santa Cruz
anti-p130 (C-20)	Santa Cruz
anti-phospho-p44/42 MAPK (9106)	Cell Signalling Technology
anti-phospho-pRb (9307)	Cell Signalling Technology
anti-pRb (IF8)	Santa Cruz
anti-TBP (58C9)	Santa Cruz
anti-TFIIB (C-18)	Santa Cruz
anti-TFIIIC110 (4286)	(Winter et al., 2000)

2.9 Preparation of total cellular RNA

Total cellular RNA was isolated from cells when approximately 80% confluent, or after 6-7 days in differentiation medium, using TRI reagent (Sigma), a solution of guanidine thiocyanate and phenol, in accordance with the manufacturer's instructions. Media was aspirated off cells grown in 10 cm tissue culture dishes and residual media removed with two washes using 5 ml ice-cold PBS. Cells from each dish were harvested by scraping in 1 ml of TRI reagent per dish and transferred to a

sterile eppendorf tube. Cells were left to stand for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform were then added to each tube and the samples vortexed for 15 seconds. The samples were then allowed to stand for a further 15 minutes at room temperature prior to being centrifuged at 13,000 rpm for 15 minutes at 4°C. This resulted in separation of the samples into three phases: a lower red organic phase containing protein, a middle white interphase containing precipitated DNA and an upper colourless, aqueous phase which contains the RNA. These upper phases were carefully removed, ensuring no contamination from the remaining phases, and transferred to fresh eppendorf tubes. Isopropanol (500 µl) was added to each of these tubes containing the aqueous RNA and thoroughly mixed by repeated inverting. Following 5 - 10 minutes incubation at room temperature to allow maximal precipitation of RNA, samples were centrifuged at 13,000 rpm 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 dH_2O (0.1% DEPC), thoroughly mixed into solution, left overnight at room temperature and then autoclaved to inactivate the remaining DEPC. The samples were vortexed briefly, subsequently microcentrifuged at 13,000 rpm for 5 minutes at 4°C and the supernatant aspirated off. Residual supernatant was removed with a P20 pipette following pulse microcentrifugation. Appropriate volumes of DEPC-dH_2O, pre-warmed to 65°C, in the range of 10 – 30 µl, were added to the RNA pellets and the samples were heated in a 65°C waterbath for 10 – 15 minutes to facilitate resuspension of the RNA. The samples were stored at –70°C.

2.10 Measuring RNA concentration

RNA concentration was determined by adding 4 µl of the RNA extract to 200 µl of 10mM Tris pH 7.6 and measuring the absorbance of the sample at 260 and 280 nm using a UV spectrophotometer. The calculation used was: RNA concentration (mg/ml) = absorbance at 260 nm x 40 x dilution factor / 1000. A ratio of absorbance at 260nm to 280nm in the range of 1.8 – 2 indicated the RNA samples were relatively free from contamination with phenol or protein.

2.11 Northern blot analysis of total cellular RNA

Typically, RNA samples of 10 – 30 µg were used in analysis, made up to a total volume of 10 µl with DEPC-dH_20. 10 µl of 2 x RNA sample buffer (1 x MOPS comprised of solutions made up with DEPC-dH_20 (20 mM MOPS pH 7.0, 8 mM sodium acetate, 1 mM EDTA pH 8.0), 4.4 M formaldehyde, 54% formamide) were added to each sample prior to heating at 65°C for 15 minutes to denature the RNA secondary structure. The samples were immediately transferred to ice to prevent any renaturation and 2 µl of 1 mg/ml ethidium bromide and 2 µl of 10 x RNA loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF) were added to each sample. Following a 20 minute pre-run at 40 V of a denaturing gel (1% agarose, 2.2 M formaldehyde, 1 x MOPS) in 1 x MOPS, samples were loaded and run for approximately 5 hours at 40 V in order to electrophoretically separate the different species of RNA according to size. The gel was visualised under a UV transilluminator in order to confirm separation and photographed. It was then

washed for 20 minutes in 20 x SSC buffer (3 M NaCl, 0.3 M sodium citrate pH 7.0) prior to capillary transfer as described in Sambrook and Russell (2001)

The transfer procedure required the prepared gel to be placed, inverted, on a bridge of Whatmann 3mm chromatography paper supported on a glass plate and suspended over a reservoir of 20 x SSC buffer. An appropriate size of Hybond N nylon membrane optimised for nucleic acid transfer (Amersham Biosciences) was pre-soaked in 20 x SSC and positioned over the gel, followed by a further two layers of pre-soaked Whatmann paper; at each stage of layering, care was taken to ensure removal of air bubbles. This arrangement was surmounted with a stack of paper towels and an appropriate weight in order to allow efficient capillary action. During transfer, the migration of the RNA from the gel to the nylon membrane is facilitated by the passive movement of the transfer solution through the gel. Plastic wrap was used to prevent a direct contact between the paper towels and the Whatmann bridge; this ensured movement of the buffer was only through the gel. In order to achieve high-transfer efficiency, the capillary action was allowed to proceed overnight. Following transfer, the RNA was fixed to the membrane by UV-crosslinking at 1200 μJ and was then washed for 5 minutes in DEPC-dH_20.

Radiolabelled DNA probes complementary in sequence to a particular RNA of interest were used to locate it on the membrane. The pol III B2 gene probe was a 0.2 kb *Eco*RI-*Pst*I fragment from pTB14 (White *et al.*, 1989) and the pol II acidic ribosomal phosphoprotein P0 (ARPP P0) probe a 1 kb *Eco*RI-*Hind*III fragment from the mouse cDNA (Hurford *et al.*, 1997). The probes were labelled using a Megaprime DNA labelling system (Amersham Biosciences) according to the random oligonucleotide priming method of Feinberg and Vogelstein (1984). This method involved the addition of 5 μl of random hexamer oligonucleotide sequences and the

appropriate volume of DEPC-dH_20 for a final volume of 50 µl in the total reaction to 25 ng of purified DNA template, which was subsequently denatured by heating at 95°C for 5 minutes. DNA synthesis is primed by the hexamer oligonucleotides which are able to anneal to the DNA during slow cooling to room temperature. Labelling was carried out at 37°C for 1 hour in 1 x reaction buffer (containing Tris-HCl pH 7.5, β-mercaptoethanol, $MgCl_2$; Amersham Biosciences) following the addition of 4 µl each of dATP, dGTP, dTTP (in Tris pH 8.0, 0.5 mM EDTA), 50 µCi of [α-^{32}P] dCTP (10 mCi/ml, 3000 Ci/mmol; Amersham Biosciences) and 2 units DNA polymerase I Klenow fragment (in 100 mM potassium phosphate pH 6.5, 10 mM β-mercaptoethanol, 50% glycerol). The reaction was stopped by heating at 80°C for 5 minutes and kept at 4°C until the nylon membrane with bound RNA had been pre-hybridised. This involved rotation in a hybridisation oven at 45°C for 45 minutes in 20 ml of hybridisation buffer (0.2 M sodium phosphate buffer pH 7.2, 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS, 45% (w/v) formamide in DEPC-dH_20). Following this, the radiolabelled probe was added to 20 ml of fresh hybridisation buffer, in which the membrane was incubated with rotation at 45°C overnight. The nylon membrane was then washed with rotation in wash buffer (40 mM sodium phosphate buffer pH 7.2, 1 mM EDTA, 1% (w/v) SDS in DEPC-dH_2O) at room temperature for 2 minutes and then twice for 15 minutes at 65°C in order to remove non-specific radioactivity before being exposed to autoradiography film overnight at -70°C. Membranes were stripped by incubating in boiling water for 5 minutes and pre-hybridised again prior to being reprobed.

2.12 Preparation of cDNAs

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

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

PCRs were carried out using Proteus II (Helena Biosciences) or TC-312 (Techne) thermal controllers. 1 μl of cDNA was amplified with 20 pmol of the appropriate primers listed in Table 2.2. Amplification reactions contained 0.5 U of *Taq* DNA polymerase (Promega) in 20 μl of 1 x *Taq* DNA polymerase buffer (Promega) containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 1.8 μCi of [α-^{32}P] dCTP (10 mCi/ml, 3000 Ci/mmol; Amersham).

PCR was performed under the following cycling parameters:

5S rRNA: 95°C for 3 minutes, 18 cycles of [95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minutes], 72°C for 5 minutes.

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

Bdp1: 95°C for 2 minutes, 25 cycles of [95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute], 72°C for 5 minutes.

HsBrf1: 95°C for 2 minutes, 25 cycles of [95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds], 72°C for 5 minutes.

Hs & MmBrf1: 95°C for 2 minutes, 25 cycles of [95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds], 72°C for 5 minutes.

MmBrf1: 95°C for 2 minutes, 25 cycles of [95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds], 72°C for 5 minutes.

HPRT: 95°C for 2 minutes, 25 cycles of [95°C for 30 seconds, 66°C for 30 seconds, 72°C for 30 seconds], 72°C for 5 minutes.

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

Reaction products were resolved on 7% polyacrylamide sequencing gels containing 7 M urea and 1 x TBE (45 mM Tris, 45 mM boric acid, 0.625 mM EDTA pH 8.0). Gels were pre-run for 30 minutes at 40 W in 1 x TBE and 1.5 µl of each sample were loaded after being boiled at 95°C for 2 minutes and quenched on ice. Electrophoresis was carried out for a further 1 hour at 40 W and the gel subsequently vacuum-dried at 80°C for 1 hour, before being exposed to autoradiography film, in order to detect the radiolabelled products.

Table 2.2 PCR primers

Gene	*Primers (5' to 3')*	*Product (bp)*
5S rRNA	GGCATACCACCCTGAACGC	107
	CAGCACCCGGTATTCCCAGG	
ARPP P0	GCACTGGAAGTCCAACTACTTC	265
	TGAGGTCCTCCTTGGTGAACAC	
Bdp1	ACCCAGTGAAGAGACCGAGA	194
	TCAGGTCAGCACCTCTTCCT	
HsBrf1	CAGCCAGAATGCATGACTTCAG	95
	AAATTCCGTGAGCCTCTTCCGCAGCG	
Hs/MmBrf1	CAGCCAGAATGCATGACTTCCG	95
	AAATTCTGTGAGCCTCTTCCGTAGCG	
MmBrf1	GGGGAGAAGAACCATGAAGT	240
	AGTGAGCTGACTGGTTGGAG	
HPRT	GTAATGATCAGTCAACGGGGGAC	186
	CCAGCAAGCTTGCAACCTTAACCA	
$tRNA^{Leu}$	GTCAGGATGGCCGAGTGGTCTAAG	88
	CCACGCCTCCATACGGAGACCAGAAGACCC	

The first row in each primer pair represents the forward primer, while the second the reverse primer. Hs: *Homo sapiens*, Mm: *Mus musculus*

2.14 Transformation of competent cells

E.coli XL-1 Blue supercompetent cells (Stratagene) were transformed for plasmid storage and propagation. Cells, which were stored at -80°C and highly temperature sensitive, were thawed on ice to prevent loss of transformation ability. 0.4 µl of β-mercaptoethanol, which enhances transformation efficiency, were added to the 50 µl of cells that were required per transformation reaction to give a final concentration of 25 mM. Typically 10 – 20 ng of plasmid DNA were then gently mixed into the chilled cells. The contents were gently tapped occasionally during a 30 minute incubation on ice, before being heat shocked at 42°C for exactly 45 seconds and then transferred to ice for a further 2 minutes. Cells were incubated at 37°C for 1 hour on

an orbital shaker (225 – 250 rpm) following the addition of 450 µl of preheated (42°C) SOC medium (LB broth, 0.04% glucose, 10 mM $MgSO_4$, 10 mM $MgCl_2$). Typically 150 µl of the transformation mixture was then plated on LB agar (2% LB, 2% agar) plates containing 50 µg/ml ampicillin or kanamycin and the plates were incubated at 37°C overnight to allow growth and colony-formation of the transformed cells.

2.15 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 the selective antibiotic (50 µg/ml ampicillin or kanamycin). This was allowed to incubate with vigorous shaking at 37°C for ~6 hours to form a mini-culture and was subsequently used to inoculate 250 ml of LB medium containing the appropriate antibiotic. Following an overnight incubation at 37°C on an orbital shaker (~300 rpm), cells were harvested by centrifugation at 6,000 *g* for 15 minutes at 4°C and plasmid DNA retrieved using the QIAGEN Plasmid Maxi Kit.

The bacterial pellet was resuspended in 10 ml of Buffer P1 (50 mM Tris pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) and then gently but thoroughly mixed with 10 ml of Buffer P2 (200 mM NaOH, 1% SDS) to initiate an alkaline lysis reaction. This reaction was allowed to proceed for 5 minutes at room temperature before neutralising the lysate by the addition of 10 ml of chilled Buffer P3 (3 M potassium acetate pH 5.5) which subsequently resulted in formation of a precipitate of potassium dodecyl sulphate. The SDS-denatured proteins and chromosomal DNA

were co-precipitated with the detergent whilst the plasmid DNA remained in solution due to a lack of close protein associations. Precipitation was enhanced by a 20-minute incubation on ice and the precipitate pelleted by centrifugation at 20,000 *g* for 30 minutes at 4°C. The supernatant containing plasmid DNA was 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). Gravity flow allowed the supernatant to pass through the anion-exchange resin to which plasmid DNA is able to tightly bind. The resin was then washed twice with 30 ml of Buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol) and the purified plasmid DNA was subsequently eluted with 15 ml of Buffer QF (1.25 M NaCl, 50 mM Tris pH 8.5, 15% isopropanol) and precipitated with 10.5 ml (0.7 volumes) of room-temperature isopropanol. This was immediately followed with a 15,000 *g* centrifugation at 4°C for 30 minutes. The plasmid DNA pellet was then washed with 70% ethanol, dried at room temperature for 5 – 10 minutes and resuspended in an appropriate volume of sterile water or TE buffer, pH 8.0 (10 mM Tris pH 8.0, 1 mM EDTA).

2.16 RNA pol III *in vitro* transcription assay

In vitro transcription of class III genes was reconstituted using 20 µg of cell extracts to provide the basal pol III transcription components. This was supplemented with 250 ng of plasmid DNA containing a specific pol III template and 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% (v/v) glycerol, 1 mM creatine phosphate, 0.5 mM each of rATP, rCTP and rGTP and 10 µCi [α-^{32}P]

UTP (400 mCi/mmol; Amersham Biosciences). Transcription components were assembled on ice and the reaction was performed at 30°C for 1 hour. In the case of assays incorporating additional reagents, a 15 minute pre-incubation at 30°C was carried out prior to adding the nucleotides required to initiate transcription. Transcription was terminated by the addition of 250 µl of 1 M ammonium acetate/0.1% SDS containing 20 µg of yeast tRNA which acts as a stabiliser for the synthesised RNA. Phenol-chloroform extraction of the samples was performed to remove protein and DNA by adding 250 µl of a 25:24:1 ratio solution of phenol/chlorophorm/isoamyl alcohol. The samples were vortexed, microcentrifuged at 13,000 rpm for 5 minutes and 200 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 repeated inversion, left at –20°C overnight before being microcentrifuged at 13,000 rpm for 20 minutes to pellet the precipitated RNA. The supernatant was carefully removed and 750 µl of 70% ethanol was added to each sample to wash the pellet. This was also carefully removed to avoid dislodging the pellet and the samples were heated at 42°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 FF) was added to each sample, which was then vortexed for at least 30 minutes to ensure the RNA was fully redissolved. 1.5 µl of each sample was loaded on a pre-run 7% polyacrylamide sequencing gel containing 7M urea and 1 x TBE (45 mM Tris, 45 mM boric acid, 0.625 mM EDTA pH 8.0) after being boiled at 95°C for 2 minutes and quenched on ice. Electrophoresis was performed at 40 W for 1hour in 1 x TBE before being dried and exposed to autoradiography film in order to detect the radiolabelled transcripts.

The plasmid templates used for *in vitro* transcription assays were as follows: pVA1 is a 221 bp *Sal*I-*Bal*I fragment of adenovirus 2 DNA containing the VA1 gene subcloned into pUC18 (Dean and Berk, 1988); pLeu is a 240 bp *Eco*RI-*Hind*III fragment of genomic DNA containing a human $tRNA^{Leu}$ gene subcloned into pAT153 (McLaren and Goddard, 1986); pMO5S1.1 is a 436 bp *Sac*I-*Nhe*I fragment of mouse genomic DNA containing a 5S gene cloned into pBluescript KS-.

2.17 Electrophoretic mobility shift assay (EMSA)

EMSAs which were carried out using a [γ-^{32}P] labelled oligonucleotide containing a consensus Myc-Max binding site [5'-GGAAGCAGAC**CACGTG**GTCTGCTTCC-3' (E-box F) annealed to the complementary 3' to 5' strand (E-box R); bold-faced letters indicated the E-box sequence]. An oligonucleotide containing a mutated E-box sequence [5'-GGAAGCAGAC**CACGGA**GTCTGCTTCC-3' (Mut F) annealed to the complementary 3' to 5' strand (Mut R); bold-faced letters indicate the mutated E-box sequence] was employed to determine specificity of binding. For use in EMSAs, oligonucleotides were 5' end-labelled using T4 polynucleotide kinase (PNK). 40 ng of E-box F or Mut F oligonucleotide were assembled on ice with 10 U of polynucleotide kinase (PNK) in 1 x PNK buffer (Promega) and following the addition of 20 µCi of [γ-^{32}P] dATP (10 mCi/ml, 3000 Ci/mmol) to give a total volume of 10 µl, the reaction was performed at 37°C for 1 hour. This was stopped by heating at 65°C for 10 minutes and was succeeded by phenol-chloroform precipitation of the labelled oligonucleotides. This was achieved by addition of 50 µl of PhOH/$CHCl_3$/IAA (25:24:1) followed by vortexing and microcentrifugation at

13,000 rpm for 5 minutes. The aqueous layer was transferred to a fresh eppendorf tube and 5 µl of 3 M sodium acetate and 125 µl of 100% ethanol added. Following a 30-minute incubation on dry ice, the oligonucleotide was pelleted by microcentrifugation at 13,000 rpm for 10 minutes. The supernatant was removed and the pellet washed by sequential addition and removal of 100 µl of 70% ethanol to ensure removal of unincorporated label. The pellet was then dried by heating at 47°C for 10 minutes before being redissolved in 20 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) by incubation at 30°C for 30 minutes. This was followed by heating at 90°C for 2 minutes in the presence of unlabelled complementary oligonucleotide (E-box R or Mut R), which was added in 2.5-fold excess (100 ng) to ensure that all labelled oligonucleotide was annealed. The sample was then allowed to cool slowly overnight, after which it was stored at 4°C. Finally, the labelled probe was diluted to 1 ng/µl using TE buffer.

Each binding reaction was performed in a total volume of 10 µl, with an optimal salt concentration range of 60 – 100 mM KCl, and contained 1 µg of poly(dI.dC), 5 µg of cell extract and 1 ng of labelled probe. A pre-incubation of 15 minutes at 30°C was carried out prior to addition of the probe, followed by a further 15 minutes at 30°C. For the competition experiments, 100 ng of unlabelled competitor oligonucleotide (E-box or Mut) were added before the pre-incubation. In the case of the supershift experiments, the reactions were assembled as previously described, and, following addition of the probe (1 ng), pre-incubated at room temperature for 15 min. Recombinant c-Myc (400 ng) or antibodies (4 or 8 µg) were subsequently added and the reactions were incubated for 1 hour at 4°C.

Analysis of the formation of protein-DNA complexes was achieved by electrophoresis of samples on a pre-run, 4% non-denaturing polyacrylamide gel in

1 x TAE buffer (40 mM Tris acetate, 1 mM EDTA pH 8.0) for 1.5 – 2 hours at 4°C. The gel was dried for 1.5 hours at 80°C and exposed to autoradiography film overnight at -70°C.

2.18 *In vitro* proteasome assay

To determine proteasomal activity of whole-cell extracts, the artificial proteasome substrate Succinyl-Leu-Leu-Val-Tyr-AMC (40 μM, Biomol International) was added to 10 μg of cell extract and incubated for 2 hours at 37°C in assay buffer (10 mM Tris-HCl pH 7.8, 1 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$). Where required, the proteasome inhibitor MG132 (50 μM, Biomol International) was added to the cell extract prior to substrate addition and incubated at 37°C for 30 minutes. At the end of the incubation period, free AMC was measured using a luminescence spectrometer (LS-50B, Perkin Elmer; excitation 343 nm, emission 437 nm) and the accompanying software (FL WinLab, Perkin Elmer). Cell extract was omitted for background measurements.

CHAPTER 3

The protein levels of the TFIIIB complex decrease following differentiation

3.1 Introduction

The study of early mammalian embryogenesis is made difficult by factors such as heterogeneity of starting populations and insufficient numbers of stem cells. These problems can be overcome by using cultured embryonal carcinoma (EC) cells. One of the cell lines that has been widely used is the murine F9 cell line, originally isolated by Bernstine *et al.* (1973). The F9 EC cells have lost the ability to differentiate spontaneously, but, when treated with various chemicals, they differentiate into endoderm-like cells, as described in Chapter 1.2.

Early experiments using mouse embryos, from unfertilised oocytes up until the late primitive streak stage (7.5 days post-coitum), showed a strong regulation of pol III transcription (Vasseur *et al.*, 1985). This was achieved by monitoring the steady-state levels of B2 transcripts, which increased substantially following fertilisation. In day 4 blastocysts, high levels of B2 transcripts could be seen in the inner cell mass, but decreased in the trophectoderm. By the late primitive streak stage, B2 production was limited to the embryonic ectoderm and mesoderm, but was severely reduced in the embryonic and extra-embryonic endoderm. Because of the difficulty inherent in such a system (heterogeneity of cells and limited amount of starting material), the F9 teratocarcinoma cell line was used to further investigate the regulation of pol III

transcription following their differentiation into endoderm-like cells. As mentioned above, this cell system closely resembles the differentiation of the inner cell mass into visceral or parietal endoderm cells (Martin, 1980). White *et al.* (1989) showed that transcription of various pol III templates, including B2 and tRNA, is decreased from EC to PE cells. Using fractionated whole-cell extracts from the two cell types, they also showed that TFIIIB, but not TFIIIC, activity is reduced in PE cells. These findings were later confirmed by Alzuherri and White (1998) using more purified TFIIIB and TFIIIC fractions. Furthermore, it was shown that protein levels of Brf1 and TBP, the only two components of mammalian TFIIIB that had been characterised at that time, decrease following differentiation (Alzuherri and White, 1998). Since then, it has become clear that mammalian TFIIIB is comprised, like its yeast counterpart, of three polypeptides, namely TBP, Brf1 (or Brf2, as discussed in the introduction) and Bdp1.

This chapter serves to demonstrate that the cell system used throughout this thesis functions as previously demonstrated. It also presents data as to the effect of cell differentiation on the levels of the TFIIIB complex.

3.2 Results

3.2.1 Differentiation of F9 embryonal carcinoma cells

F9 cells have been extensively used to study events in early embryogenesis because of their ability to differentiate into various cell types, each with different characteristics, that resemble tissues of the early mouse embryo (Figure 1.5). For example, treatment of a monolayer culture of F9 EC cells with retinoic acid (RA) and

dibutyryl cAMP induces differentiation into parietal endoderm (Strickland *et al.*, 1980). My first objective, therefore, was to verify that the F9 embryonal carcinoma (EC) cells differentiated as previously described.

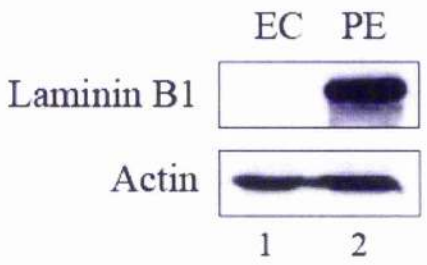
When the F9 EC cells are induced to differentiate to parietal endoderm, their morphology changes considerably. From tightly packed colonies with difficult to distinguish cell-cell boundaries, they become more rounded, move away from each other and often have processes that extend many cell diameters (Strickland *et al.*, 1980). These findings were verified by observation under a light microscope (results not shown). However, we also wanted to employ a biochemical approach to ensure that the cells were differentiating properly. One of the markers for parietal endoderm is the production of laminin (Strickland *et al.*, 1980), which the cells use, in addition to various other proteins, to form a suitable extracellular matrix. Therefore, protein extracts were prepared from undifferentiated (EC) and fully differentiated (i.e., grown in differentiation medium for 7 days – PE) cells and analysed by Western blotting, using an antibody against the β-1 chain of laminin as the marker for differentiation. Figure 3.1A shows that laminin B1 is virtually undetectable in EC cells, but is being produced at high levels following differentiation (compare lanes 1 and 2 in the top panel). The bottom panel of Figure 3.1A shows the levels of actin in the two extracts, which was used as a loading control. Identical results were obtained when a small volume of growth medium from EC and PE cultures was analysed by SDS-PAGE and immunoblotting (results not shown).

Another feature of the differentiated F9 cells is their reduced proliferation rate (Alonso *et al.*, 1991; Rosenstraus *et al.*, 1982). To verify this, and also to determine optimum passaging conditions for tissue culture, a simple experiment was conducted to establish the rate of proliferation of EC cells with or without differentiation-inducing chemicals in their growth medium. 5 x 10^4 cells were seeded on 10-cm

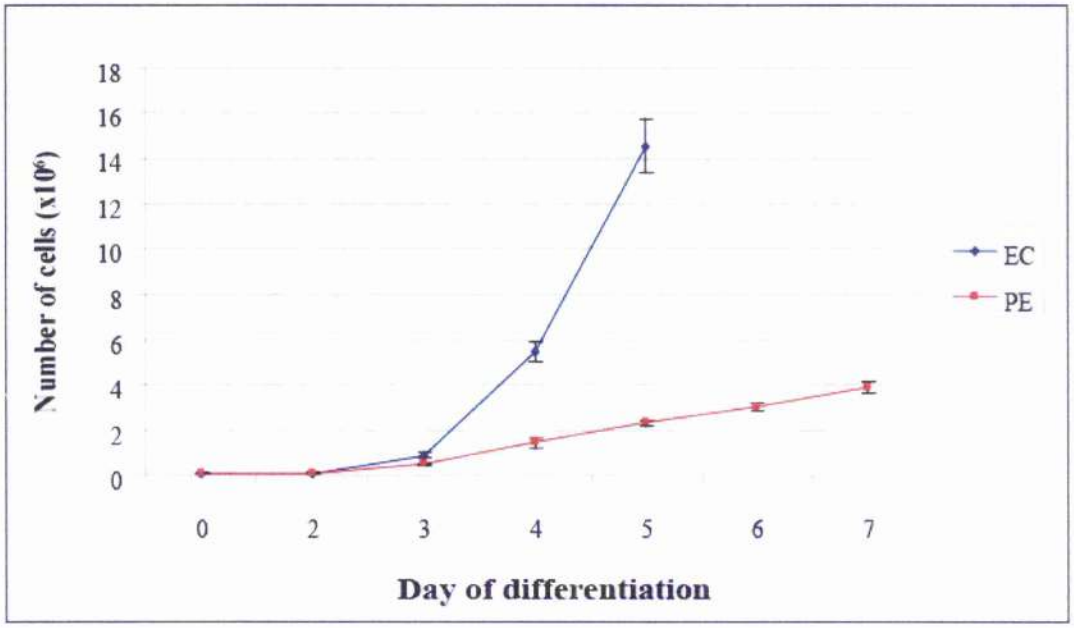
Figure 3.1 Differentiation of F9 cells

A Whole-cell protein extracts (20 μg) were prepared from undifferentiated (EC – lane 1) and differentiated (PE – lane 2) cells, resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western immunoblotting, using either an anti-laminin B1 antibody (A-1 - top) or an anti-actin antibody (C-11 – bottom). **B** Proliferation curve of uninduced cells (EC) and cells induced to differentiate to parietal endoderm (PE), over a course of 7 days. 5 x 10^4 cells were seeded per 10-cm plate on day 0 and cultured in growth medium (EC) or differentiation medium (PE). All media were renewed every two days. The values represent the mean of three experiments + standard deviation. Only viable cells were counted, as determined by trypan blue staining. **C** The data used for B were re-plotted on a semi-log graph (y-axis on logarithmic scale, x-axis on linear scale) and doubling times for the two cell types, at various stages during the differentiation, were calculated.

A



B



C

Days	*Doubling time (hours)*	
	EC	PE
0 – 2	48	42
2 – 4	8	14
4 – 7	15	51

dishes and were allowed to grow for 7 days. Half of the cells were cultivated in normal growth medium and half in differentiation medium (for composition, see Chapter 2 – Materials and Methods). Figure 3.1B shows a graphical representation of viable cell counts over a period of 7 days following plating (day 0). Uninduced F9 cells (denoted EC) grow exponentially until day 5, and to a cell count of about 1.45×10^7, following which they become over-confluent and dissociate from the culture plates in massive numbers. In contrast, F9 cells that were induced to differentiate (denoted PE) proliferate much more slowly, with a maximum cell count of about 4×10^6 on day 7, at which point the experiment was terminated. The data obtained are from three experiments, each using three 10-cm culture plates per day per condition (EC or PE). The rate at which the cells proliferate, under the two conditions, changes over the 8 days that the experiment lasted (Figure 3.1C). They initially proliferate at a very similar, very slow rate until day 2, with a doubling time (d.t.) of about 48 hours. Between days 2 and 4, the EC cells exhibit their characteristic high rate of proliferation (Rosenstraus *et al.*, 1982), with a d.t. of 8 hours, whereas the induced cells (PE) have a slower d.t. of about 14 hours. They then slow down considerably, needing on average 51 hours to double their population. The EC cells also slow down, but to a lesser extent.

3.2.2 RNA polymerase III transcription decreases during F9 cell differentiation

RNA polymerase III transcription has been previously shown to be down-regulated in this cell system (White *et al.*, 1989). Specifically, it was shown that, following differentiation of F9 EC cells to PE, the transcription of B2 genes, a rodent-specific

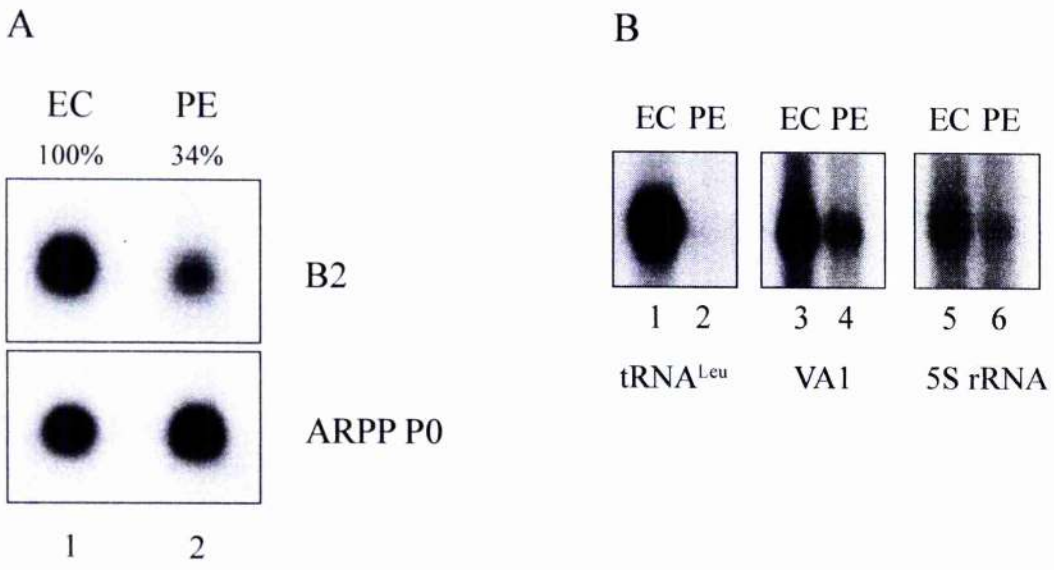


Figure 3.2 RNA polymerase III transcription is reduced in PE cells

A Total RNA (10 μg) was extracted from F9 EC (lane 1) and PE (lane 2) cells and used for Northern blot analysis with a probe against the B2 transcript. The membrane was stripped and reprobed with an ARPP P0 probe. B2 transcript levels were quantified and normalised against ARPP P0. The value obtained for EC was set as 100%. **B** Whole-cell protein extracts prepared from undifferentiated (lanes 1, 3 and 5) and differentiated (lanes 2, 4 and 6) F9 cells were used for *in vitro* transcription assays. Reactions contain 20 μg of extract and 125 ng of template, and the templates used were $tRNA^{Leu}$ (lanes 1 and 2), VA1 (lanes 3 and 4) and 5S rRNA (lanes 5 and 6).

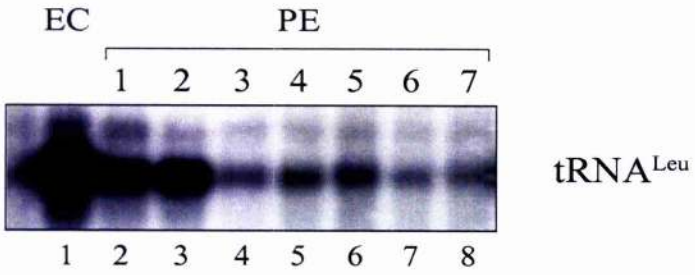
middle repetitive gene family with no known function, is greatly reduced, along with other class III genes, like those encoding tRNA. This decrease was attributed to the reduced activity of the pol III-specific general transcription factor TFIIIB, an essential component of the pol III transcription machinery. Crucially, extracts from PE cells were shown not to be deficient in TFIIIC activity, another essential component for most class III genes.

To verify these observations, the steady-state levels of the B2 transcript in total RNA extracts were compared between EC and PE cells using Northern blotting. As can be seen in Figure 3.2A, there is a significant reduction in the levels of B2 transcripts following differentiation of EC (lane 1) to PE (lane 2) cells. A pol II-transcribed gene, *ARPP P0* (acidic ribosomal phosphoprotein P0), was used as a loading control, as it has been previously shown to be constitutively expressed (Hurford et al., 1997). Quantification (using the TotalLab software) and normalisation against the loading control (ARPP P0) revealed a 3-fold reduction in the levels of the B2 transcript in PE cells.

In addition to the Northern blot analysis, *in vitro* transcription assays, described in section 2.16, were employed to directly compare the ability of cell-free extracts of EC and PE cells to transcribe pol III templates. In Figure 3.2B, we can see that transcription of a selection of class III genes, in this case $tRNA^{Leu}$, VA1 and 5S rRNA, is reduced to varying degrees following differentiation (compare lanes 1, 3 and 5 for EC with 2, 4 and 6 for PE).

Differentiation of F9 cells, as mentioned before, was achieved by adding retinoic acid and cyclic AMP to the growth medium of EC cells. To ensure that all cells are fully differentiated, cells were grown in this medium for 6 or 7 days. A question that arises is at which point during this process pol III transcription declines. To begin to answer this question, a time course experiment was conducted. Protein extracts were

A



B

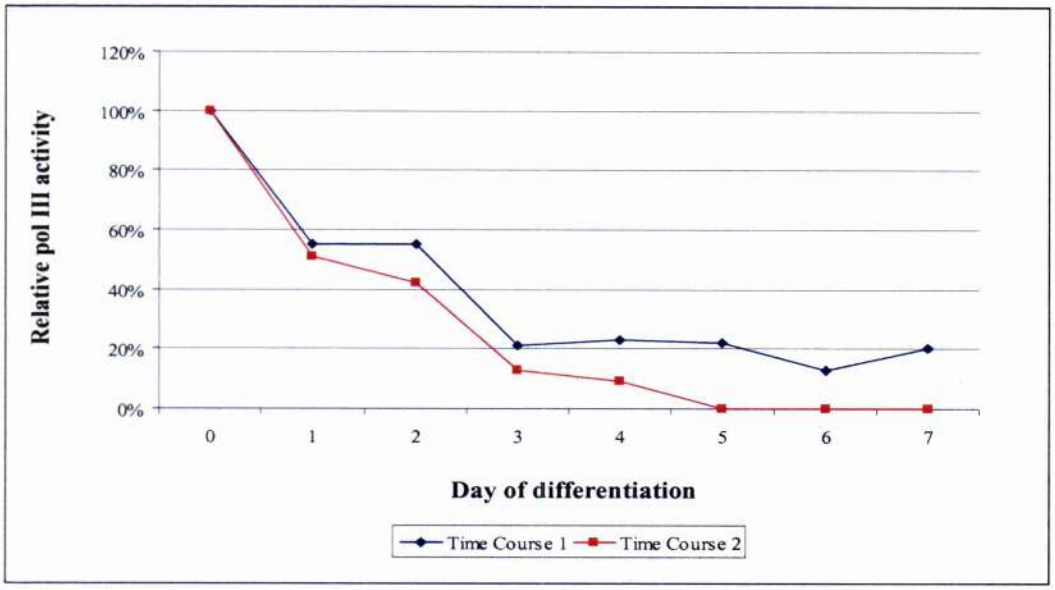


Figure 3.3 Time course of pol III down-regulation

A Cells were harvested before differentiation (lane 1) and on each day following induction of differentiation, until day 7 (lanes 2 – 8) (Time Course 1). Protein extracts prepared from these cells were used for *in vitro* transcription assays, with $tRNA^{Leu}$ as a template. Reactions contain 20 μg of extract and 250 ng of template. **B** Graphic representation of Time Courses 1 and 2. The signal obtained from differentiating cells (days 1 – 7) was quantified (TotalLab) and expressed as a percentage of that from EC cells.

prepared from each day of the differentiation process, as well as from undifferentiated cells, and were subsequently used in IVT assays. Figure 3.3A shows the *in vitro* transcription of the $tRNA^{Leu}$ template, with the extract from EC cells in lane 1 and extracts from cells on days 1 to 7 of the differentiation process in lanes 2 – 8 (Time Course 1). Figure 3.3B is a graphical representation of two independent differentiation time courses, 1 and 2. In both cases the $tRNA^{Leu}$ template was used, but similar results have been obtained with the VA1 template. There seems to be two steps in the down-regulation of pol III transcription. An initial decrease at the very start of the differentiation process, i.e., between days 0 and 1, and a secondary decrease later on, between days 2 and 3.

3.2.3 Down-regulation of TFIIIB components following differentiation

The question still remains as to the exact molecular mechanisms that cause this decrease in pol III transcription. An insight was presented in the work by Alzuherri and White (1998), where, in addition to confirming that TFIIIB, as opposed to TFIIIC, activity was limiting in PE cells, it was shown that the protein levels of two components of TFIIIB, namely Brf1 and TBP, decrease following differentiation. In contrast, the protein levels of two components of the TFIIIC complex, TFIIIC110 and TFIIIC220, did not change, further supporting the idea that TFIIIB is the limiting activity. To verify these observations, protein extracts were prepared from EC and PE (lanes 1 and 2 respectively) and analysed by Western blotting, using antibodies against Brf1 and TBP (Figure 3.4A), and against one component of TFIIIC, TFIIIC110 (Figure 3.4B). In addition, a previously unavailable antibody against

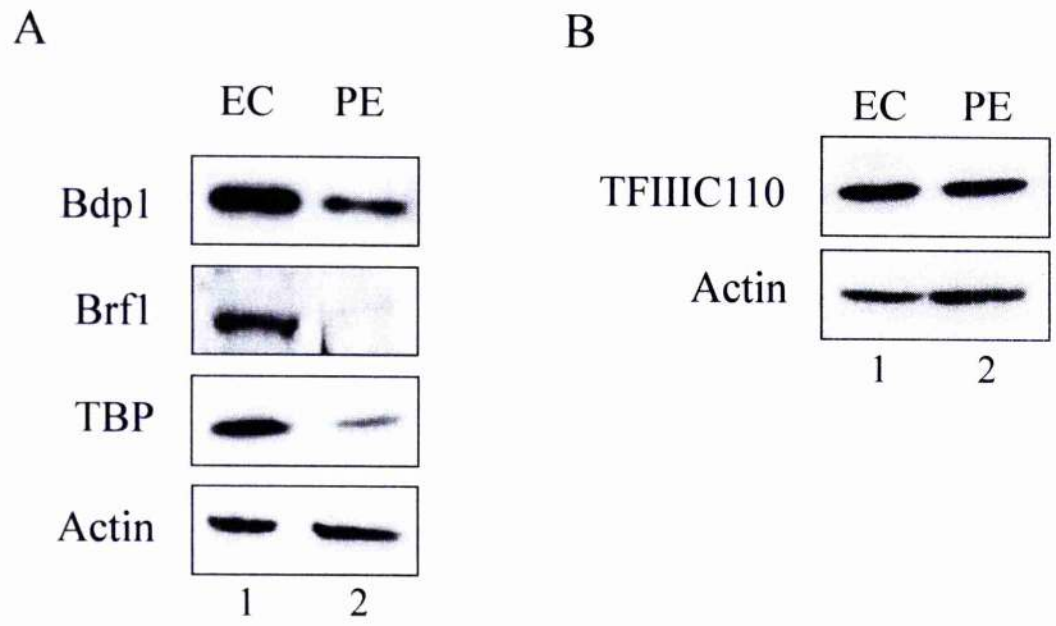


Figure 3.4 Differentiation causes a reduction in the levels of TFIIIB

A Whole cell protein extracts (50 μg) were prepared from undifferentiated (EC – lane 1) or differentiated (PE – lane 2) F9 cells, resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western immunoblotting. The membrane was cut and probed using either an anti-Bdp1 antibody (2663 – top panel), an anti-Brf1 antibody (128 – second panel), an anti-TBP antibody (58C9 – third panel) or an anti-actin antibody (C-11 – bottom panel). In the case of actin, the part of the membrane that was probed for TBP was stripped and re-probed with the anti-actin antibody. **B** As A, except that the membrane was cut in two, the top half was probed for TFIIIC110 (4286) and the bottom half for actin (C-11).

Bdp1 was used (Figure 3.4A). Actin was used as a loading control. As we can see, the levels of all three components of the TFIIIB complex, Bdp1, Brf1 and TBP, seem to decline following differentiation, when compared to the levels of actin, but TFIIIC110 levels do not change (compare lanes 1 and 2 in all panels). These results support the previous findings and show that Bdp1 levels also decline following differentiation.

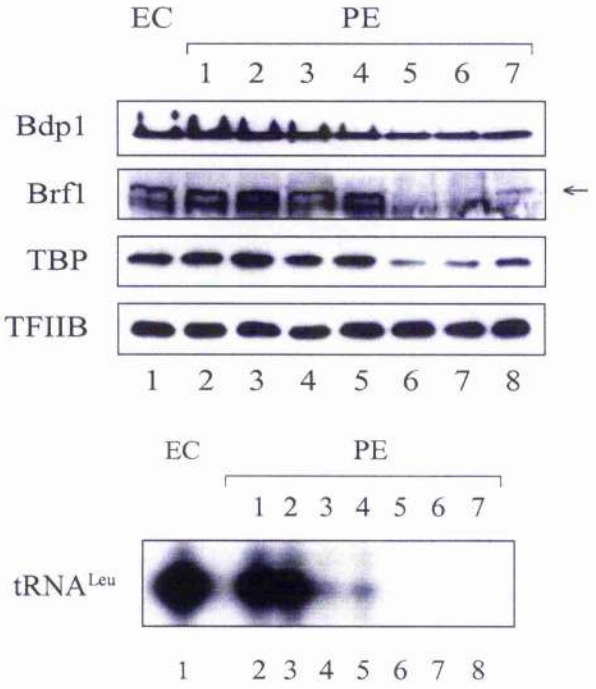
3.2.4 Time course of TFIIIB down-regulation

Since Bdp1, Brf1 and TBP are all part of the same complex, an attractive hypothesis would be that they are all under a common regulatory mechanism. A simple way of looking at this question, at least initially, was by comparing the relative levels of each protein, by Western blot analysis, throughout the differentiation process, similar to the experiment described in section 3.2.2. Whole-cell extracts were prepared before differentiation (lane 1) and on each day of the differentiation process (lanes 2 - 8) and analysed by Western immunoblotting using antibodies against Brf1, Bdp1 and TBP, while TFIIB was used as loading control (Figure 3.5A). It is immediately obvious that the levels of all three TFIIIB proteins decrease between days 4 and 5 (lanes 5 and 6), with Brf1 and TBP and, to a lesser extent, Bdp1 showing an increase towards the end of the time course. The arrow indicates the position of *in vitro* translated HA-tagged Brf1, run on the same gel. This comparison was repeated in a separate gel, with just whole-cell extract from undifferentiated cells (EC – lane 1) and *in vitro* translated HA-Brf1 (+ve - lane 2) (Figure 3.5B). Figure 3.5C shows a graphical representation of the experiment in A. The Bdp1, Brf1 and TBP bands were quantified and corrected for loading against TFIIB. Levels of the three proteins

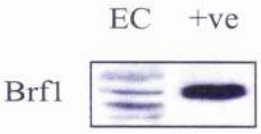
Figure 3.5 Time course of TFIIIB decrease

A Whole-cell protein extracts (20 μg) were prepared from undifferentiated F9 cells (lane 1) and cells undergoing differentiation (days 1 to 7 – lanes 2-8), resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western blotting using an anti-Bdp1 antibody (2663 – top panel), an anti-Brf1 antibody (CSH145 – second panel), an anti-TBP antibody (58C9 – third panel) or an anti-TFIIB antibody (C-18 – fourth panel), as a loading control. The arrow next to the second panel shows the position of the Brf1 band, as determined by using *in vitro* translated HA-tagged Brf1 as standard. For comparison, the Time Course 2 IVT also presented here. Lanes as in Figure 3.3A **B** Verification of the CSH145 anti-Brf1 antibody. 20 μg of whole-cell extract were resolved on a SDS – 7.8% polyacrylamide gel alongside *in vitro* translated HA-tagged Brf1 (denoted as +ve). **C** Graphical representation of A. Bdp1, Brf1 and TBP bands were quatified and normalised against the loading control (TFIIB). The values before differentiation (EC) were set at 100%. The IVT time course was analysed as before (Figure 3.3).

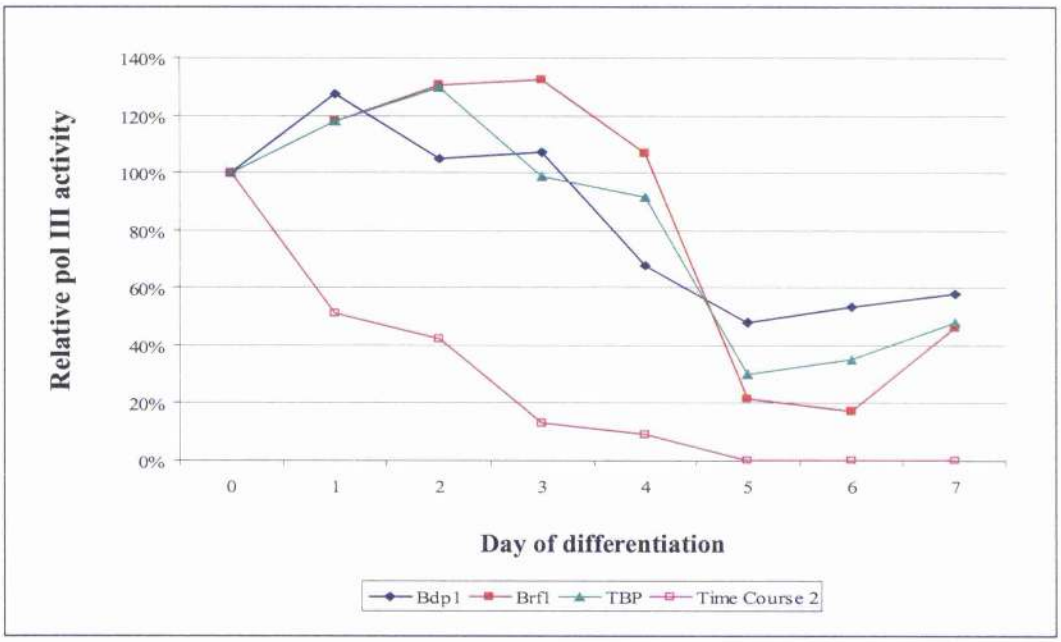
A


EC
PE
1 2 3 4 5 6 7
Bdp1
Brf1
TBP
TFIIB
1 2 3 4 5 6 7 8
EC
PE
1 2 3 4 5 6 7
tRNA^Leu
1 2 3 4 5 6 7 8

B


EC
+ve
Brf1

C


Relative pol III activity
140%
120%
100%
80%
60%
40%
20%
0%
0 1 2 3 4 5 6 7
Day of differentiation
Bdp1
Brf1
TBP
Time Course 2

were then expressed as a percentage of the EC value (set at 100%). The IVT time course was analysed as in Figure 3.3.

3.3 Discussion

3.3.1 Differentiation of F9 cells

As mentioned before, the F9 cell system has been extensively used to study events in early mouse embryogenesis, and generally as a model for the analysis of differentiation, because of its ability to differentiate into various cell types that resemble tissues of the early mouse embryo (Figure 1.5). Because of this, it was necessary to check whether or not the cells I was using behaved in a similar manner to what was previously described. Morphological examination of F9 EC cells treated with retinoic acid (RA) and dibutyryl cyclic AMP (db-cAMP) for 7 days under a light microscope revealed that they exhibited all the characteristics of terminally differentiated primitive endoderm (PE)-like cells. This includes the cells becoming more round and forming processes (data not shown), like previously described (Strickland *et al.*, 1980). This was supported by biochemical analysis of the induced cells for specific differentiation markers. Production of laminin has been shown to increase in PE cells compared to the parental EC cells or the other possible derivative, visceral endoderm (Hogan *et al.*, 1981; Strickland *et al.*, 1980). Western blot analysis of EC and PE extracts shows a dramatic increase in the production of the β-1 chain of laminin (Figure 3.1A). Increased levels of laminin β-1 were also found in the growth medium of PE cells (results not shown), suggesting that the produced laminin is indeed secreted into the medium. The formation of extracellular

matrix by PE cells was further supported by empirical observation of increased adherence to gelatinised plates, compared with EC cells. We have, therefore, two markers of differentiation, one morphological and one biochemical, which confirm that the cell system I used behaved as previously described.

F9 EC cells are known to grow very rapidly, with a doubling time of about 8-10 hours in the exponentially growing phase, due to a very short G1 phase (Rosenstraus *et al.*, 1982). Differentiation causes a lengthening of the G1 phase, with a subsequent decrease in the proliferation rate. Although I didn't analyse the cell cycle characteristics of my cells in any great detail, I did study the difference in proliferation between EC and PE cells by means of a proliferation curve, the results of which can be seen in Figure 3.1B and C. Looking at absolute number of cells (Figure 3.1B), there is not much difference between the two conditions during the first two days. At day 3, the EC population is almost double that of the PE. After that, there is an exponential increase in the number of EC cells, whereas the PE cells increase in number linearly. At day 5, which is the last day where EC cells were counted, there is a 6.3-fold difference in cell number between EC and PE. After day 5, the EC cells grew too confluent and dissociated from the plates, making further counting impossible. By plotting the same data on a semi-log graph (with the y-axis in logarithmic scale and the x-axis in linear scale – data not shown), I was able to calculate the doubling time of the cells (Figure 3.1C). The initial, slow proliferation rate could probably be attributed to the very low seeding density. When EC cells, for example, were seeded at a higher density ($1x10^6$), they exhibited their usual fast growth rate (doubling time of about 8 hours). Even in the exponential phase, between days 2 and 4, we can see a clear difference between the uninduced (EC) and induced (PE) cells, which is much more pronounced in the last 3 days. It is worth noting that the PE cells continued to proliferate until day 7, when they were reaching almost

100% confluency. If subcultured, however, they continue to proliferate, as was observed by Strickland *et al.* (1980).

3.3.2 Regulation of pol III transcription in differentiated F9 cells

Earlier work (Alzuherri and White, 1998; White *et al.*, 1989) has shown that pol III transcription is decreased when F9 EC cells differentiate into PE, and that this decrease is due to a reduction in TFIIIB activity. Initially, this was achieved by fractionating EC and PE extracts. It was already known that two activities present in phosphocellulose column fractions, TFIIIB and TFIIIC, are necessary and sufficient for pol III transcription *in vitro* (Segall *et al.*, 1980), when added to partially purified RNA polymerase III. Further purification of these crude fractions showed that TFIIIB, but not TFIIIC, activity was reduced in F9 PE cells. It was also shown, using specific antibodies against Brf1 and TBP, that the levels of those two proteins are reduced following differentiation. The reduction in pol III transcription, and in the levels of Brf1 and TBP, was confirmed by our data (Figures 3.2 and 3.4A). Furthermore, using an anti-Bdp1 antibody that was not available at the time of the previous study, we showed that the levels of the third component of the TFIIIB complex are also reduced (Figure 3.4A). In a contradictory report, however, Meissner *et al.* (1995) suggested that it is the TFIIIC activity that is limiting in F9 PE cells. They later retracted this (Meissner *et al.*, 2002), suggesting instead that the down-regulation of pol III transcription is due to a reduction in the activity of TFIIIC1, an ill-defined factor with respect to the proteins needed for reconstitution of its activity. The same group then claimed that TFIIIC1 is, in fact, Bdp1 (Weser *et al.*,

2004), and that its reduction is responsible for the down-regulation of pol III in F9 PE cells. They suggest the discrepancy between the earlier reports was due to differences in the fractionation of the cell extracts. Finally, they claimed that TBP and Brf1 are not regulated during the differentiation of F9 cells. This does not agree with the data we have obtained, as we have seen Brf1 protein and mRNA and TBP protein levels (Figures 3.4A and 4.5) decrease. Furthermore, Perletti *et al.* (2001) show that TBP is regulated via targeted proteolysis in the F9 system. Therefore, there are still some unresolved issues in this aspect of pol III regulation.

A problem that should be mentioned here is the absence of suitable internal controls for the *in vitro* transcription experiments. Errors can be introduced in this assay in a number of steps. For example, there could be differential recovery of proteins during protein extraction, as well as differential recovery of RNA during the phenol – chlorophorm precipitation step. Therefore, these results should be viewed with a certain amount of caution.

3.3.3 Timing of TFIIIB down-regulation

In Figures 3.5A and 3.5C we can see that the level of all three components of the TFIIIB complex fall between days 4 and 5 (lanes 5 and 6) after induction of differentiation. This raises the possibility that these three proteins might be co-regulated, in order, for example, to avoid having partial complexes (e.g., Brf1 and TBP without Bdp1), that might sequester other transcription factors. If, however, we compare these data with the time course of pol III transcription (Figure 3.3 and 3.5A), we notice that there is a temporal discrepancy between the reduction in the levels of TFIIIB and the down-regulation of transcription, the majority of which

occurs before day 4. A possible explanation for the discrepancy between TFIIIB protein levels and pol III transcription is that other factors that enhance or repress transcription are either up- or down-regulated in response to the differentiation of F9 EC cells. Although this idea seems to contradict earlier findings that TFIIIB activity is the limiting factor, it is quite plausible that the factor or factors that affect pol III transcription co-fractionated with TFIIIB. This hypothesis will be examined in a later chapter.

CHAPTER 4

Brf1 and Bdp1 are not degraded by the proteasome

4.1 Introduction

The ubiquitin-proteasome pathway was initially considered to be responsible for the degradation of damaged or misfolded proteins. Subsequent work has shown that it also targets a wide variety of other proteins, including cell cycle regulators, tumour suppressors, oncoproteins and transcription factors (reviewed in Pickart, 2001). In fact, it is now thought to be the major proteolytic pathway in eukaryotic cells (Figure 1.6). With such a multitude of cellular targets, it is not surprising that it is involved in the regulation of many basic cellular processes, such as differentiation and development, cell cycle and division, DNA repair and apoptosis.

Degradation by this system involves the covalent attachment of ubiquitin, a 76-amino-acid protein, to the target protein. This is achieved by the sequential action of three enzymes, which result in the attachment of a multiubiquitin chain on the target protein (Figure 1.7). The protein is subsequently recognised and degraded by the 26S proteasome, a large, multimeric proteolytic enzyme (Bochtler *et al.*, 1999).

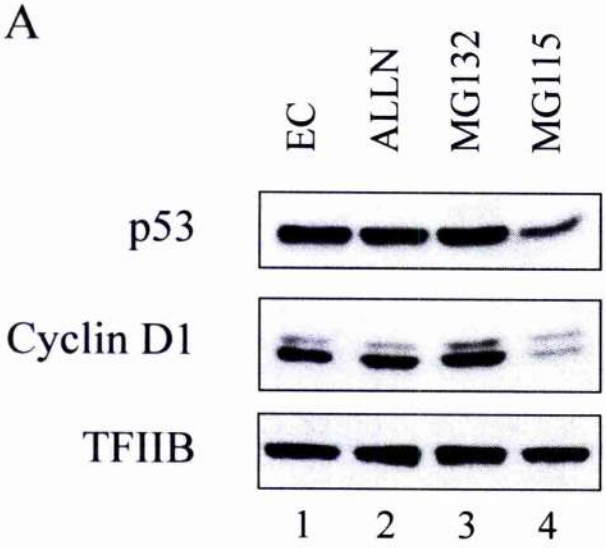
An important breakthrough in the study of the ubiquitin-proteasome pathway was the development of specific proteasome inhibitors. There are several classes of inhibitors, based on their chemical structure, and include the peptide aldehydes, peptide boronates, vinyl sulfones and lactacystin (Lee and Goldberg, 1998). Of these,

the peptide aldehydes, such as MG132, MG115 and ALLN, are the most commonly used. These molecules are substrate analogues and act by blocking, primarily, the chymotrypsin-like activity of the proteasome (Rock et al., 1994), which is thought to be the rate-limiting step in protein degradation. In addition to studying the effects that blocking the proteasome might have on intact cells, these molecules proved extremely useful in identifying proteins that are degraded by this pathway. Increase in the level of a protein, or blocking of its decrease, after treatment with an inhibitor strongly indicates proteasome-mediated degradation. For example, in the regulation of the transcription factor NF-κB, proteasome inhibition leads to the accumulation of phosphorylated IκB (Palombella et al., 1994).

Another useful tool, especially for the definition of the hydrolytic activities of the proteasome, are the artificial fluorogenic substrates. For example, succinyl-Leu-Leu-Val-Tyr-AMC has been used to define the chymotrypsin-like activity of the proteasome (Orlowski, 1990). These compounds, when hydrolysed by the proteasome, release the fluorescent group 7-amino-4-methylcoumarin (AMC), which can be easily detected using a fluorescence spectrophotometer.

This chapter looks at the effect of proteasome inhibition on the pol III transcriptional activity of F9 EC and PE cells, but also on the levels of the TFIIIB protein complex. The levels of Brf1, Bdp1 and TBP mRNA are also examined, in untreated as well as MG132-treated EC or PE cells. Initially, however, the functionality of the proteasome inhibitor MG132 and the overall activity of the proteasome in EC and PE cells were assessed.

A



B

Inhibitor	Fold increase	
	p53	cyclin D1
ALLN	2.2	3.3
MG132	1.9	2.5
MG115	2.2	2.4

Figure 4.1 **Comparison of three proteasome inhibitors**

A F9 EC cells were treated with DMSO alone (lane 1), 25 μM ALLN (lane 2), 10 μM MG132 (lane 3) or 25 μM MG115 (lane 4) for 2 hours. Whole-cell extracts (20 μg) were then prepared, resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western blotting, using antibodies against p53 (1C12 – top panel) and cyclin D1 (72-13G – bottom panel). TFIIB was used as both a negative and loading control. **B** Quantification of A. p53 and cyclin D1 was quantified using the TotalLab software, normalised against TFIIB and expressed as 'fold increase', compared to DMSO-treated EC cells.

4.2 Results

4.2.1 Proteasome inhibition in F9 EC and PE cells

To examine if proteasome inhibition has any effect on F9 EC cells, they were treated with three commonly used peptide aldehyde inhibitors, namely MG132, MG115 and ALLN. The effect of these inhibitors on the level of two proteins that have been previously shown to be degraded by the ubiquitin-proteasome pathway, p53 (Ciechanover et al., 1991; Maki et al., 1996) and cyclin D1 (Diehl et al., 1997), was then investigated. Figure 4.1A shows that levels of both proteins are increased following treatment of the cells with proteasome inhibitors (ALLN – lane 2, MG132 – lane 3, MG115 – lane 4), compared to DMSO-treated EC cells (lane 1). TFIIB was used as both negative control for proteasomal inhibition and as loading control for the electrophoresis. Quantification revealed that the efficiency of MG132 and MG115 is quite comparable, while ALLN appears to perform slightly better (Figure 4.1B). This experiment serves as a positive control for proteasome inhibition in EC cells. The question, however, is whether or not the inhibitors work equally well in differentiated PE cells. I selected MG132 for the rest of my experiments, because of its specificity and potency and because it has been previously used in this cell system (Perletti *et al.*, 2001). When whole-cell extracts from PE cells treated with MG132 were analysed by Western blotting for p53 and cyclin D1, a clear stabilisation of both proteins was observed (Figure 4.2A and B). There were no phenotypic changes in response to MG132 treatment, as determined by light microscopy (results not shown).

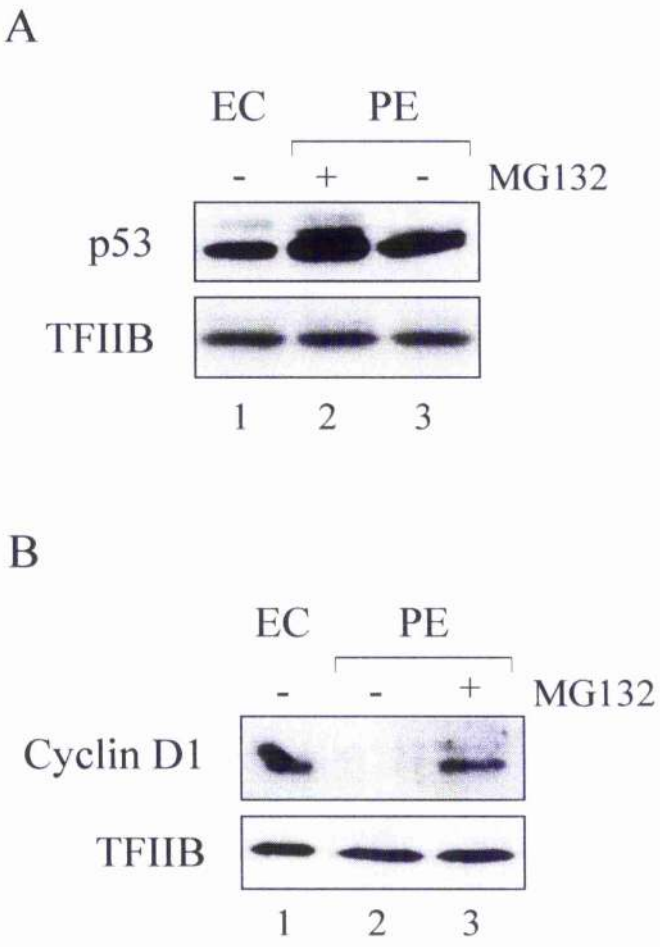


Figure 4.2 MG132 is functional in F9 PE cells

A F9 PE cells were treated with DMSO alone (lane 2) or with the proteasome inhibitor MG132 (10 μM – lane 3) for 4 hours, harvested and used for preparation of protein extracts. 20 μg were resolved on a SDS – 7.8% polyacrylamide gel, alongside 20 μg of extract from EC cells treated with DMSO for 4 hours (lane 1), and analysed by Western blotting using an anti-p53 antibody (1C12) or an anti-TFIIB antibody (C-18). **B** As A, except the loading order is different (EC – lane 1, PE – lane 2, PE + MG132 – lane 3) and the membrane was probed using an anti-cyclin D1 antibody (72-13G). TFIIB was again used as negative and loading control.

4.2.2 *In vitro proteasome assay*

Before examining the proteins of interest, i.e. Brf1, Bdp1, etc., for possible degradation by the proteasome, it was decided to investigate whether or not the ubiquitin-proteasome pathway itself is affected by the differentiation of F9 cells to parietal endoderm. This was accomplished by using an *in vitro* assay to compare the proteasomal activity of EC and PE whole-cell extracts (Figure 4.3). This assay utilises the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC), which, when cleaved by the chymotryptic-like activity of the proteasome (Stein et al., 1996), releases the fluorescent group AMC (7-amino-4-methylcoumarin). This can then be efficiently detected using a fluorescence spectrophotometer. Therefore, whole cell extracts from EC (columns 1 and 2) or PE (columns 4 and 5) cells that were treated with DMSO (columns 1 and 4) or MG132 (columns 2 and 5), were incubated with the substrate, Suc-LLVY-AMC, for 2 hours. The fluorescence was then measured, as described in Materials and Methods, and expressed as a percentage of that observed for untreated EC cells (Figure 4.3). As a positive control for proteasome inhibition by MG132, DMSO-treated EC or PE extracts were incubated with inhibitor prior to addition of the substrate (columns 3 and 4). As we can see, complete inhibition was achieved.

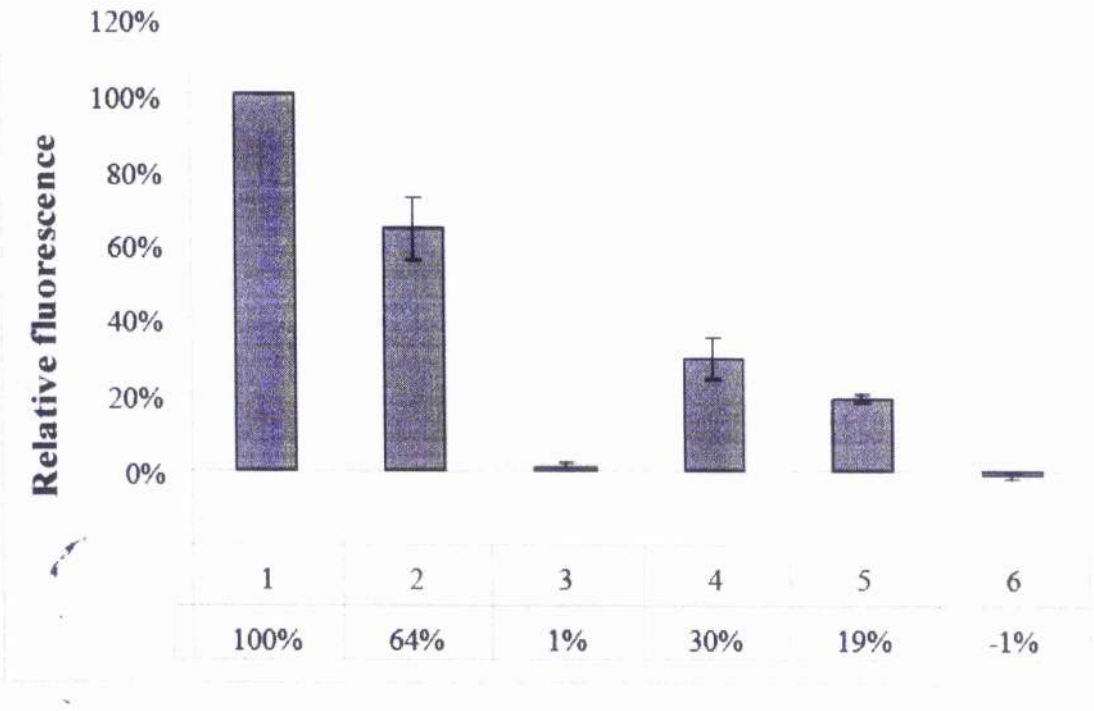


Figure 4.3 ***In vitro*** **proteasome assay**

F9 EC (columns 1 – 3) or PE (columns 4 – 6) cells were treated with DMSO (columns 1, 3, 4 and 6) or MG132 (50 μM – columns 2 and 5) for 4 hours prior to harvesting. Resulting whole cell extracts (10 μg) were then incubated with DMSO (columns 1, 2, 4 and 5) or MG132 (50 μM – columns 3 and 6) for 30 min at 37°C, before adding the artificial proteasome substrate Suc-LLVY-AMC (40 μM) and incubating for 2 hours at 37°C. The fluorescence of released AMC was measured using a fluorescence spectrophotometer (LS-50B, Perkin Elmer) and expressed as percentage of that from untreated EC cells. The values represent the average of four (for EC) or three (for PE) experiments, ± standard deviation, with background values (substrate alone) subtracted.

4.2.3 *Proteasome inhibition does not affect the levels of Brf1 and Bdp1, nor pol III transcription*

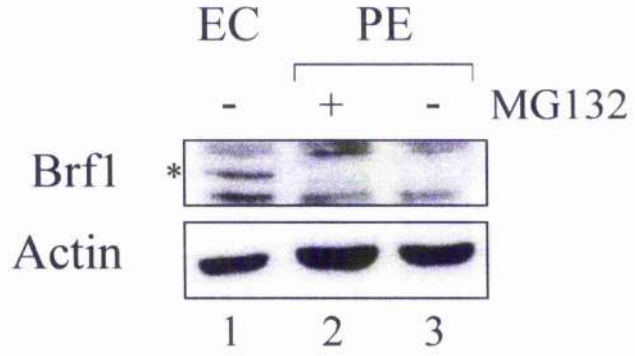
Having established that MG132 could indeed block the proteasome, both in EC and PE cells, leading to the accumulation of known degradation targets, p53 and cyclin D1, its effects on the TFIIIB components, Brf1 and Bdp1, were investigated. Figure 4.4A shows Western blot analysis of extracts from EC (lane 1) or PE cells treated with MG132 (lane 2) or DMSO (lane 3). Probing with the anti-Brf1 antibody (top panel) reveals that Brf1 levels are not stabilised by proteasome inhibition (the asterisk indicates the position of Brf1, verified by running *in vitro* translated protein alongside it – results not shown). The actin blot (bottom panel) serves both as negative and loading control. Similarly, Bdp1 levels are also unaffected by MG132 treatment (Figure 4.4B). F9 EC (lanes 1 and 2) or PE (lanes 3 and 4) cells were treated with either DMSO (lanes 1 and 3) or MG132 (lanes 2 and 4). Whole-cell extracts from these cells were analysed by Western blotting with an anti-Bdp1 antibody (top panel), revealing no stabilisation of the protein either before (lanes 1 and 2) or after (lanes 3 and 4) differentiation. Again, actin was used as both loading and negative control.

RNA polymerase III transcription is regulated by a variety of mechanisms (White, 1998; White, 2004a). Therefore, it is possible that the proteasome, by influencing one of those mechanisms, might have an indirect effect on pol III transcription. This hypothesis was tested by comparing the capacity of extracts from control or MG132-treated cells to transcribe a pol III template *in vitro*. Figure 4.4C shows a representative experiment, using whole-cell extracts from EC (lanes 1 and 2) or PE (lanes 3 and 4) cells, that were treated with MG132 (lanes 1 and 3) or DMSO (lanes

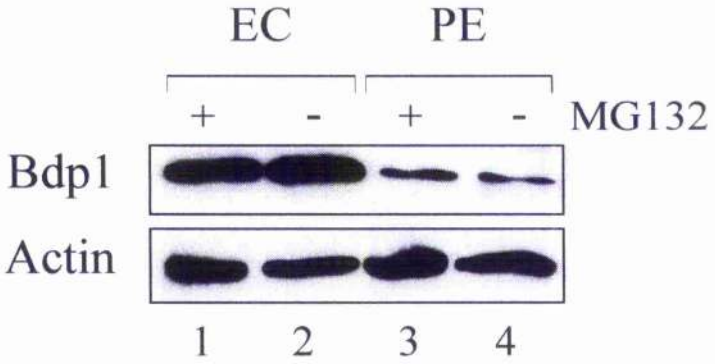
Figure 4.4 Effect of proteasome inhibition on TFIIIB levels and pol III transcriptional activity

A Whole-cell extracts (20 μg) from EC (lane 1) or PE (lanes 2 and 3) cells, treated with DMSO (lane 1 and 3) or 25 μM MG132 (lane 2) for 4 hours prior to harvesting, were resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western blotting using either an anti-Brf1 antibody (145 – top panel) or an anti-actin antibody (C-11 – bottom panel). The asterisk next to the top panel indicates the position of Brf1, as determined by using *in vitro* translated HA-tagged Brf1 as standard. **B** Whole-cell extracts (20 μg) from EC (lanes 1 and 2) or PE (lanes 3 and 4) cells, treated with DMSO (lane 1 and 3) or 25 μM MG132 (lanes 2 and 4) for 4 hours prior to harvesting, were resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western blotting using antibodies against Bdp1 (2663 – top panel) or actin (C-11 – bottom panel). **C** Protein extracts (20 μg) from EC (lanes 1 and 2) or PE (lanes 3 and 4) cells, treated with DMSO (lane 2 and 4) or 25 μM MG132 (lanes 1 and 3) for 4 hours prior to harvesting, were used to *in vitro* transcribe a VA1 template (250 ng).

A


EC
PE
- + - MG132
Brf1
*
Actin
1 2 3

B


EC
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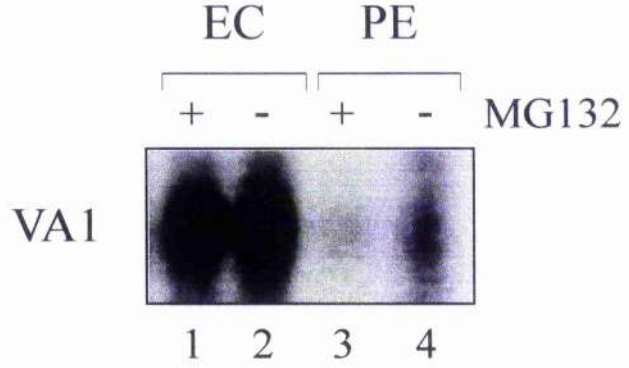
+ - + - MG132
Bdp1
Actin
1 2 3 4

C

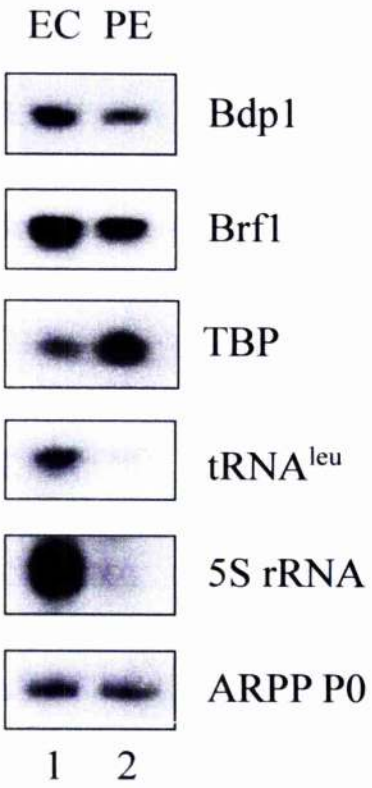

EC
PE
+ - + - MG132
VA1
1 2 3 4

2 and 4), to transcribe a VA1 template. As we can see, there is no effect of transcription before (lanes 1 and 2) or after (lanes 3 and 4) differentiation. The apparent decrease in the MG132-treated PE extract (lane 3) was not reproducible.

4.2.4 RT-PCR analysis of TFIIIB components

In addition to a post-translational control mechanism, there could be other levels at which the differentiating cells could regulate the amount of TFIIIB, and therefore pol III transcription, since it seems to be the limiting factor in this system (Alzuherri and White, 1998; White *et al.*, 1989). The possibility of regulation at the transcriptional or post-transcriptional level was therefore examined, by comparing the abundance of Bdp1, Brf1 and TBP mRNAs in EC and PE cells. This was accomplished by reverse transcription-PCR (RT-PCR), where cDNAs are prepared by reverse transcription of total RNA extracted from cells. Specific PCR primers are then used to amplify the sequence of interest, using a radioactively-labelled nucleotide for increased sensitivity. When RNA extracts from EC and PE cells were analysed in this way (Figure 4.5A), a small, but consistent, decrease in the levels of Bdp1 and Brf1 mRNAs (top two panels) was observed following differentiation of cells (EC – lane 1, PE – lane 2). Surprisingly, TBP mRNA levels were found to increase following differentiation (Figure 4.5A). The primers that were used in these PCR reactions were designed to only amplify the mature transcript, so there is no way to make a distinction between transcriptional and post-transcriptional regulation. The transcript levels of two pol III products, namely $tRNA^{Leu}$ and 5S rRNA (third and fourth panels – Figure 4.5A) were also examined, as a positive control for the down-regulation of pol III transcription in PE cells. The levels of primary tRNA transcripts offer an

A



B

mRNA	Fold change
Bdp1	0.59
Brf1	0.66
TBP	1.63

Figure 4.5 RT-PCR analysis of EC and PE RNA

A cDNAs were generated by reverse transcription of total RNA (3 μg), extracted from undifferentiated (EC – lane 1) and differentiated (PE – lane 2) F9 cells, and were amplified by PCR using primers for Bdp1 (top panel), Brf1 (second panel), TBP (third panel), $tRNA^{Leu}$ (fourth panel), 5S rRNA (fifth panel) and ARPP P0 (bottom panel). **B** The levels of Bdp1, Brf1 and TBP mRNA (from 4, 3 and 2 experiments, respectively) were quantified, normalised against ARPP P0, averaged and expressed as 'fold change' in response to differentiation.

accurate picture of the rate of ongoing transcription (Cormack and Struhl, 1992), since the intron sequences are processed and degraded very fast. The primers used in this case were designed to amplify part of the intron sequence, thus revealing the levels of the primary $tRNA^{Leu}$ transcript. This, therefore, shows that pol III transcription is dramatically reduced following differentiation, as was observed previously using other techniques (Figure 3.2; White *et al.*, 1989). Similarly, 5S rRNA expression is markedly reduced following differentiation. The effects seen here are specific, since the levels of ARPP P0 mRNA (bottom panel) remain unchanged following differentiation. Quantification of 4 (for Bdp1), 3 (for Brf1) or 2 (for TBP) experiments revealed that there is a 0.59-fold decrease in Bdp1 mRNA levels and a comparable 0.66-fold decrease in Brf1 mRNA levels in PE cells, compared to EC cells, while levels of TBP increase by an average of 1.6-fold (Figure 4.5B).

4.2.5 Time course analysis of TFIIIB mRNAs

To try to address a question posed previously, about the possible co-regulation of the TFIIIB components, we analysed RNAs extracted from cells throughout the differentiation process using RT-PCR. Similar to the protein time course, RNA was extracted from EC cells (Figure 4.6 – lane 1) and from differentiating cells over a course of 7 days (Figure 4.6 – lanes 2-8). The resulting cDNAs were used in PCR reactions with primers for Bdp1 (top panel), Brf1 (second panel) and TBP (third panel). As we can see, Bdp1 and Brf1 mRNA levels show a similar pattern during differentiation, with a rapid decrease by day 2 (lanes 1-3) and then an increase, peaking at day 4 (lanes 4 and 5), after which the levels decrease again (lanes 6 and 7,

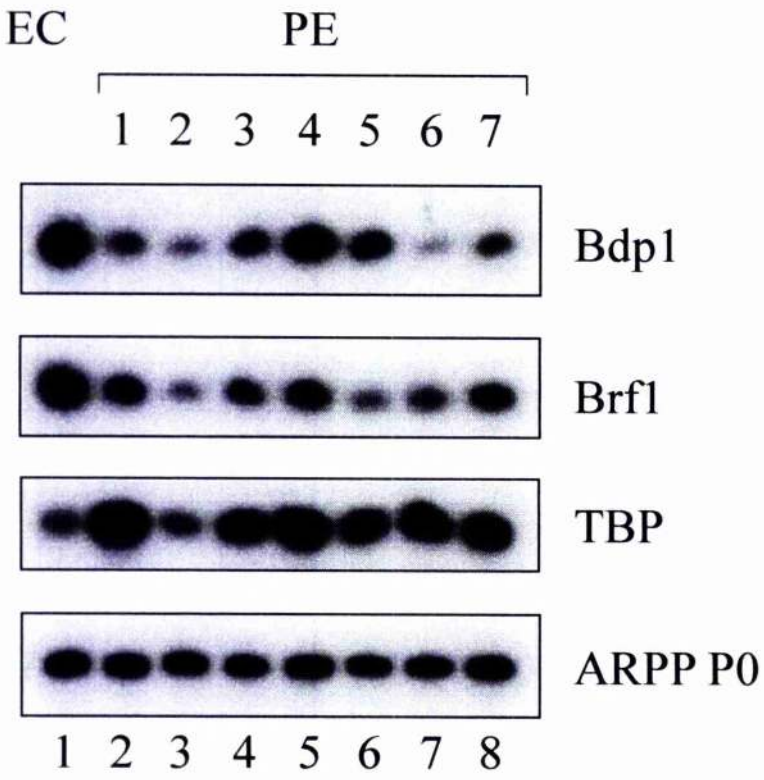


Figure 4.6 Time course analysis of differentiating cells using RT-PCR

cDNAs were generated by reverse transcription of total RNA (3 μg) extracted from undifferentiated F9 cells (lane 1) and cells undergoing differentiation (days 1 to 7 – lanes 2-8) and were amplified by PCR using primers for Bdp1 (top panel), Brf1 (second panel), TBP (third panel) and ARPP P0 (bottom panel).

although there is a difference between Brf1 and Bdp1 at this point). Finally, there is an increase at the very last day of the time course (lane 8). On the other hand, TBP mRNA levels show a markedly different pattern during the same time period. There is an initial and strong increase between days 0 and 1 (lanes 1 and 2), presumably due to the increased requirement of the cells for polII transcription when entering the differentiation process. With the exception of day 2 (lane 3), TBP levels are maintained at high levels, higher than in EC cells, throughout the differentiation process. As before, this behaviour seems to be specific, as the levels of the control transcript (ARPP P0, bottom panel) remain relatively unchanged.

4.2.6 mRNA levels of the TFIIIB complex are affected by proteasome inhibition

Because of the wide-ranging effects blocking the proteasome might have, we decided to check whether MG132 treatment affected the mRNA levels of Brf1, Bdp1 and TBP. As we saw above, Brf1 and Bdp1 mRNA levels fall following differentiation of F9 cells (Figure 4.5A), whereas TBP mRNA levels seem to increase (Figure 4.5A). The previous observations were confirmed, i.e., Bdp1 and Brf1 mRNA show a reduction, while TBP shows a slight increase (compare lanes 1 and 2 of Figure 4.7A). In EC and PE cells, however, that had been treated with MG132 (lanes 3 and 4, respectively), there seems to be a reversal of the reduction of Bdp1 and Brf1 mRNAs, now showing a slight increase or no change, respectively, when cells differentiate. TBP levels show the same pattern as before. Like previously, ARPP P0 was used as a control pol II transcript. Figure 4.7B shows PCR analysis of the same cDNAs for two pol III transcripts, B2 and 5S rRNA. As before, there is a down-

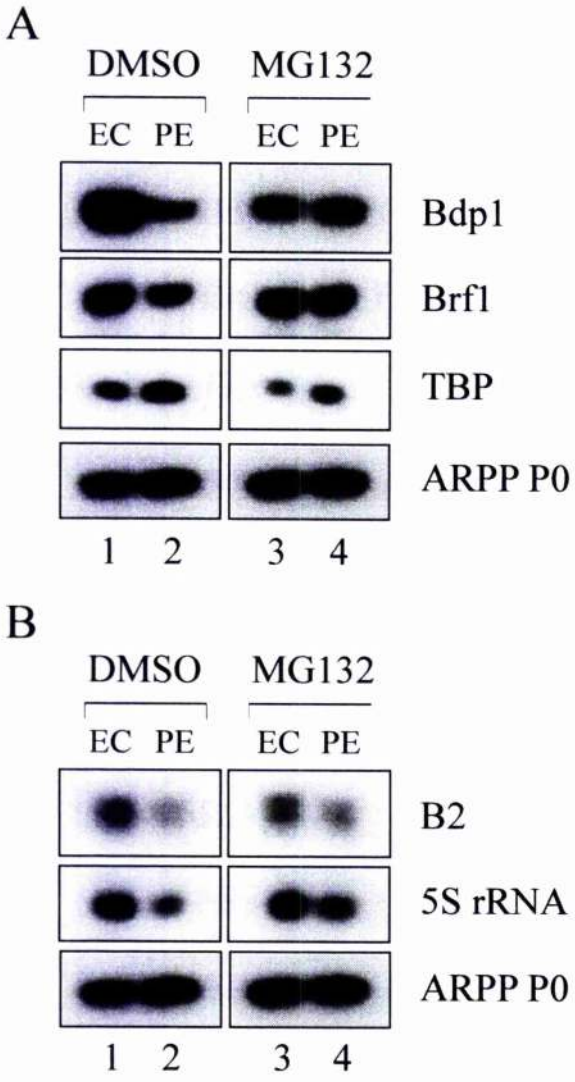


Figure 4.7 Effect of proteasome inhibition on the mRNA levels of various genes

cDNAs were generated by reverse transcription of total RNA (3 μg), which was extracted from EC (lanes 1 and 3) or PE (lanes 2 and 4) cells, treated with DMSO alone (lanes 1 and 2) or 25 μM MG132 (lanes 3 and 4) for 4 hours. Primers for Bdp1, Brf1, TBP and ARPP P0 (**A**) and B2 and 5S rRNA (**B**) were used for PCR analysis of the cDNAs.

regulation in the transcription of both genes following differentiation (compare lanes 1 and 2). When the cells are treated with the proteasome inhibitor (lanes 3 and 4, for EC and PE respectively), *B2* and *5S rRNA* transcript levels exhibit the same pattern as the control cells, i.e., their transcription is not affected by the presence of MG132.

4.3 Discussion

4.3.1 Proteasome inhibition in F9 EC and PE cells

Before looking at TFIIIB protein levels, and how they might be affected by inhibition of the proteasome, we needed to ensure that the inhibitor was functional in both EC and PE cells, i.e. that it entered the cell and demonstrably blocked the proteasome. To this end, the levels of two known targets of the ubiquitin-proteasome pathway, p53 (Maki et al., 1996) and cyclin D1 (Diehl et al., 1997) were first examined. Both proteins are stabilised in the presence of MG132, both in EC and PE cells (Figures 4.1A and 4.2). Furthermore, comparison between three peptide aldehyde inhibitors, ALLN, MG115 and MG132, revealed no significant differences in their ability to stabilise p53 and cyclin D1 (Figure 4.1B). For further experiments, one of the inhibitors was selected, namely MG132, primarily because it had been used previously in F9 cells (Perletti *et al.*, 2001). These data suggest that the proteasome inhibitors tested, and in particular MG132, can enter EC and PE cells and block the proteasome, but to what extent? To try to answer this question, we employed *in vitro* proteasome assays to directly measure the activity of the proteasome in whole-cell extracts from EC and PE cells. This assay makes use of an

artificial, fluorogenic substrate, which, when degraded by the proteasome, releases a fluorescent group, AMC, that can be easily measured using a fluorimeter. Data from these assays are presented in a graphical view in Figure 4.3. The first thing we can observe is that proteasomal activity in untreated PE cells (column 4) is reduced by about 70% compared to untreated EC cells (column 1). This suggests a previously undescribed down-regulation of the overall activity of the proteasome during F9 cell differentiation, although if it is reduction in the actual protein levels or due to inhibition is still unknown. Another important observation is that treating the cells with MG132 (columns 2 and 5) only slightly reduces proteasome function (36% in EC and 11% in PE cells). The proteasome is still responsive to inhibition by MG132, however, as complete inhibition is seen if the protein extracts are incubated with MG132 prior to addition of the substrate (columns 3 and 6). A possible explanation for this is that this modest inhibition is sufficient to stabilise the proteins examined (p53 and cyclin D1). Alternatively, one could argue that the proteasome is adequately inhibited in the cell (i.e. before protein extraction), but, because of the reversible nature of the inhibition by MG132, it is derepressed during protein extraction and the course of the assay.

4.3.2 Brf1 and Bdp1 protein levels and pol III transcription in MG132-treated PE cells

Figures 4.4A and B show no stabilisation of Brf1 and Bdp1, respectively, following proteasome inhibition by MG132. Although this strongly suggests that these two proteins are not regulated by the ubiquitin – proteasome pathway, the possibility cannot be wholly excluded. The extracts that were used in these experiments were

from cells grown in differentiation medium for 7 days. If the cells receive a signal to down-regulate pol III transcription by degrading TFIIIB at an earlier day, combined with the reduced transcription of Brf1 and Bdp1 (Figure 4.5) and the down-regulation of other pol III activators (or up-regulation of repressors – discussed in a later chapter), treatment of the cells with MG132 on the seventh day after induction would likely have little demonstrable effect on Brf1 and Bdp1 levels. One thing that might argue against such a hypothesis is that, in the work of Perletti *et al.* (2001) on TBP degradation, a clear stabilisation of the protein can be seen even after eight days following induction of differentiation. Furthermore, MG132 stabilises TBP even in undifferentiated F9 cells, which is not the case for Brf1 (data not shown) or Bdp1 (Figure 4.4B – compare lanes 1 and 2).

Somewhat more surprising is the fact that blocking the proteasome has no effect on pol III transcription, in EC or PE cells (Figure 4.4C), considering the plethora of proteins that is regulated by this pathway and also the variety of mechanisms that have an impact on pol III regulation. For example, p53 has been shown to repress pol III transcription through TFIIIB (reviewed in Brown et al., 2000), yet, even in MG132-treated EC cells, where TFIIIB is still abundant (Figure 3.4A) and p53 stabilised (Figure 4.1 – compare lanes 1 and 3), pol III transcription levels are unaffected (Figure 4.4C – compare lanes 1 and 2 for EC and 3 and 4 for PE cells).

4.3.3 Co-regulation of TFIIIB components

In the previous chapter, the possibility of coordination in the down-regulation of the TFIIIB subunits was raised. As we saw in section 4.2.3 of this chapter, proteasome inhibition has no effect on TFIIIB levels, strongly suggesting that they are not

degraded through the ubiquitin – proteasome pathway. Time course analysis TFIIIB mRNA revealed that Brf1 and Bdp1 mRNA levels follow a similar pattern over the 8 days (Figure 4.6). TBP, on the other hand, behaves rather differently than Brf1 and Bdp1. Although TBP mRNA levels through the differentiation period show a few similarities with Brf1 and Bdp1, if we just take the beginning and end of the time course (Figure 4.5A), we see that TBP mRNA levels actually increase in PE cells compared to EC cells.

Surprisingly, changes in Brf1 and Bdp1 mRNA levels do not appear to reflect the changes seen in protein levels (Figure 3.5A). This could reflect the difference in sensitivity between the two methods used. It is also worth noting that the two analyses were performed on extracts (protein or RNA) from different time courses, which might influence the results observed. Furthermore, the PCR analysis was only performed once. Further experiments are needed to ensure that the pattern observed is accurate.

4.3.4 RT-PCR analysis of MG132-treated cells

As described above (section 4.2.6), Brf1 and Bdp1 mRNA levels decrease following differentiation. RT-PCR analysis of EC and PE cells that had been treated with MG132, however, revealed that proteasome inhibition actually affects Bdp1 and Brf1 transcript levels (Figure 4.7A). From the reduction seen in control cells (compare lanes 1 and 2 for EC and PE respectively), we now see an increase in Bdp1 mRNA, while Brf1 stays relatively unchanged (lanes 3 and 4). TBP mRNA levels, on the other hand, are not affected by MG132 treatment, as they show the same pattern in both conditions (Figure 4.7A – row 3). These data agree with those obtained from the

mRNA time course (Figure 4.6), where *BRF1* and *BDP1* show a similar pattern of expression, different from that of *TBP*. A possible explanation for the effect of MG132 on Brf1 and Bdp1 mRNA levels is that proteasome inhibition could stabilise a transcription factor (or factors), involved in *BDP1* and *BRF1* expression, that is normally kept at low levels in PE cells. Alternatively, the half-life of the mRNA might be affected, with a protein that stabilises Brf1 and Bdp1 mRNA increasing in levels due to proteasomal inhibition. An interesting observation is that the increased mRNA level is not reflected in the overall protein levels. This could be attributed, for example, to regulation at the translational level. Thus, higher levels of mRNA would not lead to increased protein synthesis.

Two pol III-transcribed genes, *5S rRNA* and *B2*, were also tested (Figure 4.7B). There appears to be no effect of the proteasome inhibitor on the transcription of the two genes (compare lanes 1 and 2 with 3 and 4), which agrees with the absence of an effect of proteasome inhibition on pol III transcription seen in *in vitro* transcription assays (Figure 4.4C).

CHAPTER 5

Over-expression of Brf1 does not rescue pol III transcription

5.1 Introduction

Because most pol III-transcribed genes are essential for normal cellular metabolism (e.g., 5S rRNA for ribosome biogenesis, tRNA for translation and U6 small nuclear RNA for mRNA processing), pol III transcription is under strong regulation in response to a variety of external stimuli (reviewed in White, 2002). For example, during the cell cycle, pol III transcription is activated during the G_1/S transition, due to release from pRb-mediated repression, and repressed at mitosis (White et al., 1995a; White et al., 1995b; White et al., 1996). Pol III transcription is also repressed by p53 (Cairns and White, 1998). Both tumour suppressors, pRb and p53, bind to and inactivate TFIIIB. TFIIIB, and consequently pol III transcription, is also activated directly by the oncogene product c-Myc (Gomez-Roman *et al.*, 2003) and the kinases CK2 (Johnston *et al.*, 2002) and ERK (Felton-Edkins *et al.*, 2003a). All of these suggest that TFIIIB is the pivotal initiation factor in the pol III system (Figure 1.4).

Most pol III promoters in higher eukaryotes (and all in yeast) require a TFIIIB complex comprised of Bdp1, Brf1 and TBP. Consequently, many studies have tried to determine which of these components are most important for pol III regulation.

Work in yeast has shown that Brf1 is the limiting factor (Schramm and Hernandez, 2002), though the situation in mammals is still unclear.

To try to address some of the questions raised earlier about Brf1 regulation in differentiating F9 cells, I decided to establish an F9 cell line stably expressing HA-tagged human Brf1, driven by the constitutively active human cytomegalovirus immediate early promoter. The HA tag will be of use in monitoring the protein levels through differentiation, and the different promoter structure might help address the question of transcriptional control.

5.2 Results

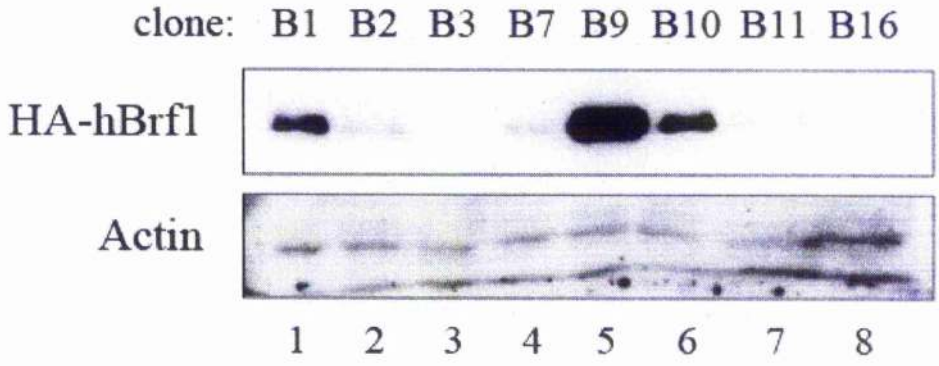
5.2.1 HA-hBrf1 is overexpressed in stably transfected cells.

As described in the Materials and Methods chapter, F9 EC cells were transfected using the Superfect protocol with either the pcDNA3.1-HA-hBrf1 plasmid or the empty vector pcDNA3.1-HA. They were subsequently grown in G418-containing medium, to select for those cells that had taken the plasmid up. Of the resulting G418-resistant clones, several were selected and grown further. Extracts from these cells were analysed by Western immunoblotting for expression of HA-hBrf1. Clones from cells that were originally transfected with the hBrf1-containing plasmid were called B1, B2, etc. (B for Brf1) and those with the empty vector C1, C2, etc. (C for control cells). Figure 5.1A shows the expression levels of exogenous HA-hBrf1 (top panel) in clones B1, B2, B3, B7, B9, B10, B11 and B16. Actin was used as loading control. Of these we can see that clones B1, B9 and B10 express HA-hBrf1 at high levels, with B9 being the highest. Clones B2, B7 and B11 also express HA-hBrf1,

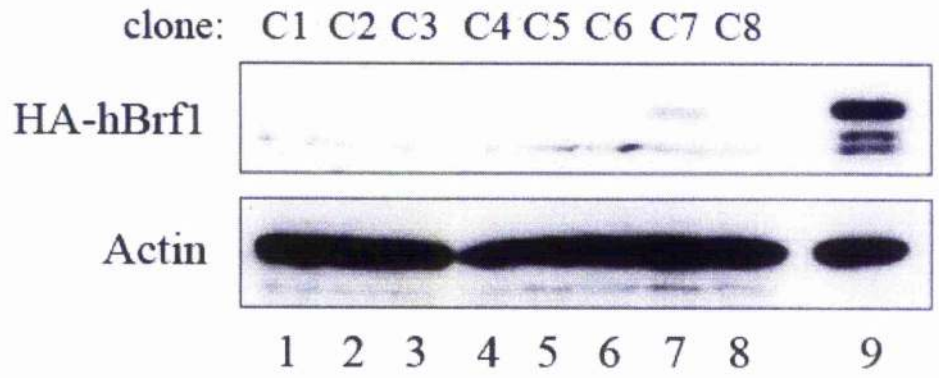
Figure 5.1 Screening of G418-resistant clones

G418-resistant clones, containing either the pcDNA3-HA-hBrf1 plasmid or the empty vector (generated as described in Materials and Methods), were used to prepare whole-cell extracts, which were resolved in a SDS – 7.8% polyacrylamide gel and analysed by Western immunoblotting for Brf1 levels. **A** Protein extracts (50 μg) from clones stably transfected with the HA-hBrf1 plasmid (B1-3, 7, 9-11, 16) were probed with an anti-HA antibody (F-7 – top panel) and an anti-actin antibody (C-11 – bottom panel). **B** and **C** G418-resistant clones, stably transfected with the control vector (C1-8) were probed with an anti-HA antibody (F-7 – top panel in B) or an anti-Brf1 antibody (128, which can recognise both exogenous and endogenous Brf1 – top panel in C). Actin was used as loading control. In B, *in vitro* translated HA-Brf1 was used as positive control (lane 9).

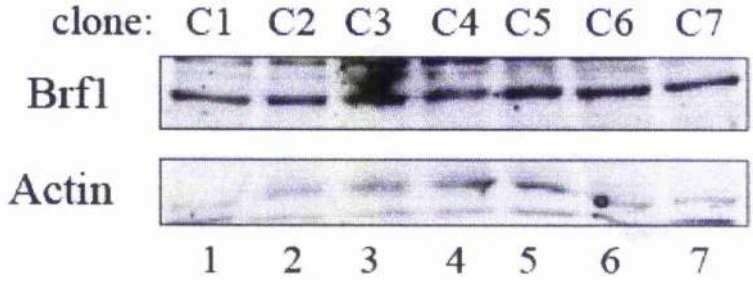
A


clone: B1 B2 B3 B7 B9 B10 B11 B16
HA-hBrf1
Actin
1 2 3 4 5 6 7 8

B


clone: C1 C2 C3 C4 C5 C6 C7 C8
HA-hBrf1
Actin
1 2 3 4 5 6 7 8 9

C


clone: C1 C2 C3 C4 C5 C6 C7
Brf1
Actin
1 2 3 4 5 6 7

but at very low levels. No signal was observed with clones B3 and B16 (see Discussion in this chapter about clone B16). The three high expressing clones, B1, B9 and B10, were therefore used for further experiments.

Eight clones of vector-transfected cells (C1 – C8) were also tested (Figure 5.1B and C) in a similar manner. When probed for HA-hBrf1 (top panel in 5.1B), C7 gave a weak signal and was therefore discarded. This was possibly due to contamination with HA-hBrf1-expressing cells during tissue culture. The rest were probed for total Brf1 (top panel in 5.1C), which revealed no significant differences between the clones. Any one of them could therefore be used as a control cell line.

For these stable cell lines to be useful, it was important to show that the total levels of Brf1, both protein and mRNA, are higher in the HA-hBrf1 cells compared to the controls, as the cells could compensate for the overexpression of exogenous Brf1 by reducing endogenous Brf1 production. Figure 5.2A shows that this is not the case, as the B9 clone shows higher levels of total Brf1 protein than both untransfected cells and a control clone (compare lane 2 with lanes 1 and 3). The antibody used here against Brf1 (145) recognises both the mouse and the human protein. Quantification revealed an almost 4-fold increase in the total protein levels of Brf1. Similar analysis was carried out with the other two clones that express high levels of HA-hBrf1 (B1 and B10 – results not shown). When RT-PCR analysis was carried out (Figure 5.2B), using cDNAs from two HA-hBrf1 clones (B9 and B10) and two control clones (C1 and C8) and primers that recognise both human and mouse sequences, it was revealed that the HA-hBrf1 clones contain higher levels of the Brf1 mRNA than the control clones (compare lanes 1 and 2 with 3 and 4). The pol II-transcribed gene *ARPP P0* was used as internal control.

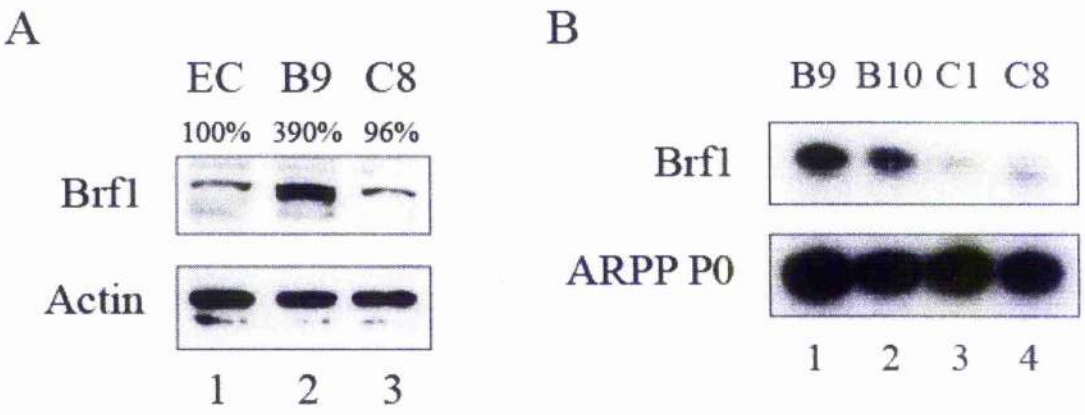


Figure 5.2 Total Brf1 levels in HA-hBrf1 clones

A Whole-cell extracts (20 μg) from either untransfected cells (EC – lane 1), HA-hBrf1 cells (B9 – lane 2) or control cells (C8 – lane 3) were resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western blotting using an anti-Brf1 antibody (145, which recognises both exogenous and endogenous Brf1 – top panel) or an anti-actin antibody (C-11 – bottom panel), as loading control. Total levels of Brf1 were quantified (TotalLab), normalised to actin and expressed as a percentage of EC. **B** cDNAs were generated from total RNA (3 μg) extracted from HA-hBrf1 cells (B9 and B10 – lanes 1 and 2) or control cells (C1 and C8 – lanes 3 and 4), and analysed by PCR using primers for Brf1 (top panel) or ARPP P0 (bottom panel). The Brf1 primers used here amplify the same sequence from both mouse and human Brf1.

5.2.2 *Overexpression of Brf1 does not affect the proliferation rate of F9 EC cells*

Previous work in the laboratory has shown that mouse embryonic fibroblasts overexpressing Brf1 proliferate twice as fast as control cells (Marshall and White, in preparation). We, therefore, wondered if overexpressing Brf1 in F9 EC cells would result in faster proliferation. To investigate this, a simple proliferation experiment was performed, in which cells from either an HA-hBrf1 clone (B9) or a control clone (C8) were plated at a density of $1x10^4$ and numbers of viable cells monitored over a 6 day-period. A graphical representation of the average of three independent experiments, with the same cell lines, can be seen in Figure 5.3. It is immediately obvious that there is no difference in the proliferation rates of Brf1 overexpressing cells, compared to control cells.

5.2.3 *Levels of HA-hBrf1 are not affected by differentiation or MG132 treatment*

The primary reason for establishing these stable cell lines was to allow for easier observation of Brf1 during the differentiation of F9 cells, courtesy of the HA tag at its N-terminus. Western immunoblotting, using an antibody against the HA tag of hBrf1, revealed that, when HA-hBrf1 cells (B1 clone) were induced to differentiate, no reduction in the levels of the exogenous protein were observed (Figure 5.4A lanes 3 and 4). Similarly, when HA-hBrf1 was monitored throughout the differentiation process by means of a time course, no variation in its levels was

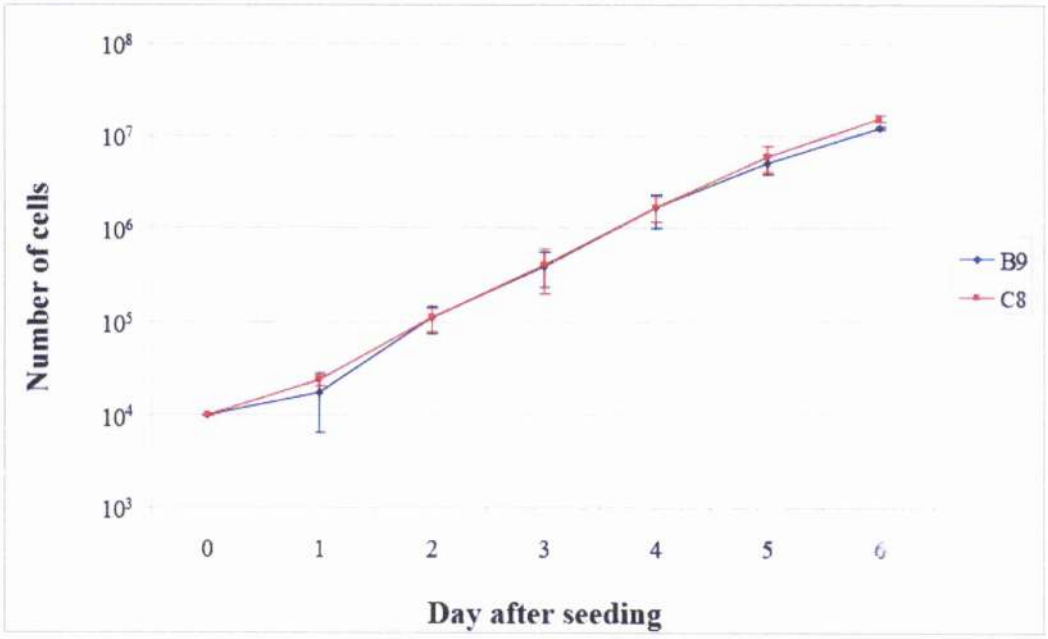


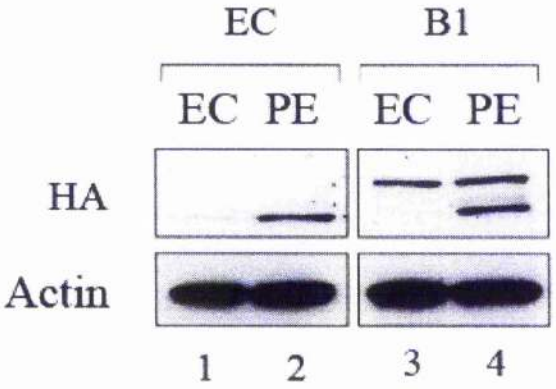
Figure 5.3 Proliferation curves of HA-hBrf1 and control cells

HA-hBrf1 (B9) or control (C8) cells were plated at a density of $1x10^4$ on day 0 and cultured in growth medium. Viable cell counts, determined by trypan blue staining, were taken in triplicate every day for 5 or 6 days after seeding. The results from three independent experiments were averaged and plotted on a semi-logarithmic graph, ± standard deviation.

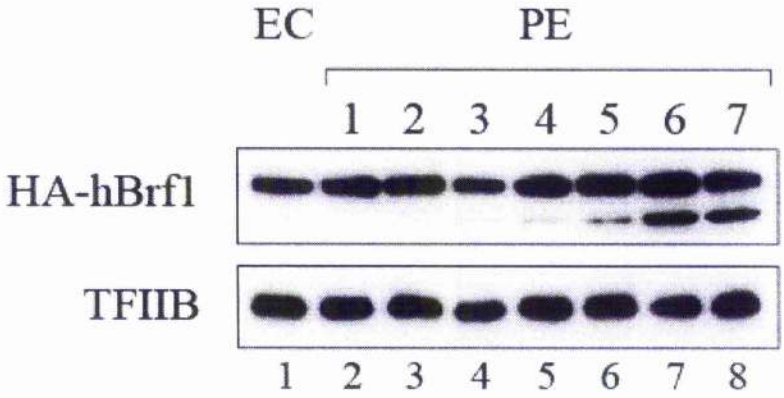
Figure 5.4 Levels of HA-hBrf1 in differentiated, stably transfected cells

A Whole-cell extracts (50 μg) from untransfected (lanes 1 and 2) or clone B1 (lanes 3 and 4) cells, before (EC – lanes 1 and 3) and after (PE – lanes 2 and 4) differentiation were resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western blotting using an anti-HA antibody (F-7 – top panel) or an anti-actin antibody (C-11 – bottom panel) as loading control. **B** Whole-cell extracts (20 μg) from EC B9 cells (lane 1) or from each day during differentiation of these clones (lanes 2-8) were analysed as in A, except that TFIIB was used as loading control (antibody C-18). **C** Total RNA (3 μg), extracted from B9 (lanes 1 and 3) or C8 (lanes 2 and 4) clones, before (lanes 1 and 2) or after (lanes 3 and 4) differentiation, was used to generate cDNAs by reverse transcription. Levels of Brf1 were determined by PCR analysis, using primers that recognise only the human mRNA. HPRT was used as internal control. B refers to the B9 clone and C to the C8 clone.

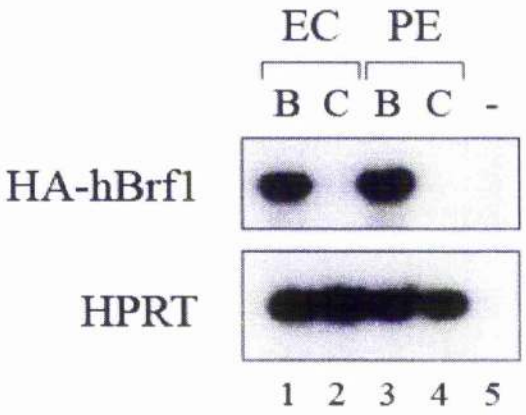
A


EC
B1
EC PE EC PE
HA
Actin
1 2 3 4

B


EC
PE
1 2 3 4 5 6 7
HA-hBrf1
TFIIB
1 2 3 4 5 6 7 8

C


EC PE
B C B C -
HA-hBrf1
HPRT
1 2 3 4 5

observed (Figure 5.4B). The band that appears below HA-hBrf1 in Figure 5.4A and B is thought to be a cross-reacting protein and is discussed later in this chapter.

A small reduction in the mRNA levels of Brf1 has been previously observed (Figure 4.5), suggesting a possible transcriptional regulation in response to differentiation. Although under the control of a different promoter, i.e. the constitutively active CMV promoter, it was interesting to examine whether or not exogenous Brf1 mRNA was regulated in response to differentiation, either at transcriptional or post-transcriptional level. RNA was extracted from B9 and C8 clones, before and after differentiation. RT-PCR analysis using primers that recognise only human Brf1 (and therefore only the exogenous Brf1 in these cells) revealed no reduction in HA-hBrf1 levels following differentiation (Figure 5.4C -- compare lane 1 with lane 3 in the top panel). HPRT, the product of a constitutively expressed gene, was used as an internal control.

Experiments using COS7 cells (from monkey kidney tumours) suggested that HA-hBrf1 might be ubiquitinated and degraded by the proteasome (data not shown). Similar experiments in F9 cells lead to an interesting observation. When a plasmid containing the ubiquitin gene, under the control of the CMV promoter, was transfected into an undifferentiated HA-hBrf1 clone (EC-B9), the protein levels of both Bdp1 and HA-hBrf1 were reduced (Figure 5.5A). When, however, EC or PE B9 cells were treated with the proteasome inhibitor MG132, no effect on the levels of HA-hBrf1 was observed (Figure 5.5B, compare lanes 1 and 2 for EC and 3 and 4 for PE).

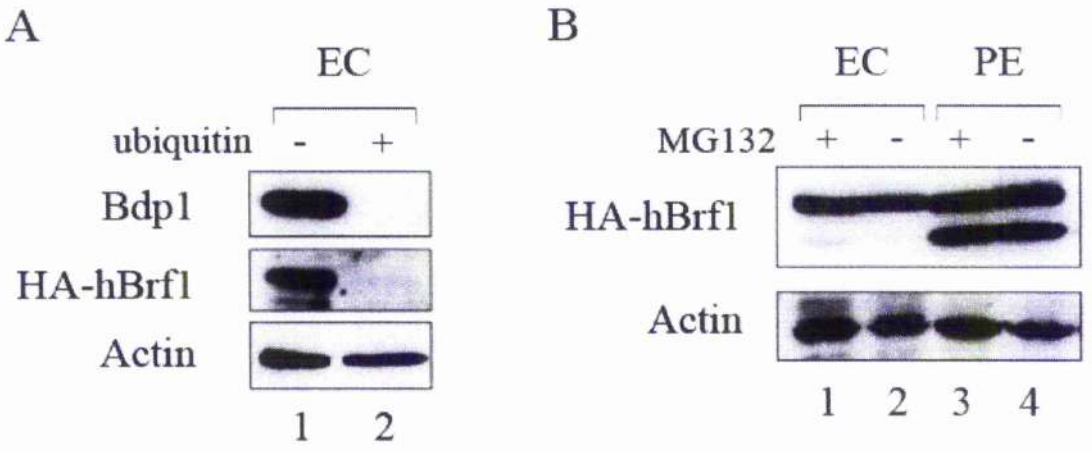


Figure 5.5 Stability of HA-hBrf1 in EC and PE cells

A EC B1 clones were transiently transfected with a vector containing histidine-tagged ubiquitin, under the control of a constitutive promoter, using the Superfect protocol (see Materials and Methods). Cell lysates were prepared from these cells, resolved on a SDS – 7.8% polyacrylamide gel and transferred onto PVDF. The membrane was subsequently probed for Bdp1 (antibody 2663 – top panel) and HA-hBRF1 (HA probe, F-7 – middle panel). Actin was used as both loading and negative control. **B** B9 cells were treated with DMSO (lanes 1 and 3) or the proteasome inhibitor MG132 (50 μM – lanes 2 and 4) for 4 hours prior to harvesting and protein extraction. Whole-cell extracts from these cells were analysed by Western blotting using an anti-HA antibody (F-7 – top panel) or an anti-actin antibody (C-11 – bottom panel).

5.2.4 Effect of HA-hBrf1 overexpression on pol III transcription

The fact that HA-hBrf1 levels did not decline following differentiation raised the possibility that pol III transcription might also be affected in PE cells. First, however, we needed to examine if overexpression of HA-hBrf1 had any effect on pol III transcription in undifferentiated cells. To do this, *in vitro* transcription assays were carried out, using extracts from untransfected EC cells or stably transfected B9 or C8 clones. Figure 5.6A shows that pol III transcription of a $tRNA^{Leu}$ template is elevated in the B9 clones compared to untransfected or control cells (compare lane 2 with lanes 1 and 3). When these cell lines were induced to differentiate, however, a reduction in pol III transcription could still be observed, similar to that for untransfected F9 cells (see Figure 3.2B). As we can see in Figure 5.6B, transcription of a $tRNA^{Leu}$ template is reduced in differentiated B9 cells (compare lanes 1 and 2), even though they contain comparable Brf1 levels to the undifferentiated cells (Figure 5.4). The control cells, C8, show a similar reduction. As in the case of the wild-type F9 cells (see Figure 4.4C), proteasome inhibition does not affect pol III transcription following differentiation (Figure 5.6C). EC (lanes 1 and 2) or PE (lanes 3 and 4) B9 cells were treated with MG132 (lanes 1 and 3) or DMSO (lanes 2 and 4) for 4 hours prior to harvesting and protein extraction. *In vitro* transcription assays of a $tRNA^{Leu}$ template using these extracts show no changes in pol III transcription in response to MG132 treatment (compare lane 1 with lane 2 and lane 3 with lane 4). Since these extracts are obtained at the start and the end of the differentiation process, i.e. days 0 and 7 respectively, there is no indication as to the levels of pol III transcription in the intervening period. To address this, extracts were prepared before induction and at

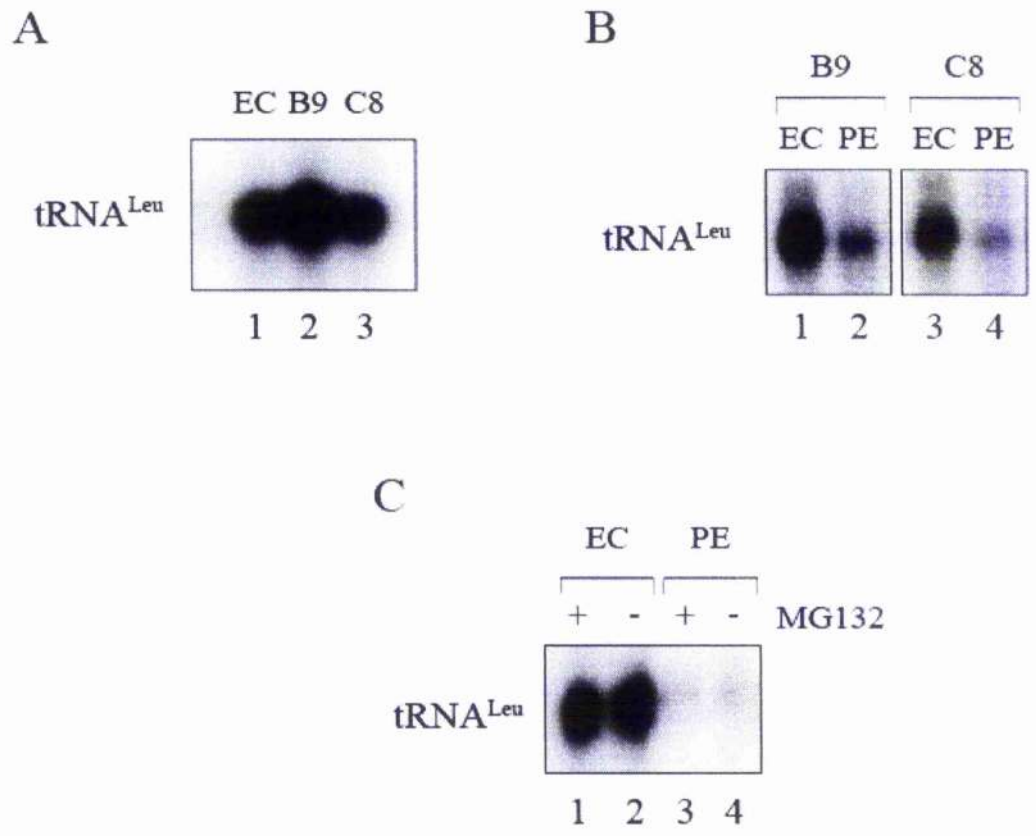


Figure 5.6 Effect of Brf1 over-expression on pol III transcription

A Whole-cell extracts (20 μg) from untransfected EC cells (lane 1) and B9 (lane 2) or C8 EC clones were used to *in vitro* transcribe a $tRNA^{Leu}$ template (250 ng). **B** *In vitro* transcription of a $tRNA^{Leu}$ template (125 ng) by EC (lanes 1 and 3) or PE (lanes 2 and 4) whole-cell extracts from B9 (lanes 1 and 2) or C8 (lanes 3 and 4) clones. **C** EC (lanes 1 and 2) or PE (lanes 3 and 4) B9 cells were treated with MG132 (50 μM – lanes 1 and 3) or DMSO (lanes 2 and 4) for 4 hours. Whole-cell extracts from these cells were used to *in vitro* transcribe a $tRNA^{Leu}$ template (250 ng).

each day during differentiation of HA-hBrf1 clones. Figure 5.7A shows the *in vitro* transcription of $tRNA^{Leu}$ using extracts from B9 cells, although the experiment was repeated with B1 and B10 cells as well. We can see here that transcription remains at high levels until day 5, compared to day 2 in untransfected F9 cells (Figure 3.3A), although there is a small reduction between days 2 and 3. The same experiment was performed with the B1 and B10 clones, the autoradiographs were quantified (TotalLab) and the results averaged and presented graphically in Figure 5.7B (blue line). From the graph we can see an initial reduction by about 20% for the first two days following induction of differentiation, and then a further decline between days 3 and 5. RNA polymerase III transcription levels reach a minimum level at day 6 of the differentiation. The red line represents the same experiment done with untransfected F9 cells (also presented in Figure 3.3B), and is included here for easier comparison.

5.2.5 Recombinant hBdp1 does not restore pol III transcription following differentiation

As was discussed previously, when F9 EC cells are induced to differentiate, pol III transcription is down-regulated due to a reduction in one or more activities found in a protein fraction containing the TFIIIB complex (Alzuherri and White, 1998). The HA-hBrf1 clones we made contain high levels of Brf1 even after differentiation, but if there is no free Bdp1, the only other pol III-specific component of TFIIIB, then the additional Brf1 might not become incorporated into functional complexes. PE extracts from B9 clones were, therefore, pre-incubated with recombinant human Bdp1 (rhBdp1) and then used these for *in vitro* transcription of a $tRNA^{Leu}$ template. In Figure 5.8, we can see that rhBdp1 has no effect on pol III transcription when it is

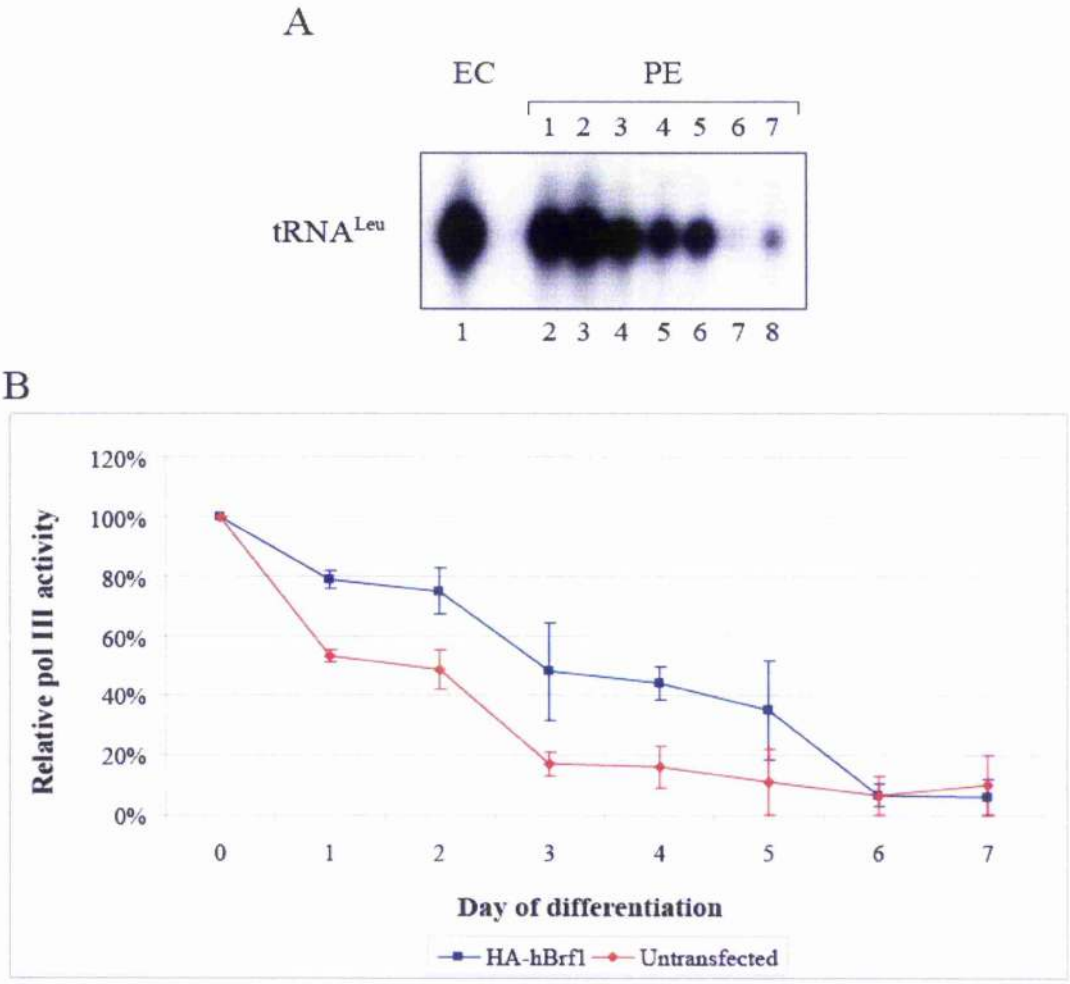


Figure 5.7 Time-course of pol III down-regulation in HA-hBrf1 cells

A B9 cells before (lane 1) and during differentiation (lanes 2-8) were used to prepare whole-cell protein extracts. 20 µg of the extracts were then used for the *in vitro* transcription of a $tRNA^{Leu}$ template (250 ng). **B** Graphical representation of three independent time-course experiments (blue line), ± standard deviation. The down-regulation of pol III transcription in untransfected F9 cells is also shown here (red line), for easier comparison (see also Figure 3.4B).

used to supplement extract from Brf1-overexpressing cells (compare lanes 3 and 4 with lane 2). EC extracts from the same cells (lanes 1 and 5) indicate the level of transcription before differentiation.

5.3 Discussion

5.3.1 Establishment of an HA-hBrf1 overexpressing cell line

It is known that expression of some viral proteins in early mouse embryogenesis is severely restricted or absent, and that it is activated subsequently during development (Kelly and Condamine, 1982). As a further testament to their close resemblance to the inner cell mass (ICM) of the early mouse embryo, F9 EC cells also exhibit this block to viral expression. Viruses whose expression is limited in F9 cells include the polyoma and SV40 viruses (members of the polyomaviridae family), the retroviruses Moloney murine leukaemia virus and mouse mammary tumour virus, as well as murine cytomegalovirus (belonging to the herpesviridae family). This was cause for some concern, since the HA-hBrf1 construct had been cloned into the pcDNA3.1 plasmid, which contains the human cytomegalovirus promoter and enhancer sequences. Transient transfections demonstrated that HA-hBrf1 was indeed expressed in F9 EC cells (results not shown). This was verified when G418-resistant clones were screened for expression of HA-hBrf1 (Figure 5.1A). The differences observed in expression could be the result of the efficiency of transfection, the number of plasmids integrated in the genomic DNA and the site of those integrations. For that reason, although the B9 clone was predominantly used, most of

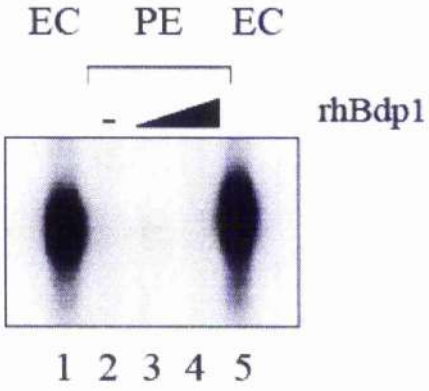


Figure 5.8 Recombinant Bdp1 add-back

Bacterially expressed recombinant human Bdp1 (rhBdp1) was assessed in its ability to restore pol III transcription of PE extracts from HA-hBrf1 cells (B9 clone). PE extracts were preincubated with 0, 1.5 and 3 µl of rhBdp1 and subsequently used to *in vitro* transcribe a $tRNA^{Leu}$ template (lanes 2-4, respectively). EC extracts from the same cell line were used for comparison (lanes 1 and 5). 20 µg of cell extract and 250 ng template were used in the assay.

the experiments were repeated with the other two high-expressing clones (i.e. B1 and B10). Another thing to note is that clone B16 showed an extremely high level of expression during the first screening stage. As can be seen in Figure 5.1A (lane 8), this expression was subsequently lost. Therefore, whenever new clones were brought up from liquid nitrogen storage, and at various times thereafter if grown for long periods (more than a month), HA-hBrf1 expression was verified by Western blot analysis.

5.3.2 Proliferation rate of F9 EC cells overexpressing HA-hBrf1

As discussed in the introduction, TFIIIB is under intense regulatory control by a number of factors and environmental conditions, most of which focus on the Brf1 subunit of TFIIIB. Furthermore, work in mouse embryonic fibroblasts has shown that regulated overexpression of Brf1 leads to an increase in the proliferation rate of the cells (Marshall and White, in preparation). HA-hBrf1 overexpression in F9 EC cells results in increased pol III transcription (Figure 5.6). This suggests either that human Brf1 can functionally interact with the mouse pol III machinery (particularly TBP and Bdp1, to form TFIIIB), or that it can sequester inhibitory factors, for example pRb and p53. When the proliferation rate of the cells was assessed, however (Figure 5.3), no difference was observed between HA-hBrf1 and control cells. This suggests that pol III activity in F9 EC cells is not rate limiting for progression through the cell cycle. This is not surprising, considering the transformed status of the cells and the very high proliferation they exhibit normally (Rosenstraus *et al.*, 1982).

5.3.3 HA-hBrf1 levels in response to differentiation and proteasome inhibition

In the case of wild-type, untransfected F9 cells, we previously saw that differentiation causes a small reduction in the mRNA levels of Brf1 and Bdp1 (Figure 4.5), which is accompanied by a larger decline in protein levels (Figure 3.4). However, since we are dealing with different techniques (with different sensitivities), direct comparisons can be misleading. Nevertheless, these data, combined with the lack of stabilisation of these proteins by the proteasome inhibitor MG132 (Figure 4.4A and B), suggest that Brf1 and Bdp1 regulation may occur at transcriptional, post-transcriptional or translational level. Establishing a Brf1-overexpressing F9 cell line allowed us to investigate this further, since HA-hBrf1 is controlled by the constitutively active human CMV promoter. The possibility of transcriptional control can be thus examined, since it is likely to have different transcription factor requirements from endogenous Brf1 (this is not a certainty, since regulation of *BRF1* expression has not been studied).

As we can see in Figure 5.4, HA-hBrf1 levels are not reduced following differentiation either at protein (A and B) or mRNA level (C). In comparison, endogenous Brf1 is reduced at both levels (Figures 3.4 and 4.5). These data further argue against regulation of Brf1 at protein level, considering the similarity between the mouse and human Brf1 (almost 90% identity). Exogenous Brf1 mRNA is also not affected by differentiation (Figure 5.4C, compare lanes 1 and 3), suggesting that transcriptional (or post-transcriptional) regulation is different for endogenous and exogenous *BRF1*.

The lower band in Figure 5.4A and B appears to be a protein, whose expression is induced during differentiation, that cross-reacts with the HA antibody. This is supported by the fact that it can be seen even in untransfected F9 PE cells (Figure 5.4A, lanes 2).

The data obtained from the experiment where ubiquitin was transfected into an HA-hBrf1 clone (Figure 5.5A) were quite surprising, since Brf1 and Bdp1 were not stabilised by MG132 in F9 EC cells (Figure 4.4B for Bdp1 and data not shown for Brf1). Overexpression of ubiquitin, however, seems to drive the degradation of HA-hBrf1 and Bdp1 in the B1 clone that was used for the experiment. Although not shown in Figure 5.5A, this degradation was somewhat blocked by MG132, but levels for both proteins were still lower than the mock-transfected cells. A possible explanation for this can be found in the fact that ubiquitin is thought to be a limiting factor for the proteasome pathway (Hay, R. T., personal communication). Its overexpression, therefore, could allow the degradation of proteins that would, under physiological conditions, not be targeted to the proteasome. This also suggests that both of these proteins contain the necessary signals for proteasomal degradation. Nevertheless, proteasome inhibition in the same cells, without the overexpression of ubiquitin, does not lead to stabilisation of HA-hBrf1 (Figure 5.5B), before or after differentiation.

5.3.4 RNA polymerase III transcription in HA-hBrf1 clones

We saw that EC extracts from B9 cells have higher transcriptional activity than either control or untransfected F9 EC cells (Figure 5.6A) and that HA-hBrf1 protein levels do not decrease following differentiation (Figures 5.4A and B). It was therefore

surprising to see that, following differentiation of the B9 cells, pol III transcription of the same template ($tRNA^{Leu}$) decreased (Figure 5.6B – compare lanes 1 and 2). This decrease was similar to that observed using extracts from the C8 cells (lanes 3 and 4 in Figure 5.6B). Based on these data, it would seem that, in F9 PE cells, Brf1 is no longer limiting for pol III transcription. Bdp1 also decreases in differentiated cells. The extra HA-hBrf1 might, therefore, not be able to form functional TFIIIB complexes due to absence of Bdp1. When B9 PE extracts were pre-incubated with recombinant hBdp1 and used for IVT assays, however, no restoration of transcription was observed (Figure 5.8). These data suggest that TFIIIB is not limiting in F9 PE cells. This appears to contradict earlier findings (Alzuherri and White, 1998; White *et al.*, 1989), that TFIIIB is the limiting activity in F9 PE cells. Since those findings were based on protein fractionation, it is possible that another pol III activator, which co-fractionates with TFIIIB, is limiting in PE cells. This possibility will be discussed in the following chapter.

When we look at the down-regulation of pol III transcription over the whole of the 7 days that the cells are induced to differentiate, we see that there a difference exists between untransfected and HA-hBrf1 clones (Figure 5.7B – red and blue lines, respectively). Although the level of pol III transcription is similar between the two cell lines at the end of the differentiation period (days 6 and 7), the decline is delayed until day 4 or 5. Of particular note is that the initial decrease (days 1 and 2) is only about 20%, compared to around 50% in the untransfected cells, which seems to be responsible for the delay observed later on. The large error bars for the HA-hBrf1 cells could be due to the fact that different clones (B1, B9 and B10) were used for the experiments, in order to get a more accurate picture, and these express HA-hBrf1 at different levels. It, therefore, seems that overexpression of Brf1 does have an effect on differentiating F9 cells, but only until a certain time point, i.e. around day 4 or 5

after induction of differentiation. After that, other factors that affect pol III transcription might become limiting.

CHAPTER 6

Regulation of other pol III regulators during differentiation

6.1 Introduction

In Section 1.1.6, a number of key regulatory proteins were presented, all of which seem to exert their effect through TFIIIB. A diagram is presented in Figure 1.4. It is immediately obvious that the proteins involved encompass a large number of regulatory pathways, which, in response to different stimuli, can activate or repress pol III transcription.

It has been previously shown that TFIIIB is activated at the G1/S transition during the cell cycle (White *et al.*, 1995a), at about the same time that the retinoblastoma protein (pRb) is inactivated (Weinberg, 1995), raising the possibility that the two events might be connected. Subsequent work showed that overexpression of pRb in transfected cells leads to reduced pol III activity (Chu *et al.*, 1997; White *et al.*, 1996), and that recombinant Rb has the ability to repress pol III transcription *in vitro* (Larminie *et al.*, 1997; White *et al.*, 1996). When the *Rb* gene was inactivated by site-directed mutagenesis in mice, tRNA and 5S rRNA synthesis increased by about 5-fold (White *et al.*, 1996), and extracts obtained from these mice exhibited a specific increase in TFIIIB activity (Larminie *et al.*, 1997). Furthermore, co-immunoprecipitation and pull-down experiments showed that pRb physically interacts with TFIIIB (Chu *et al.*, 1997; Larminie *et al.*, 1997). All these data pointed

to a specific effect of pRb on pol III transcription, the mechanism of which became clear a few years later, when Sutcliffe *et al.* (2000) showed that pRb exerts its repressive effect by disrupting the interactions of TFIIIB with TFIIIC and the polymerase itself.

The retinoblastoma protein, pRb, belongs to a small family of structurally and functionally related proteins, collectively referred to as the pocket proteins (reviewed in Grana *et al.*, 1998; Mulligan and Jacks, 1998). Because of those functional similarities, the possibility of involvement of p107 and p130 in the regulation of pol III transcription was examined (Sutcliffe *et al.*, 1999). Similar to pRb, overexpression of p107 and p130 was found to repress pol III transcription in transfected cells, while recombinant p107 and p130 inhibited pol III transcription *in vitro* (Sutcliffe *et al.*, 1999). Co-immunoprecipitation experiments showed that endogenous p107 and p130 associated with the Brf1 subunit of TFIIIB. In addition, p107 and p130 co-fractionated with TFIIIB activity, suggesting a stable interaction (Sutcliffe *et al.*, 1999). Fibroblasts from *p107*$^{-/-}$ *p130*$^{-/-}$ double knockout mice exhibited increased pol III activity, as was the case when p107 and p130 were specifically inactivated by the E7 oncoprotein of human papillomavirus (Sutcliffe *et al.*, 2000). Therefore, p107 and p130, like pRb, have the ability to bind to TFIIIB and repress transcription by pol III.

Although no direct comparisons of pRb, p107 and p130, as far as protein levels are concerned, have been made between F9 EC and PE cells, it has been shown that pRb and p107 E2F-associated activity is low in EC but elevated in PE cells (Partridge and La Thangue, 1991; Zamanian and La Thangue, 1993). In addition, it has been shown that the *Rb* gene is induced in a variety of mouse cell lines, other than F9, following differentiation (Coppola *et al.*, 1990; Endo and Goto, 1992; Richon *et al.*, 1992;

Slack *et al.*, 1993). No reports deal with the third member of the family, p130, in F9 cells.

As well as pRb, p53, an unrelated tumour suppressor (reviewed in Levine, 1997), also has the ability to repress transcription by pol III. Chesnokov *et al.* (1996) originally showed that wild-type p53 can inhibit transcription of some class III genes, both *in vitro* and *in vivo*. These observations were later extended to include other genes transcribed by pol III (Cairns and White, 1998). Furthermore, it was shown that p53 exerts its effect by binding to TFIIIB through the TATA-binding protein, disrupting the interactions of TFIIIB with TFIIIC and pol III (Crighton *et al.*, 2003). Since TFIIIC is responsible for recruiting TFIIIB to most pol III-transcribed genes, p53 induction leads to a reduction in TFIIIB, and therefore pol III, present at class III promoters (Crighton *et al.*, 2003). Conversely, promoter occupancy, as well as tRNA and 5S rRNA synthesis, increases in fibroblasts obtained from p53-knockout mice (Cairns and White, 1998; Crighton *et al.*, 2003).

Testicular teratocarcinoma cells, from which the F9 cell line is derived, never contain *p53* gene mutations, even though these tumours express high levels of p53 protein (Lutzker and Levine, 1996). Specifically, F9 EC cells have been shown to contain wild-type p53 (Pennica *et al.*, 1984), the levels of which, both mRNA and protein, decrease following differentiation into parietal endoderm-like cells (Dony *et al.*, 1985; Oren *et al.*, 1982). However, it has been reported that, although p53 is present in F9 EC cells, it is functionally inactive (for example Mayo and Berberich, 1996; Schmidt-Kastner *et al.*, 1998), but is activated in response to UV irradiation (Mayo and Berberich, 1996) or other DNA damaging agents (Lutzker and Levine, 1996). Furthermore, RA-induced differentiation results in increased p53-mediated transcriptional activity, even though p53 levels decrease (Lutzker and Levine, 1996).

The first indication that CK2 might be involved in pol III transcription came from work in yeast, where it was shown that it stimulates the transcription of the *5S rRNA* and *tRNA* genes (Hockman and Schultz, 1996). Further work in yeast demonstrated that CK2 phosphorylates the TBP component of TFIIIB, thus enhancing its recruitment to promoters of class III genes and activating pol III transcription (Ghavidel and Schultz, 1997; Ghavidel and Schultz, 2001; Hockman and Schultz, 1996).

Work in mouse and human cells showed that CK2 activity is equally important in mammalian cells, since its inhibition resulted in a reduction of pol III transcription both *in vivo* and *in vitro* (Johnston *et al.*, 2002). It was also shown that, like in yeast, CK2 activity affects recruitment of TFIIIB to class III promoters by TFIIIC (Johnston *et al.*, 2002), a step that, at least in yeast, is rate-limiting for pol III transcription (Rameau *et al.*, 1994). However, while, in yeast, TBP alone is phosphorylated efficiently by CK2 *in vitro* (Ghavidel and Schultz, 1997), all three human TFIIIB components, TBP, Brf1 and Bdp1, are phosphorylated directly by CK2 *in vitro* (Johnston *et al.*, 2002). Overall, these data suggest that CK2 involvement in pol III transcription is maintained through evolution, consistent with the very high degree of conservation that is displayed by CK2.

Studies concerning CK2 in F9 cells have focused on the effects that chemotherapeutic agents, like cisplatin and carboplatin, have on its levels and activity, especially in relation to p53 and Mdm2 as part of the apoptotic response (e.g. Siemer *et al.*, 1999 and references therein). No investigations have been made into the effects of cell differentiation on CK2 levels and activity.

The mitogen-activated protein kinase (MAPK) pathway, of which the Ras/ERK pathway is but a branch, exists in all eukaryotes and controls fundamental cellular processes, such as proliferation, differentiation, survival and apoptosis. It was not

surprising, therefore, to find that ERK plays a role in the regulation of RNA polymerase III, the output of which (e.g. tRNA and 5S rRNA) is essential for growth. Serum induction of quiescent cells results in an immediate increase in pol III transcription, which is mediated by the ERK pathway, as inhibition of kinase just upstream of ERK (MEK) by PD98059, or Ras (by FTI-277), causes a reduction in the expression of class III genes (Felton-Edkins *et al.*, 2003a). Further experiments showed that ERK binds to and phosphorylates Brf1, thus increasing TFIIIB binding to TFIIIC (similar to CK2), but also to the polymerase. As before, inhibition of MEK activity by a chemical reagent (U0126) significantly reduced this effect (Felton-Edkins *et al.*, 2003a). Activation of pol III transcription by ERK is, therefore, conditional on its prior activation (phosphorylation) by MEK.

As mentioned before, differentiation of F9 cells from EC to PE is a two-step process, with the cells initially adopting a primitive endoderm-like morphology and characteristics, induced by retinoic acid. Subsequently, in response to elevated cyclic AMP levels, they differentiate into parietal endoderm-like cells (Figure 1.5). The Ras/ERK pathway has been shown to play an important role in both of these processes. Specifically, it was shown that RA-induced differentiation into primitive endoderm is accompanied by activation of endogenous Ras and ERK, whereas differentiation into parietal endoderm has the opposite effect, with Ras and ERK activity being reduced (Verheijen *et al.*, 1999). Furthermore, expression of constitutively active forms of Ras, Raf and MEK in F9 EC cells were shown to induce differentiation toward a primitive endoderm-like phenotype, while inhibiting the subsequent differentiation to parietal endoderm (Verheijen *et al.*, 1999).

The myc/max/mad network is a group of transcription factors that have been shown to greatly affect cell behaviour (for reviews see Amati *et al.*, 1998; Bouchard *et al.*, 1998; Facchini and Penn, 1998; Grandori *et al.*, 2000). c-Myc, and its relatives N-

and L-Myc, are proto-oncogenes, whose expression generally correlates with cell proliferation. Through dimerisation with Max, they are able to bind to particular DNA sequences (Myc E-boxes) and activate expression of target genes. Max can also bind another small family of proteins, Mad1, Mad2 (or Mxi1), Mad 3 and Mad4. Mad-Max heterodimers exert the opposite effect to Myc-Max dimers, repressing the transcription of target genes, using the same E-box sequences as Myc-Max. So, in effect, Myc and Mad family members compete with each other for binding of Max, to activate or suppress expression of genes containing E-box sequences.

Myc is known to be involved in regulating cell growth through induction of a number of growth-related genes (Dang, 1999), transcribed by RNA polymerase II. It has been recently shown, however, that c-Myc directly activates RNA polymerase III, through interactions with the TFIIIB complex (Gomez-Roman *et al.*, 2003). Furthermore, it is present at tRNA and 5S rRNA genes in several cell types (Felton-Edkins *et al.*, 2003b; Gomez-Roman *et al.*, 2003). Fibroblasts obtained from c-Myc knockout mice, as well as Myc-depleted HeLa cells, exhibit increased pol III activity, while overexpression leads to decreased transcription of *tRNA* and *5S rRNA* genes (Felton-Edkins *et al.*, 2003b; Gomez-Roman *et al.*, 2003). These data suggest that c-Myc contributes significantly to pol III transcription in normal cycling cells, expanding the involvement of c-Myc in cell growth control.

Because of its importance in cell growth and differentiation, the behaviour of c-Myc during differentiation of F9 EC cells has been studied extensively. Induction of differentiation by retinoic acid and dibutyryl cAMP results in a strong down-regulation of c-*myc* mRNA at an early stage, within 24 hours following induction (Dean *et al.*, 1986; Dony *et al.*, 1985). The rate of transcription of the *myc* gene, however, is not affected by retinoic acid and cAMP (Dony *et al.*, 1985), suggesting a post-transcriptional method of regulation. Further work suggested that c-*myc* mRNA

down-regulation is related to growth arrest rather than differentiation. Decreased proliferation of F9 EC cells, in the absence of differentiation, was accompanied by reduced c-*myc* mRNA levels (Dean *et al.*, 1986), whereas cells constitutively expressing c-*myc* became completely differentiated upon treatment with retinoic acid and cAMP, although they exhibited prolonged proliferation once they were fully differentiated (Schulz and Gais, 1989).

Contrary to the situation with c-Myc, the volume of data for other proteins in this network in F9 cells is quite limited. Nevertheless, it has been shown that differentiation to PE causes a gradual reduction in Max protein levels, although there is little variation to *max* mRNA, suggesting a post-transcriptional regulation (Larsson *et al.*, 1997). The same authors also state that *mad1* mRNA was undetectable in F9 EC or PE cells.

This chapter examines the protein levels of these regulatory proteins in differentiating F9 cells and the possible implications for the regulation of transcription by RNA polymerase III.

6.2 Results

6.2.1 The pocket proteins, pRb, p107 and p130, are differentially regulated during F9 cell differentiation

The relative levels of the three pocket proteins, pRb, p107 and p130, were investigated by Western immunoblotting of extracts from F9 EC and PE cells. As we can see in Figure 6.1A, p107 and p130 levels do not change following differentiation (top two panels). In contrast, pRb levels are strongly up-regulated in PE cells (third

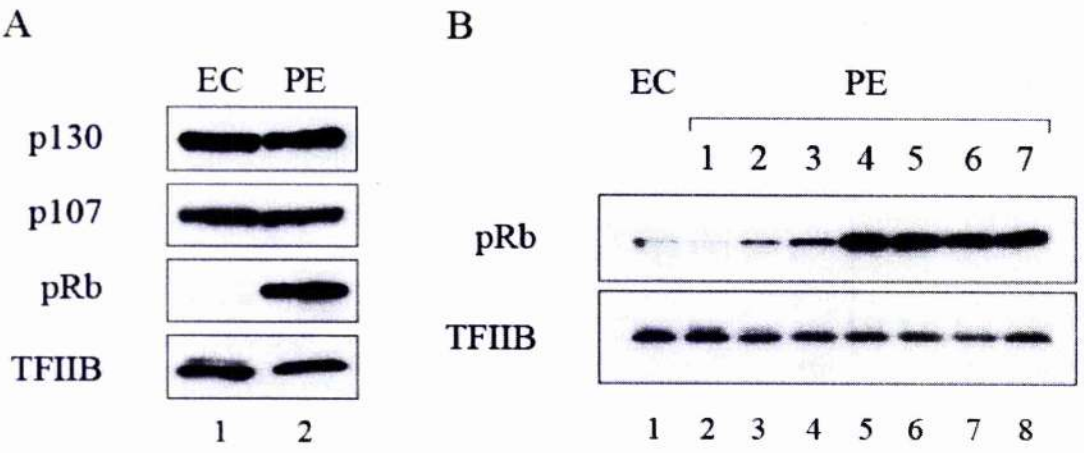


Figure 6.1 The pocket proteins are differentially regulated in differentiating F9 cells

A Whole-cell extracts (20 μg) from EC (lane 1) or PE (lane 2) cells were resolved on a SDS – 7.8% polyacrylamide gel, transferred onto PVDF membrane and analysed by Western blotting using antibodies against p130 (C-20 – top panel), p107 (C-18 – second panel), pRb (IF8 – third panel) and TFIIB (C-18 – bottom panel). **B** Whole-cell extracts (20 μg) were prepared from EC (lane 1) or differentiating (day 1-7 – lanes 2-8) cells and resolved on a SDS – 7.8% polyacrylamide gel. Following transfer onto PVDF membrane, Western blot analysis was performed, with antibodies against pRb (IF8 – top panel) and TFIIB (C-18 – bottom panel).

panel). This up-regulation seems to start around day 2 after induction, with maximum pRb levels being reached by day 4 (Figure 6.1B – top panel). It has been previously shown that cyclin D- and E-dependent phosphorylation of pRb abolishes interaction with TFIIIB (Scott *et al.*, 2001). The observed increase in pRb levels would, therefore, be meaningless, in the context of pol III transcription, if the proteins became phosphorylated throughout the differentiation process. I, therefore, examined the levels of an important regulator of pRb phosphorylation, cyclin D1. In Figure 6.2A we can seen that, in the same extracts used for Figure 6.1B, cyclin D1 levels fall dramatically after the first day of differentiation (compare lanes 2 and 3). To directly examine the phosphorylation state of pRb, cell lysates (see Materials and Methods) from EC and differentiating cells were analysed by Western immunoblotting, using an antibody against pRb phosphorylated at serine 773 (Figure 6.2B). Phosphorylation at this site requires cyclin D1 *in vivo* (Geng *et al.*, 2001). As we can observe, Ser773 phosphorylation is reduced significantly within 24 hours of induction of differentiation (compare lanes 1 and 2), with a further reduction evident between days 2 and 3 (lanes 3 and 4). Figure 6.2C is a graphical representation of phospho-pRb levels, taking the levels in the EC cells as 100%.

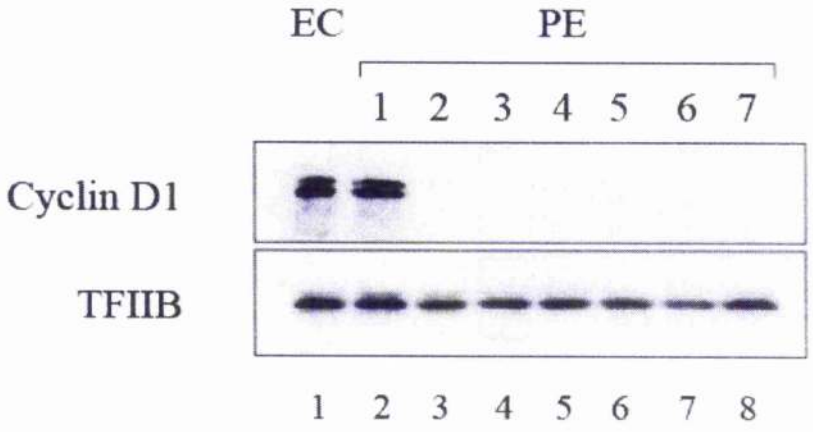
6.2.2 Protein levels of the tumour suppressor p53 do not change when F9 EC cells differentiate into PE

Contrary to previous reports (Oren *et al.*, 1982), p53 protein levels do not seem to change following induction of differentiation. As we can see in Figure 6.3, Western blot analysis of extracts from EC or differentiating cells reveals that p53 levels are constant throughout the 7-day period, when compared to the loading control, TFIIB.

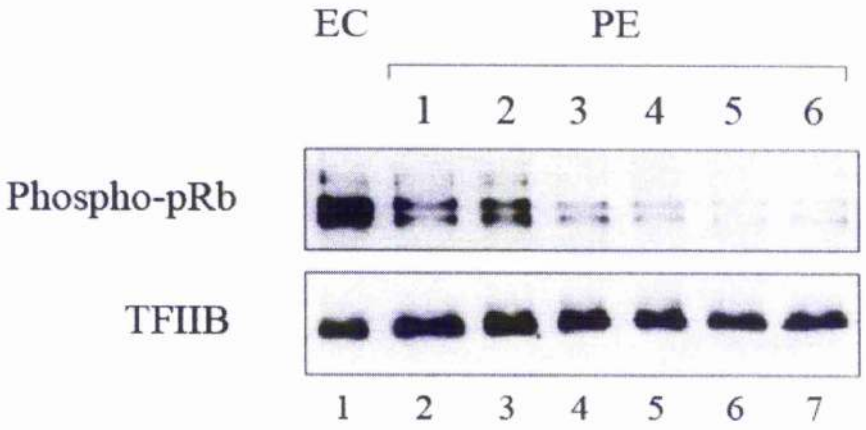
Figure 6.2 Dephosphorylation of pRb

A Whole-cell extracts (20 μg) were prepared from EC (lane 1) or differentiating (day 1-7 – lanes 2-8) cells and resolved on a SDS – 7.8% polyacrylamide gel. Following transfer onto PVDF membrane, Western blot analysis was performed, with antibodies against cyclin D1 (72-13G – top panel) and TFIIB (C-18 – bottom panel). **B** Cell lysates (20 μg) were prepared from EC (lane 1) or differentiating (day 1-6 – lanes 2-7) cells and analysed as in A, using an antibody against the phosphor-pRb (9307 – top panel). TFIIB was used as loading control (bottom panel). **C** Graphical representation of the phosphorylation status of pRb during differentiation. The signal obtained from differentiating cells (B, lanes 2-6) was quantified by densitometry (TotalLab) and expressed as a percentage of that from EC cells (B, lane 1).

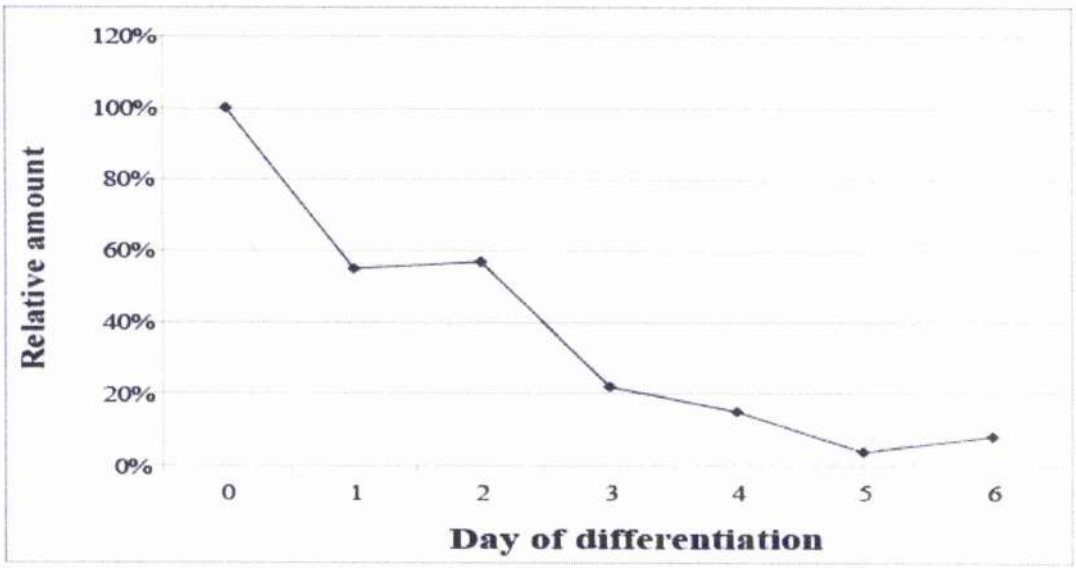
A


EC
PE
1 2 3 4 5 6 7
Cyclin D1
TFIIB
1 2 3 4 5 6 7 8

B


EC
PE
1 2 3 4 5 6
Phospho-pRb
TFIIB
1 2 3 4 5 6 7

C


120%
100%
80%
60%
40%
20%
0%
Relative amount
0 1 2 3 4 5 6
Day of differentiation

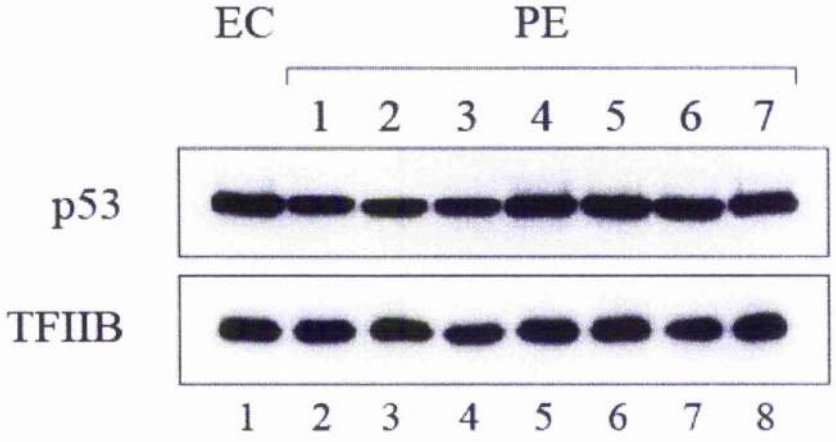


Figure 6.3 p53 levels remain unchanged during differentiation

Whole-cell extracts were prepared from undifferentiated EC cells (lane 1) or at each day following induction of differentiation (PE 1-7 – lanes 2-8). 20 μg were then resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western blotting, using an anti-p53 (1C12 – top panel) or an anti-TFIIB (C-18) antibody.

6.2.3 *Levels of the catalytic subunits of casein kinase 2 (CK2) are reduced in PE cells*

Previous work concerning CK2 in F9 cells has focused on the involvement of the kinase in the apoptotic response, especially in relation to p53 (e.g. Siemer *et al.*, 1999). Since CK2 has been previously shown to also have an effect on pol III transcription (Johnston *et al.*, 2002), I decided to investigate its levels during F9 cell differentiation.

As we can see in Figure 6.4A, both catalytic subunits of CK2, α and α', are down-regulated at the protein level in differentiating cells, although down-regulation of the α subunit seems to precede that of α' by about 2 days. The levels of the regulatory β subunit do not change in response to differentiation (Figure 6.4B).

6.2.4 *Active, but not total ERK, is down-regulated in PE cells*

F9 EC or PE cells were harvested in a lysis buffer that enables the detection of phosphorylated proteins (see Materials and Methods) and analysed by Western immunoblotting. Figure 6.5A shows a comparison of the levels of total vs. active (i.e. phosphorylated) ERK. While total ERK levels do not change following differentiation, it is evident that active ERK levels are reduced. TFIIB was used as loading control. The reduction observed in PE extracts is similar to that observed when F9 EC cells are treated with the MEK inhibitor PD98059 (Figure 6.5B). MEK (a MAPK kinase) phosphorylates and activates ERK, and its inhibition leads to reduction in phosphorylated ERK (middle panel in Figure 6.5B), while it has no

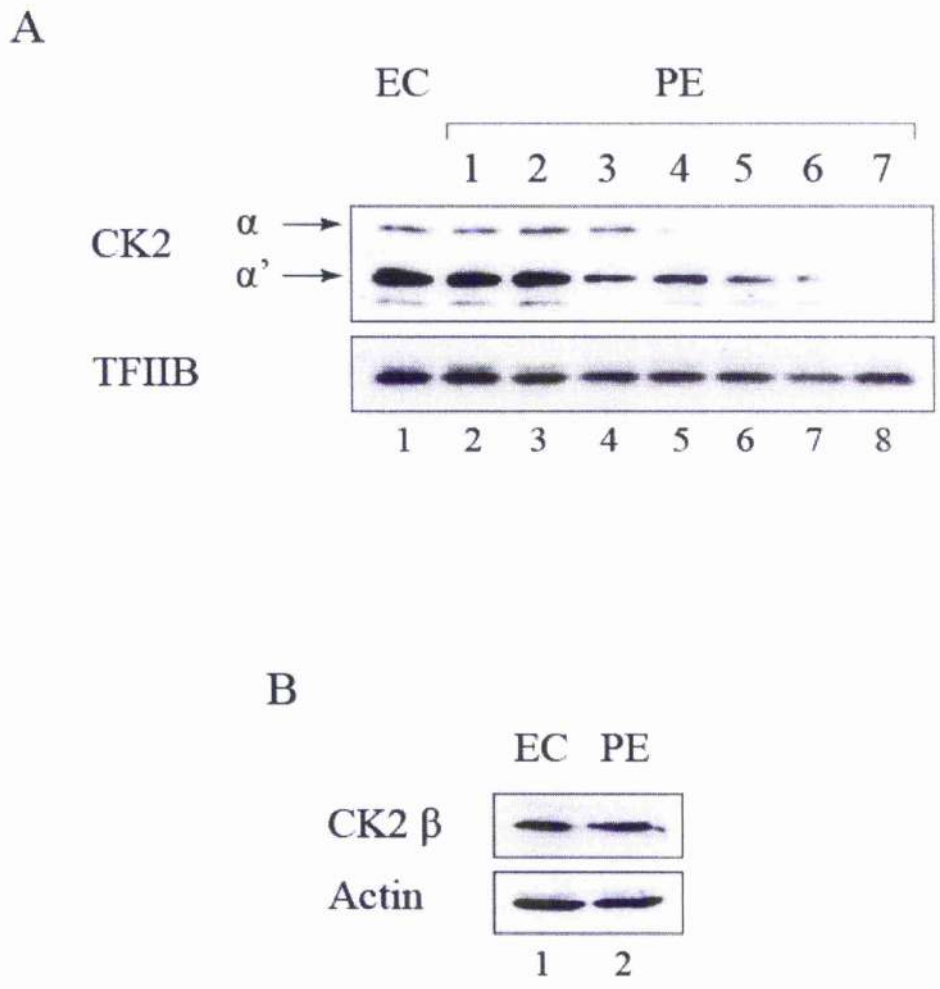


Figure 6.4 CK2 protein levels are reduced during differentiation

A Whole-cell extracts were prepared from undifferentiated EC cells (lane 1) or at each day following induction of differentiation (PE 1-7 – lanes 2-8). 20 μg were then resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western blotting, using an anti-CK2α antibody (H-286, which cross-reacts with α' – top panel) and an anti-TFIIB antibody (C-18) as loading control. **B** Western blot analysis of EC or PE extracts (20 μg – lanes 1 and 2 respectively) using an anti-CK2β antibody (top panel) and an anti-actin antibody (C-11 – bottom panel) as loading control.

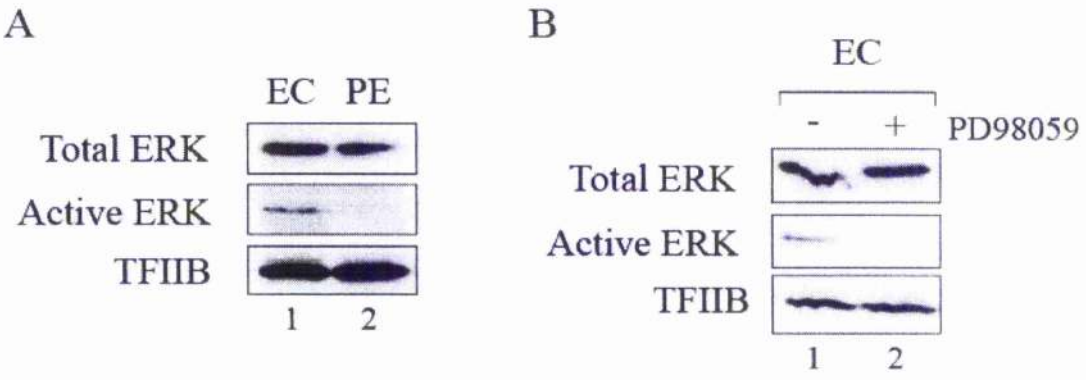


Figure 6.5 Levels of active ERK are reduced in F9 PE cells

A F9 EC (lane 1) and PE (lane 2) cells were harvested in lysis buffer (see Materials and Methods). 20 μg of the cell lysates were resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western immunoblotting, with antibodies against total ERK (9102 – top panel) or active (i.e., phosphorylated) ERK (9106 – middle panel). TFIIB (antibody C-18) was used as loading control. **B** F9 EC cells were treated with DMSO or the MEK inhibitor PD98059 (50 μM) for 6 hours, harvested in lysis buffer and resolved on a SDS – 7.8% polyacrylamide gel. Western immunoblotting was performed using antibodies against total ERK (9102 – top panel), phosphorylated ERK (9106 – middle panel) and TFIIB (C-18 – bottom panel).

effect on total ERK levels (Figure 6.5B, top panel) or TFIIB (bottom panel), which was used as loading control.

6.2.5 Members of the myc/max/mad network are down-regulated in PE cells

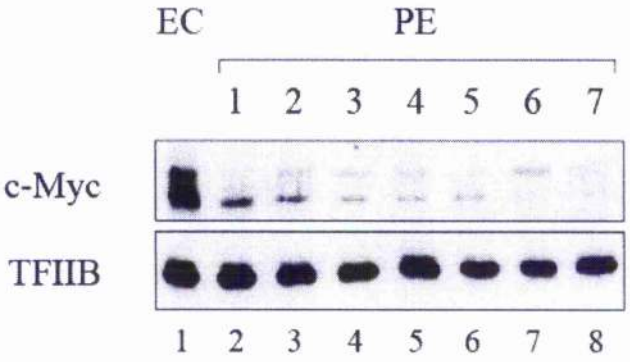
In addition to its function as a transcription factor when complexed with Max, c-Myc directly activates pol III transcription through interactions with TFIIIB (Gomez-Roman *et al.*, 2003). It has been previously demonstrated that c-Myc is strongly down-regulated within a few hours after induction of differentiation. To verify this, extracts from differentiating cells were analysed by Western immunoblotting for c-Myc protein levels. As we can see in Figure 6.6A, there is a sharp decrease in c-Myc within 24 hours of differentiation induction. The two bands observed are both c-Myc, representing the p67 (top band) and p64 (bottom band) forms of the protein, which arise through initiation of translation at two different start codons (Hann and Eisenman, 1984). A graphical representation of the decline in c-Myc levels can be seen in Figure 6.6C. Note that both bands (p64 and p67 c-Myc) were included in the measurements, since both have an effect in pol III transcription.

F9 EC and PE extracts were also analysed for other members of the myc/max/mad network, the results of which can be seen in Figure 6.6B. Max protein levels decline only slightly (top panel), whereas the decrease in the levels of Mads 2, 3 and 4 is much more striking. No Mad1 could be detected by Western blotting in F9 cells, EC or PE, which agrees with previous observations (Larsson *et al.*, 1997). It is also noteworthy that, in the EC extracts tested, Mad3 levels were considerably lower than either Mad2 or Mad4 (results not shown). This difference is not obvious in

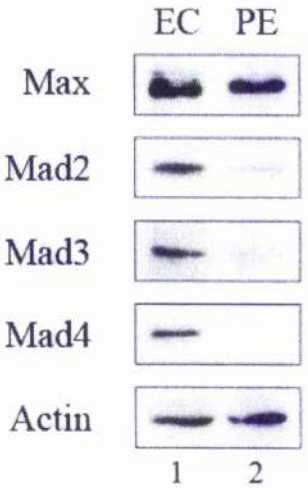
Figure 6.6 Levels of the Myc/Max/Mad network proteins in F9 EC and PE cells

A Whole-cell extracts were prepared from undifferentiated EC cells (lane 1) or at each day following induction of differentiation (PE 1-7 – lanes 2-8). 20 µg were then resolved on a SDS - 7.8% polyacrylamide gel and analysed by Western blotting, using an anti c-Myc (N-262 – top panel) or an anti-TFIIB antibody (C-18 – bottom panel). **B** Whole-cell extracts (20 µg) from EC (lane 1) or PE (lane 2) cells were resolved on a SDS – 7.8% polyacrylamide gel and analysed by Western blotting, using antibodies against Max (C-17 – top panel), Mad2 (G-16 – second panel), Mad3 (H-206 – third panel) and Mad4 (N-19 – fourth panel). Actin was used as loading control (bottom panel). **C** Graphical representation of the reduction in c-Myc levels. The signal obtained from differentiating cells (B, lanes 2-6) was quantified by densitometry (TotalLab) and expressed as a percentage of that from EC cells (B, lane 1). Note that both bands, representing the p64 and p67 forms of c-Myc, were considered in this analysis.

A


EC
PE
1 2 3 4 5 6 7
c-Myc
TFIIB
1 2 3 4 5 6 7 8

B


EC PE
Max
Mad2
Mad3
Mad4
Actin
1 2

C

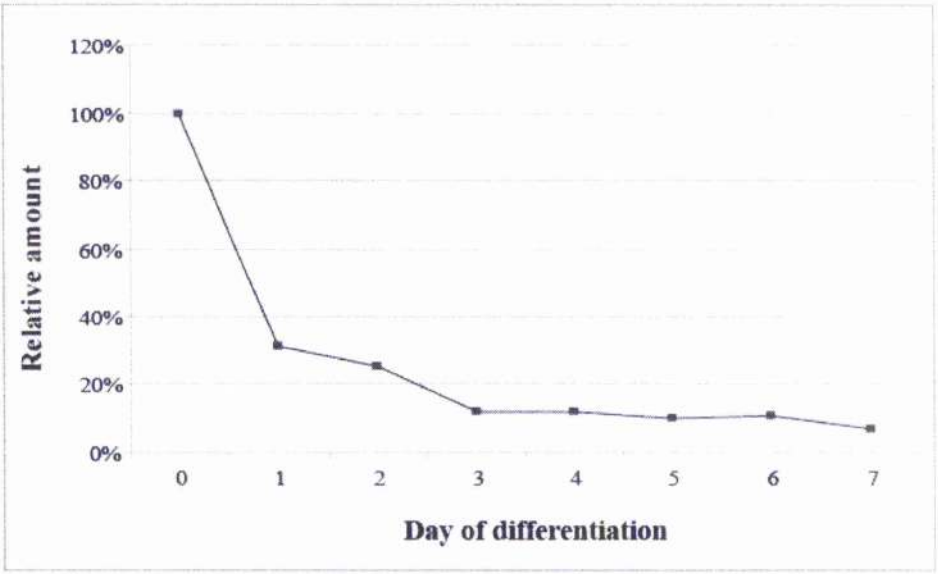

120%
100%
80%
60%
40%
20%
0%
Relative amount
0 1 2 3 4 5 6 7
Day of differentiation

Figure 6.6B, because different exposure times were used to obtain a similar EC signal. The possibility, however, that the Mad3 antibody is poorer than the Mad2 and Mad4 antibodies cannot be excluded.

6.2.6 Recombinant c-Myc can partially restore pol III transcription in PE extracts

To test whether or not increased c-Myc levels could enhance pol III transcription in F9 cells after differentiation, PE extracts were pre-incubated with recombinant c-Myc and then utilised in an *in vitro* transcription assay, using $tRNA^{Leu}$ as template. As we can see in Figure 6.7, increasing amounts of c-Myc lead to increased pol III transcription (compare lanes 2, 3 and 4). Transcription, however, is not restored to the levels seen in EC extracts (lanes 1 and 5), even when higher amounts of recombinant c-Myc were used (results not shown).

In the previous experiment, the recombinant c-Myc was incubated with the extract prior to addition of the template and nucleotides. There is, therefore, no way of knowing which stage of transcription is activated. To investigate this, c-Myc or buffer were added to F9 PE extracts either 15 min prior to addition of the $tRNA^{Leu}$ template (Figure 6.8 – lanes 1 and 2), simultaneously with the addition of the template (lanes 3 and 4), or 15 min after the addition of the template (lanes 5 and 6). Subsequently, nucleotides were added to allow transcription. The last two lanes in this experiment represent the ability of c-Myc to activate transcription of a pre-formed initiation complex. We can clearly see that addition of c-Myc at any of the three time-points results in increased transcription, suggesting that it might act before or after pre-initiation complex assembly.

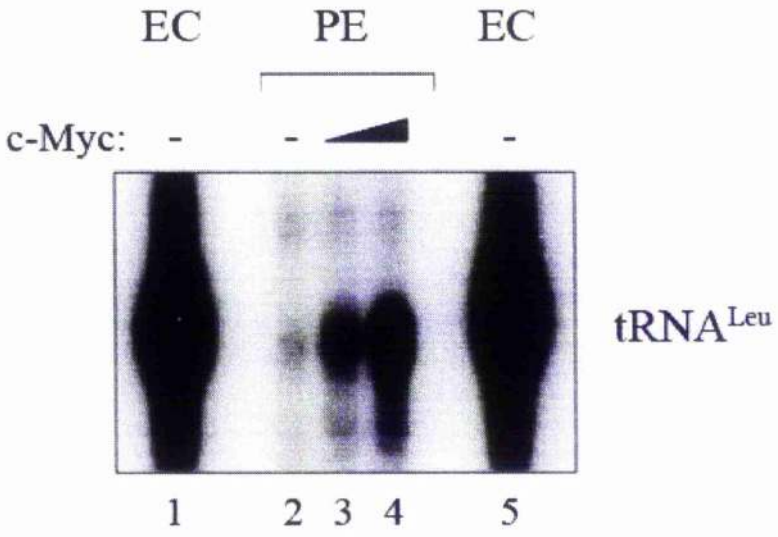


Figure 6.7 Recombinant c-Myc restores pol III transcription in PE extracts

F9 PE extracts (20 μg) were incubated with 0, 100 ng or 200 ng (lanes 2, 3 and 4, respectively) of recombinant c-Myc for 15 min at 30°C and subsequently used for the *in vitro* transcription of a tRNA^{Leu} template (125 ng). EC extracts (20 μg) are also shown (lanes 1 and 5) for comparison.

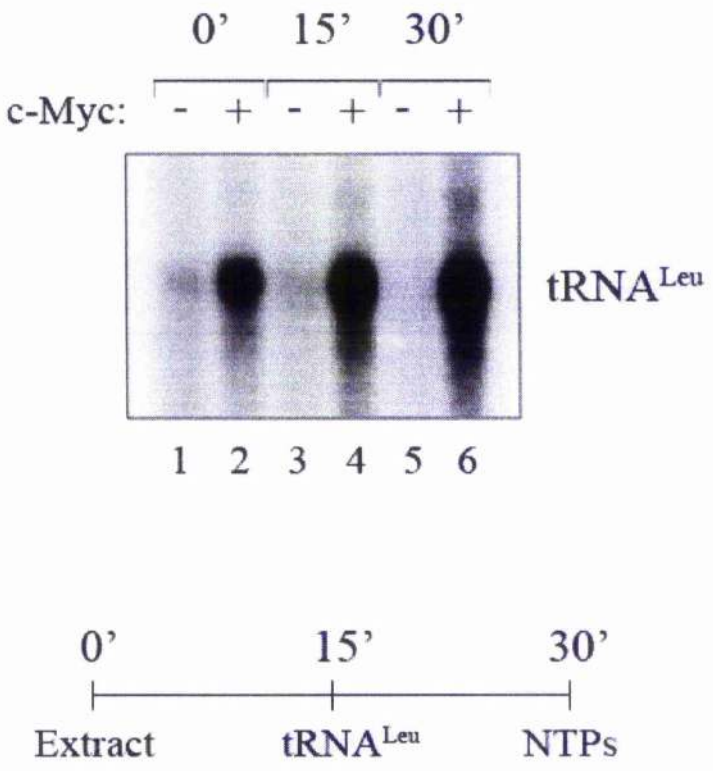


Figure 6.8 Recombinant c-Myc can activate pol III transcription before or after assembly of the pre-initiation complex

F9 PE extract (20 µg) was pre-incubated for 15 min at 30°C before the addition of pLeu (125 ng). After a further 15 min, nucleotides were added and transcription was allowed to proceed. Buffer (lanes 1, 3 and 5) or recombinant c-Myc (200 ng – lanes 2, 4 and 6) were added to the reactions at the indicated times.

6.2.7 *The DNA-binding activity of c-Myc is reduced in PE cells*

Electrophoretic mobility shift assays (EMSAs) were employed to study the DNA-binding capacity of Myc-Max heterodimers in F9 EC or PE extracts. To do this, oligonucleotides containing either a consensus Myc-Max binding site (E-box oligonucleotide), or a mutated E-box, were designed (for sequence details see Materials and Methods). When EC or PE extracts were incubated with radiolabelled E-box oligonucleotide, a single band was observed, marked by an arrow, the signal of which decreases when PE extract was used (Figure 6.9A, compare lanes 2 and 3). This band was not detected when the extracts were incubated with the mutant oligonucleotide (Figure 6.9A, lanes 5 and 6). In addition to this strong band, two more bands can be detected lower in the gel, marked by asterisks in Figure 6.9A. To determine if they are specific or not, excess of unlabelled E-box or mutant oligonucleotides were added to reactions containing radiolabelled E-box probe and EC or PE extracts (Figure 6.9B). Predictably, the top band disappeared when E-box competitor was added, but not when the mutated oligonucleotide is used (compare lanes 1 and 2 with 3 and 4). The lower of the weaker bands is not affected by any of the competitor sequences, suggesting that it is due to non-specific binding. On the other hand, the middle band seems to disappear in lanes 1 and 2, i.e. when the E-box competitor is used. This might indicate another type of protein-DNA complex, and will be discussed later.

The competition experiments established that the prominent band seen in the previous figure had specificity for an E-box Myc/Max recognition sequence, but did not establish that c-Myc was part of the complex. In order to verify this, I employed two methods. Recombinant c-Myc was added to some reactions, to test if the

intensity of the band would increase. To the rest, anti-myc or control antibodies were added in order to supershift the bands (i.e. form antibody-protein-DNA complexes). Figure 6.10 shows a typical experiment. In lanes 2 and 3 EC or PE extracts were used, similar to figure 6.9A (lanes 2 and 3). Like before, we can see a decrease in complex formation in PE cells, compared to EC. When recombinant c-Myc is added to the reactions (lanes 4 and 5), both EC and PE bands increase in intensity (compare lane 4 with lane 2 and lane 5 with lane 3). The presence of an anti-myc (lanes 6-9), but not of a control (anti-fos – lanes 10-13), antibody leads to the appearance of a slower-migrating band (marked with an asterisk in Figure 6.10), with a concomitant decrease in the initial band. We can therefore conclude that the prominent band seen in these two figures (6.9 and 6.10) contains c-Myc, probably as a Myc-Max-DNA complex.

6.3 Discussion

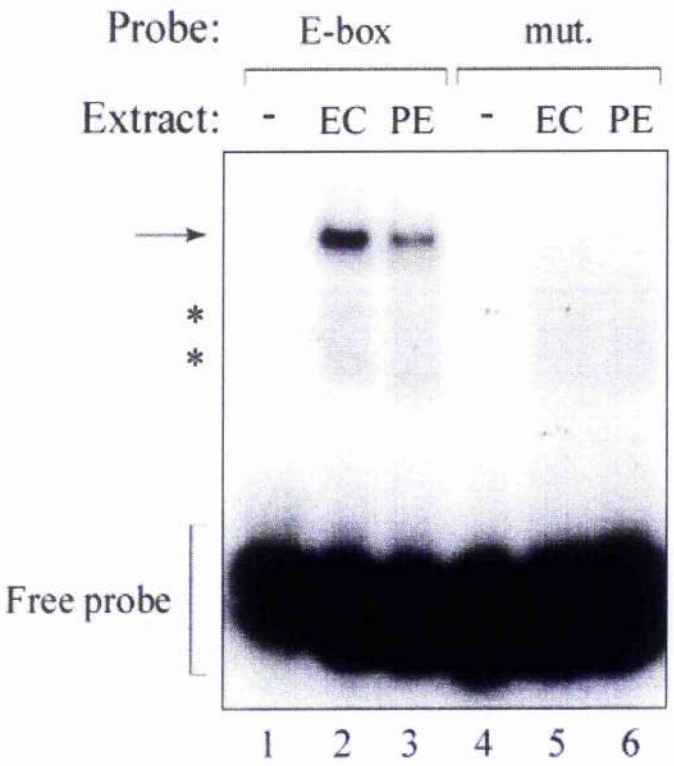
6.3.1 pRb/p107/p130

It has been previously reported that the E2F-associated activity of pRb and p107, i.e. their ability to influence transcription by RNA polymerase II, is elevated in F9 PE cells (Partridge and La Thangue, 1991; Zamanian and La Thangue, 1993). Examining the protein levels of these proteins, I found that pRb is strongly induced during the differentiation of F9 cells (Figure 6.1A – third panel and Figure 6.1B – top panel). On the other hand, p107 levels remain unchanged during this process (Figure 6.1A – second panel), which might argue against the previous findings. Since, however, the reports mentioned previously look at E2F-associated activity, as

Figure 6.9 Binding to a consensus E-box sequence is reduced in PE extracts

A Electrophoretic mobility shift assay (EMSA) using 1 ng of radiolabelled E-box (lanes 1-3) or mutated E-box (lanes 4-6) oligonucleotides, 1 μg of poly[dI-dC] competitor and 5 μg of EC (lanes 2 and 4) or PE (lanes 3 and 5) whole-cell extract, or no extract (lanes 1 and 4). **B** Reactions contain 1 ng of radiolabelled E-box oligonucleotide, 1 μg of poly[dI-dC] competitor and 5 μg of F9 EC (lanes 1 and 3) or F9 PE (lanes 2 and 4) extract. Unlabelled E-box (lanes 1 and 2) or mutant E-box (lanes 3 and 4) oligonucleotides (100 ng) were also added.

A


Probe:
E-box
mut.
Extract:
-
EC
PE
-
EC
PE
*
*
Free probe
1
2
3
4
5
6

B

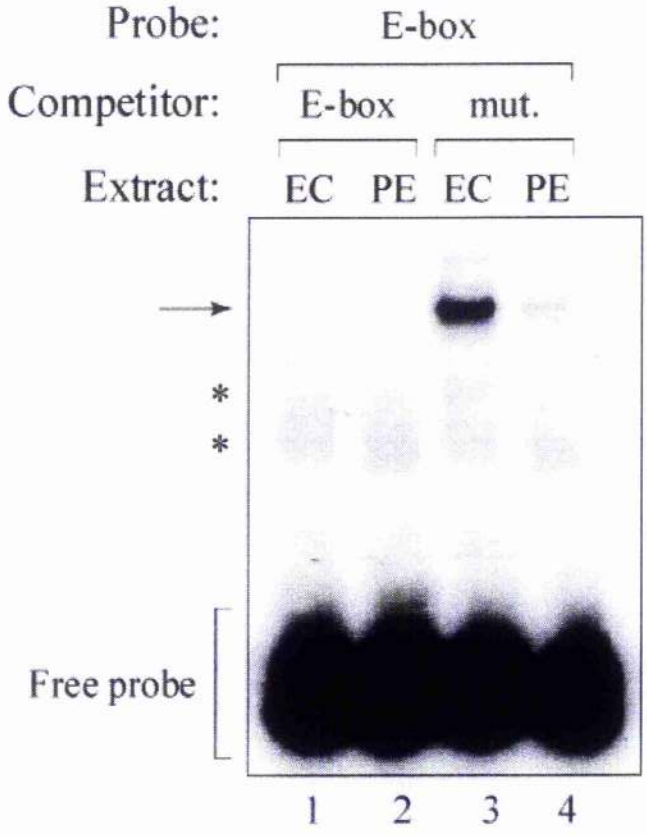
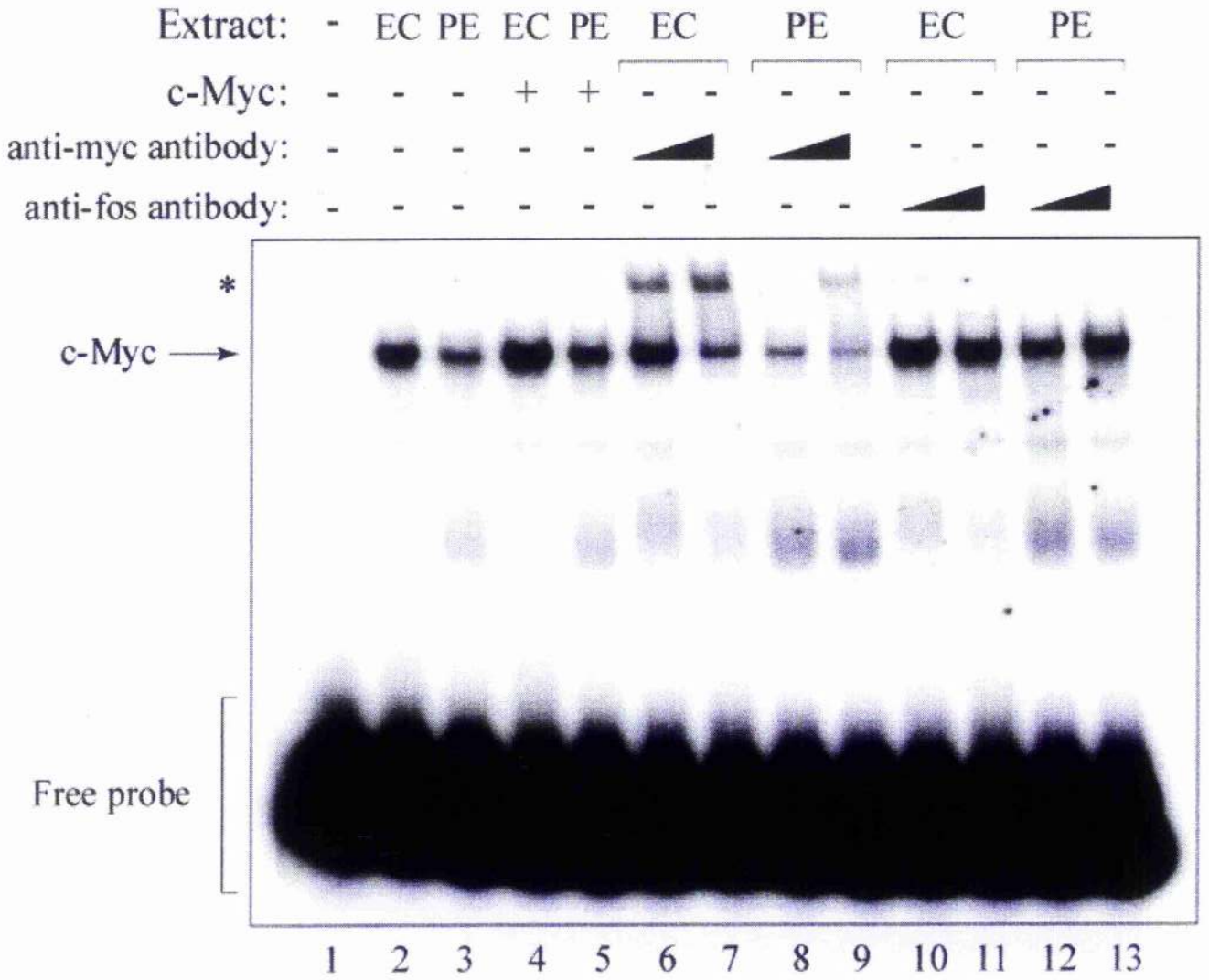

Probe:
E-box
Competitor:
E-box
mut.
Extract:
EC
PE
EC
PE
*
*
Free probe
1
2
3
4

Figure 6.10 Binding to a consensus E-box oligonucleotide increases in the presence of recombinant c-Myc

Electrophoretic mobility shift assay (EMSA) containing 1 ng of radiolabelled E-box oligonucleotide, 1 μg of poly[dI-dC] competitor, 5 μg of F9 EC (lanes 2, 4, 6, 7, 10 and 11) or F9 PE (lanes 3, 5, 8, 9, 12 and 13) extract, or no extract (lane 1). Reactions were supplemented with 400 ng of recombinant c-Myc (lanes 4 and 5) or an anti-myc antibody (4 μg in lanes 6 and 8, 8 μg in lanes 7 and 9) or a control anti-fos antibody (4 μg in lanes 10 and 12, 8 μg in lanes 11 and 13).


Extract: - EC PE EC PE EC PE EC PE
c-Myc: - - - + + - - - - - - - -
anti-myc antibody: - - - - - ▲ ▲ - - - -
anti-fos antibody: - - - - - - - - - ▲ ▲
*
c-Myc
Free probe
1 2 3 4 5 6 7 8 9 10 11 12 13

opposed to protein levels, one must also look at the behaviour of pRb/p107/p130-regulatory proteins, such as the D-type cyclins, which mediate the Cdk4/6-dependent phosphorylation and inactivation of pRb (reviewed in Zheng and Lee, 2001) and p107/p130 (reviewed in Classon and Dyson, 2001) in the G1 phase of the cell cycle. When cyclin D1 levels were monitored, it was observed that the protein is quickly and strongly down-regulated upon induction of differentiation (Figure 6.2A). This is in agreement with a previous report, which shows that cyclin D1, D3 and E levels are reduced in PE cells (Li *et al.*, 2004; Li *et al.*, 1999). Surprisingly, both groups also show that the levels of cyclin D2 increase following differentiation. There are indications, however, that cyclin D2 may function as a negative regulator of cell growth (Meyyappan *et al.*, 1998). These data show that the proteins that normally inactivate p107 (i.e. cyclins D and E) are down-regulated, possibly leading to the elevated p107 activity observed previously (Zamanian and La Thangue, 1993). A similar situation might be envisaged for p130, the protein levels of which are also unchanged in PE cells (Figure 6.1A – top panel), although its regulation in the F9 system has not been reported previously.

One of the many targets of the pocket proteins is the TFIIIB complex, as was described previously. By binding to and inactivating this protein complex, pRb, p107 and p130 can repress transcription by pol III (reviewed in White, 2004b). Down-regulation by the pocket proteins, therefore, could be one of the mechanisms contributing to the reduction in pol III activity when F9 cells are induced to differentiate. Although the induction of pRb seen in Figure 6.1B does not explain the initial reduction in pol III transcription observed between days 0 and 1 (Figure 3.3B), it could enhance the inhibition of pol III transcription at a later time. More important than overall levels, however, is the phosphorylation status of pRb, since only the underphosphorylated form can bind TFIIIB and repress pol III transcription (Scott *et*

al., 2001). In particular, it was demonstrated that cyclin D- and E-dependent phosphorylation stimulates pol III transcription. Direct examination of the phosphorylation status of pRb revealed that phosphorylation at serine 773 decreases during differentiation (Figure 6.2B). Since this site is phosphorylated in a cyclin D-dependent manner, there is a strong possibility that the observed reduction might lead to activation of pRb and repression of pol III. Significantly, the kinetics of the dephosphorylation (Figure 6.2C) resemble closely the reduction in pol III transcription seen earlier (Figure 3.3B). Although this is by no means absolute proof that elevation in pRb activity is solely responsible to the observed down-regulation of pol III transcription, pRb might play a significant role in the early decrease in transcription seen during the first few days.

6.3.2 p53

As mentioned in the introduction of this chapter, F9 EC cells were reported to contain high levels of wild type p53 protein (Pennica *et al.*, 1984), the levels of which, both mRNA and protein, decline following differentiation into parietal endoderm (Dony *et al.*, 1985; Oren *et al.*, 1982). The p53 protein, however, seems to be inactive in EC cells, whereas it becomes activated after retinoic acid-induced differentiation (Lutzker and Levine, 1996). DNA damage by UV irradiation or chemical agents also activate p53 in EC and PE cells (Lutzker and Levine, 1996; Mayo and Berberich, 1996).

Western blot analysis of differentiating F9 cells showed that p53 protein levels persist throughout the process (Figure 6.3), contradicting previous reports. p53 can still be stabilised by a proteasome inhibitor (MG132), as seen in Figures 4.1 and

4.2A, which suggests that turn-over of the protein is not affected. Although proteasome inhibition has a multitude of effects, p53 stabilisation could lead to repression of pol III transcription, as shown previously (reviewed in White, 2004b). This is not the case, however, as extracts from MG132-treated EC or PE cells do not exhibit increased pol III activity (Figure 4.4C). This would be consistent with the reports showing that p53 is not functional in EC cells, but inconsistent with p53 becoming activated by RA-induced differentiation (Lutzker and Levine, 1996). A possible explanation for this could be that, over time, the cells I used acquired a mutation that, first, reversed the differentiation-induced decrease in protein levels and, second, eliminated the proteins ability to influence pol III transcription. This possibility needs to be examined further, by looking at p53 activity in differentiating F9 cells, in relation to both pol II and pol III transcription.

6.3.3 CK2

CK2 protein levels and activity have not been previously described in F9 PE cells. Here I have seen down-regulation of both catalytic subunits, α and α', at protein level (Figure 6.4A). The regulatory subunit β, on the other hand, does not decrease following differentiation (Figure 6.4B). The effect of CK2 on RNA polymerase III transcription in mammalian cells has been reported previously (Johnston *et al.*, 2002). Specifically, CK2 binds to and phosphorylates TFIIIB, thus activating it. Therefore, one could imagine that CK2 might be directly involved in the down-regulation of pol III transcription in PE cells, although an indirect involvement through one of CK2's many targets cannot be ruled out at this point.

6.3.4 ERK

Another kinase that has been shown to affect pol III transcription is ERK, a mitogen-activated protein kinase (MAPK). In response to serum induction, ERK becomes phosphorylated and activated by MEK and in turn it binds to TFIIIB, through Brf1. This leads to phosphorylation of Brf1 and activation of TFIIIB and subsequently pol III transcription (Felton-Edkins *et al.*, 2003a). When F9 EC cells were induced to differentiate into PE, I observed a reduction in active ERK levels, while total protein levels were unaffected (Figure 6.5A). This is in agreement with an earlier report showing that ERK activity is reduced when F9 cells differentiate into PE (Verheijen *et al.*, 1999). Therefore, the reduced levels of active ERK could be an additional factor in the down-regulation of pol III transcription in F9 PE cells.

6.3.5 Myc/Max/Mad network

One of the earliest events following induction of differentiation in F9 cells is the reduction in the levels of c-*myc* mRNA (Dean *et al.*, 1986; Dony *et al.*, 1985). These two groups showed that mRNA levels are reduced to almost zero very early in the differentiation process, within 24 hours of addition of the inducing chemicals. They did not, however, examine the protein levels in differentiating cells, although the short half-life of this protein (Hann *et al.*, 1985) means that the protein is unlikely to persist for more that 2-3 days. It was, therefore, surprising to see that PE extracts 5 or 6 days following induction of differentiation contain some c-Myc (Figure 6.6A). A more recent report shows that, although c-*myc* mRNA is strongly down-regulated by day 2 of differentiation, very low levels of transcript can be seen even at day 5

(Larsson *et al.*, 1997). A striking feature seen in Figure 6.6A is the two bands that react with the anti-myc antibody. These most likely represent the p64 and p67 forms of c-Myc. p64 is considered to be the predominant form of c-Myc, initiating from an AUG start codon in exon 2. The p67 form contains an N-terminal extension, resulting from translation initiation from an alternative codon (CUG) located in exon 1. A report by Hann *et al.* (1994) suggests that the two forms of c-Myc differentially regulate (pol II) transcription, by using different DNA-binding sites. c-Myc-mediated activation of pol III transcription, however, is unaffected by the DNA-binding activity of c-Myc, since the TFIIIB binding site lies within the N-terminal part of the protein. Both forms of c-Myc, therefore, should have a similar effect on pol III transcription, although this has not been specifically examined. As a result, both proteins are included in the measurements represented in Figure 6.6C. Taken together, we can see that there is a sharp decrease in c-Myc within 24 hours of induction of differentiation, similar to what has previously been reported, which could play, together with pRb discussed earlier, a significant role in the initial decrease in pol III transcription.

Reduction in c-Myc levels was verified by EMSA, using a consensus E-box sequence as probe (Figures 6.9 and 6.10 – lanes 2 and 3). Once more, although a clear decline in Myc/Max binding to the probe can be seen, it is not abolished in PE extracts. It is worth noting that both forms of c-Myc, p64 and p67, bind consensus E-boxes with similar efficiency (Hann *et al.*, 1994). The identity of the prominent band was verified by addition of specific antibodies to the reaction. The anti-Myc, but not the control anti-Fos, antibody supershifts the band (Figure 6.10 - compare lanes 6-9 with lanes 10-13), suggesting that the band seen contains a Myc-DNA complex. Curiously, two different Max antibodies failed to supershift the band (data not shown). This could be due to the epitopes recognised by the antibodies being

obscured when Max is bound to the DNA probe. Another indication that the band observed is a Myc-containing complex comes from the fact that addition of recombinant c-Myc leads to an increase in band intensity, without any change in mobility (Figure 6.10 - compare lanes 4 and 5 with lanes 2 and 3).

Studies so far have revealed a clear link between *Mad* expression and terminal differentiation (for a review see Grandori *et al.*, 2000). Here, however, I see that the levels of three of the Mad proteins, Mad2, 3 and 4, decline following differentiation (Figure 6.6B). It has been previously shown that *mad3* expression is confined to the S phase of proliferating cells (Queva *et al.*, 1998), so the fact that Mad3 protein levels are reduced in PE cells is not entirely surprising. It would be interesting to see at which day after induction do the levels of Mad2 and Mad4 decrease. Mad1 was not detected in EC or PE cells, which is in agreement with the observation that *mad1* mRNA is absent from F9 cells (Larsson *et al.*, 1997). In the same report, it was described that Max protein levels gradually decline during differentiation. Although to a lesser extent, I also observe a slight reduction in Max protein levels (Figure 6.6B – top panel). Since *max* appears to be expressed at a constant rate, both in differentiating F9 (Larsson *et al.*, 1997) and other cell types (Berberich *et al.*, 1992), this opens the possibility of post-transcriptional control of this protein.

In agreement with the proposed direct activation of pol III transcription by c-Myc (Gomez-Roman *et al.*, 2003), addition of recombinant c-Myc to PE extracts partly restores pol III transcription in *in vitro* assays (Figure 6.7). Activation of pol III transcription by c-Myc is not influenced by formation of the pre-initiation complex, as revealed in Figure 6.8. This suggests that c-Myc does not enhance initiation complex assembly, but acts at a later stage. Note that in PE extracts, all three subunits of the TFIIIB complex are reduced (Figure 3.4A). We must therefore

assume that enough TFIIIB remains to serve as a target for c-Myc activation, although an indirect role for c-Myc cannot be excluded.

6.3.6 Concluding remarks

Although it has been previously suggested that TFIIIB is the limiting factor in differentiating F9 cells, and that its down-regulation is responsible for the marked reduction in pol III transcription in PE cells (Alzuherri and White, 1998), experiments in previous chapters have cast some doubts. For example, time course analysis revealed that the reduction in TFIIIB protein levels occur after the initial reduction in pol III transcription (compare Figures 3.5 and 3.3). Therefore, although one cannot exclude that reduction in TFIIIB levels have an impact on pol III transcription, additional factors must be responsible for the observed down-regulation in the first few days after induction of differentiation. A possible explanation for the discrepancy could reside in the fact that fractionated extracts were used to demonstrate that TFIIIB activity, as opposed to TFIIIC, was limiting in F9 PE cells. It has since been shown that c-Myc, pRb, p107, p130 and p53 co-fractionate with TFIIIB (Cairns and White, 1998; Gomez-Roman *et al.*, 2003; Larminie *et al.*, 1997; Sutcliffe *et al.*, 1999), although in those cases extract from HeLa cells was used. It is therefore quite plausible, indeed almost certain, that one or more of these proteins were included in the TFIIIB fraction used in reconstitution assays (Alzuherri and White, 1998).

One of the most likely candidates for the initial reduction in pol III activity is c-Myc, considering that c-*myc* mRNA levels decline within hours following induction of differentiation, and the effect of recombinant c-Myc on pol III transcription in *in*

vitro transcription assays (Figures 6.7 and 6.8). Since numerous c-Myc targets are involved in protein synthesis (Shiio *et al.*, 2002), involvement of c-Myc in regulation of pol III transcription, whose output is essential for the protein synthetic machinery, would place it in a perfect position to globally regulate cell growth, and therefore proliferation, in differentiating F9 cells.

CHAPTER 7

Discussion

Expression of pol III transcripts, as monitored by *in situ* hybridisation, appears to be tightly regulated in the early stages of mouse embryonic development (Vasseur *et al.*, 1985). As a result, approximately 7.5 days post-coitum, pol III activity is high in the ectoderm and mesoderm layers, but low in endodermal tissues.

The differentiation of the ICM to endodermal cell types in the early mouse embryo can be mimicked in culture by the F9 cell line. Under particular culture conditions, the stem cells of this line, called embryonal carcinoma (EC) cells, can differentiate into visceral or parietal endoderm-like cells (Hogan *et al.*, 1981; Strickland *et al.*, 1980). As shown in Figure 1.5, the first common step of the differentiation pathway is the formation of primitive endoderm-like cells, as a result of treatment with retinoic acid alone (Grover and Adamson, 1986). White *et al.* (1989) showed previously that the activity of pol III during the differentiation of EC cells to parietal endoderm (PE)-like cells resembles that observed *in vivo*, when the ICM differentiates to form (parietal) endoderm. Complementation experiments demonstrated that crude fractions containing TFIIIB could restore transcription in extracts from PE cells, whereas similar fractions containing TFIIIC did not have this effect (White *et al.*, 1989). It was, therefore, proposed that TFIIIB activity decreases, and becomes limiting for pol III transcription, as F9 cells differentiate.

Further purification of the crude TFIIIB and TFIIIC fractions verified the previous observations, i.e. that TFIIIB activity is limiting in F9 PE cells (Alzuherri and White,

1998). It was also shown that the protein levels of one of the TAF components of TFIIIB, Brf1, declined during differentiation, suggesting a possible mechanism for the observed decrease in pol III transcription. Importantly, addition of recombinant Brf1 did not restore transcription in PE cell extracts, indicating that Brf1 is not the only factor for pol III transcription that is deficient in PE cells. Interestingly, significantly less TBP was found in the TFIIIB fraction, whereas TBP levels were unaffected by differentiation in fractions containing SNAPc, TFIID or SL-1 (Alzuherri and White, 1998). At the time of that study, the behaviour of the third subunit of TFIIIB, Bdp1, could not be examined.

My studies initially focused on the regulation of the TFIIIB complex in response to differentiation. Brf1 levels were seen to decline, as was observed previously (Alzuherri and White, 1998). Using an antibody previously unavailable, I showed that Bdp1 protein levels also decrease following differentiation. I also noted a decrease in the overall levels of TBP, which has been described previously (Alzuherri and White, 1999) and attributed to a decrease in the TBP levels contained within the TFIIIB fraction (Alzuherri and White, 1998). Furthermore, a recent report shows that TBP is targeted for ubiquitin-mediated degradation in response to differentiation (Perletti *et al.*, 2001). These data are in disagreement with the report by Meissner *et al.* (1995), which claims that no significant differences in the overall levels of TBP could be observed between EC and PE extracts.

Based on the observations of Perletti *et al.* (2001) that TBP is degraded by the proteasome, I examined whether or not Brf1 and Bdp1 levels are also regulated by this pathway. Treatment of cells with proteasome inhibitors, however, failed to stabilise either Brf1 or Bdp1, before or after differentiation, suggesting that they are not targeted for degradation. As a control, two known targets of proteasomal

degradation were used, p53 and cyclin D1, both of which responded as expected. Importantly, proteasome inhibition did not affect the levels of pol III transcription in PE cells. This will be discussed in more detail later on.

An alternate mechanism for down-regulating Brf1 and Bdp1 could involve regulation of the two genes at transcriptional or post-transcriptional level. Therefore, the mRNA levels of each gene were examined using RT-PCR. This analysis revealed that both mRNA species declined following differentiation. It is worth noting that the design of the primers for the RT-PCR allows the detection of mature mRNA, and, therefore, the results represent the 'steady-state' levels of the mRNA species, i.e. their total levels at the time the RNA was prepared. This means that the down-regulation observed in PE extracts could be due to reduced transcription of the genes or increased turnover / reduced stability of the mRNAs themselves during the differentiation process. Additionally, translational regulation cannot be ruled out. TBP mRNA levels, on the other hand, do not decrease, and in fact appear to increase, following differentiation. This supports the proposed post-translational regulation of TBP during differentiation (Perletti *et al.*, 2001).

The presence of Brf1, Bdp1 and TBP in the TFIIIB complex in a 1:1:1 stoichiometry opens up the possibility of co-ordinate regulation of the three proteins, as incomplete complexes could be harmful to the cell. It has already been mentioned that this is probably not the case for TBP, since it seems to be regulated differently at the mRNA level. But what of Brf1 and Bdp1? Time-course analysis of the differentiation process revealed that the levels of both proteins decrease significantly at the same time point, i.e. between days 4 and 5. It is interesting that TBP is also down-regulated at the same time, suggesting that there might be some sort of co-ordination, or that it is a general effect associated with the reduction in proliferation rate. In support of a co-regulatory mechanism, RT-PCR analysis of RNA extracts throughout

the differentiation process revealed the same pattern of change for Brf1 and Bdp1 mRNAs. This pattern, however, is not accurately reflected in the protein levels. This could be due to translational regulation, as mentioned before, or simply due to the difference in sensitivities between the two methods used (Western immunoblotting vs. RT-PCR).

It has been demonstrated that TFIIIB activity is limiting in PE cells. This could indicate either a reduction in TFIIIB levels or changes in the activity of its regulators in response to differentiation. Using the same protein extracts in *in vitro* transcription assays, I observed that there is a significant decrease in pol III transcription very early, i.e. within 24 hours of induction of differentiation, with a further decrease between days 2 and 3. As both of these events precede the reduction in TFIIIB protein levels, it seems quite likely that reduction in TFIIIB levels is not responsible for the initial decrease in pol III transcription.

In parallel with these experiments, I constructed an F9 cell line that stably expresses HA-tagged human Brf1 (HA-hBrf1). Following differentiation of these cells, the levels of the exogenous protein were monitored. I found that HA-hBrf1 levels did not decrease in PE cells. They were similarly unaffected by treatment of the cells with proteasome inhibitors, either before or after differentiation. These data support the idea that Brf1 regulation is not post-translational, since the exogenous gene is under the control of a different promoter (the human cytomegalovirus promoter) than endogenous *BRF1*. It is worth noting here that differentiated derivatives of embryonal carcinoma cells exhibit enhanced expression of some viral genomes compared to the undifferentiated cells. One of these is the murine cytomegalovirus (reviewed in Kelly and Condamine, 1982). Therefore, possible HA-hBrf1 degradation by the proteasome in PE cells could be masked by increased expression

of the exogenous gene driven by the human CMV promoter. If that was the case, however, proteasome inhibition would have a pronounced effect on the levels of HA-hBrf1. I have also observed, by RT-PCR analysis, that synthesis of human (i.e. exogenous) *Brf1* mRNA does not change following differentiation. Furthermore, other reports (e.g. Zamanian and La Thangue, 1993) have demonstrated that constructs containing the human CMV promoter and enhancer sequences function with similar or equal activity in EC and PE cells. It is, therefore, my conclusion that the endogenous and exogenous *BRF1* genes are regulated differently in response to differentiation.

Another interesting observation came from examining the levels of pol III transcription in stably-transfected EC and PE cells. I found that the observed down-regulation of pol III in PE cells was not rescued by the increased levels of HA-hBrf1. This observation indicates that Brf1 is not the limiting factor in F9 PE cells, in contrast to yeast, where it has been shown that Brf1 is limiting for pol III transcription both *in vitro* and *in vivo* (reviewed in White, 2002). The possibility that the human protein is not functional in a murine environment is countered by the fact that pol III transcription is enhanced in F9 EC stables, compared to untransfected or mock-transfected cells. It is not possible to distinguish at this point, however, whether this increase is due to direct activation of pol III transcription by HA-hBrf1 or to de-repression of the endogenous protein, by sequestration of inhibitory factors. In addition, human and mouse Brf1 are almost 90% identical, making it quite likely that one can substitute for the other.

This is not to say, however, that overexpression of HA-hBrf1 has no effect on pol III transcription in differentiating F9 cells. Time course analysis of pol III activity, using the *in vitro* transcription of a $tRNA^{Leu}$ template as an indicator, revealed that its down-regulation is somewhat delayed, at least during the first 5 days.

A possible explanation for the inability of HA-hBrf1 to rescue pol III transcription in PE cells might be linked to the concurrent down-regulation of the other TAF in TFIIIB, i.e. Bdp1. However, addition of recombinant human Bdp1 to extracts from HA-hBrf1-expressing cells does not restore transcription. As with HA-hBrf1, it is not guaranteed that human Bdp1 can substitute for its murine homologue in functional pre-initiation complexes. The two proteins, however, are approximately 62% identical, including 100% identity in the SANT domain and 84% identity in the region just upstream of it.

The intricate pathways that cells use to regulate pol III transcription are slowly being elucidated (reviewed in White, 2004a). For example, two unrelated tumour suppressors, p53 and pRb, can reduce the transcriptional capacity of pol III, while oncogenic factors, like c-Myc and ERK, have the opposite effect. At the heart of these regulatory networks lies TFIIIB, directly and indirectly up- or down-regulated (Figure 1.4). In most cases, these changes in TFIIIB activity are reflected in the output of pol III. Since many of these regulatory proteins physically interact with TFIIIB, it is possible that they are contained in the same protein fractions as the transcription factor. This has been shown to be the case for c-Myc, pRb/p107/p130 and p53 (Cairns and White, 1998; Gomez-Roman *et al.*, 2003; Larminie *et al.*, 1997; Sutcliffe *et al.*, 1999). Therefore, it is possible that the reduced activity observed in TFIIIB-containing fractions from PE cells could be attributed to one or more of these proteins, rather than TFIIIB itself.

The regulation of these proteins during F9 differentiation reported previously in the literature is covered elsewhere. My own observations support some of these findings. pRb protein levels increase markedly following differentiation, while levels of cyclin D1, an important down-regulator of pRb, are reduced. Importantly, the inactive,

hyperphosphorylated form of pRb also decreases during differentiation. The latter, in particular, occurs with kinetics strongly resembling the down-regulation of pol III transcription itself, as measured by *in vitro* transcription experiments. pRb is therefore expected to be active, and present at high levels, in PE cells, perhaps a contributing factor to the reduced proliferation rate. As it is a known repressor of pol III transcription, and also found in TFIIIB fractions, it is plausible that it is involved in the down-regulation of pol III transcription in response to differentiation. The levels of the other two pocket proteins, p107 and p130, do not seem to change much during differentiation. Both of these proteins, however, are phosphorylated, and inactivated, by cyclin-dependent kinases, and as such their activity might increase as cells differentiate and cyclin levels fall.

Contrary to previous reports, I have found that p53 levels are unaffected during the differentiation of F9 EC cells to PE. p53 has been shown to be inactive in EC cells, increasing in transcriptional activity as the cells differentiate. Although class III do not contain p53-binding sites, the DNA-binding activity of p53 is essential for the repression of pol III transcription. It is, therefore, possible that activation of p53 could contribute to the repression of pol III transcription. An argument against this is that although proteasome inhibitors stabilise p53 in PE cells, they have no effect on pol III transcription.

The Ras/ERK pathway has been shown to play an important role in F9 cell differentiation (Verheijen *et al.*, 1999). Thus, although the activity of Ras (and hence ERK) increases in the initial steps of differentiation – formation of primitive endoderm – it decreases during parietal endoderm formation. My own results verify this. Although I did not examine the situation in primitive endoderm, PE cells have reduced levels of phosphorylated (active) ERK, even though total levels of ERK are

unchanged. Since ERK directly activates pol III transcription, it is likely to contribute to its down-regulation in PE cells.

Another kinase that activates TFIIIB is CK2. I have found that the levels of the catalytic subunits, α and α', decline during differentiation. The α subunit, however, seems to decline earlier than α', which persists until late in the time period monitored (day 6). Combined with the fact that the levels of the regulatory subunit do not change, CK2 might be active after the observed reduction in pol III transcription.

Finally, an important regulator of pol III activity that has recently come to light is the oncoprotein c-Myc. It has been previously reported that c-Myc is strongly down-regulated following differentiation. I have observed a similar response, with the protein levels of c-Myc rapidly declining following induction of differentiation. This decrease, therefore, will quite likely have an impact on pol III transcription. This is supported by the observation that adding recombinant c-Myc into PE extracts partly restores transcription of a $tRNA^{Leu}$ template, in a dose-dependent manner. Interestingly, c-Myc activates pol III transcription irrespective of the timing of its addition, i.e. before or after pre-initiation complex (PIC) assembly. It is, therefore, possible that, rather than PIC assembly and the first round of transcription, c-Myc enhances downstream events, e.g. recycling of the polymerase and re-initiation.

I have also noted that, although Max levels are not significantly altered, the levels of Mad family proteins decrease markedly in PE cells. This was unexpected, since decreased proliferation is often associated with high levels of Mad proteins (reviewed in Grandori *et al.*, 2000).

Taking all these data together, it seems that most regulators of pol III transcription are affected by differentiation in such a way as to bring about a decrease in pol III transcription. Thus, pRb is up-regulated and activated, p107 and p130 activity probably enhanced, the activity of the kinases CK2 and ERK decreased and the oncogene c-Myc down-regulated. In addition, there is a moderate change in the expression levels of *BRF1* and *BDP1* mRNA, and a clear reduction in the levels of the two proteins (and TBP). The decrease in pol III transcription is, therefore, inevitable. The question that remains, however, is which of the factors, if any, is more important than any other. To begin to answer this question, we must look at the temporal control of the various processes, taking the time course of pol III transcription down-regulation as the benchmark.

The initial reduction in pol III transcription, observed within 24 hours of induction, could be attributed to the reduction in c-Myc levels and the activation (dephophosphorylation) of pRb. The second observable reduction in pol III transcription occurs between days 2 and 3. A possible candidate for this is the pocket proteins. pRb phosphorylation decreases further and, although the levels of p107 and p130 do not change, it is quite likely that their activity also increases. This is supported by the observed reduction in cyclin D1 levels. Similarly, Li *et al.* (1999) showed that cyclin D3 levels fall during the first few days after induction. This would result in a decrease in cyclin D-dependent kinase activity, and thus activation (hypophosphorylation) of the pocket proteins. Other regulators of Cdk activity, like the Cdk inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ are targets of c-Myc. Therefore, the down-regulation of c-Myc will also have a strong impact on pRb/p107/130 activity. It seems plausible that the combination of these two pathways is sufficient for the early down-regulation of pol III transcription, occurring before the reduction in Brf1, Bdp1 and TBP levels. A supporting fact for the level of activation of pRb in response to

differentiation comes from studying the cell cycle characteristics of the differentiating cells. Rosenstraus *et al.* (1982) demonstrated that the most pronounced changed in the cell cycle is a lengthening of the G1 phase, exit from which is determined to a large extent by pRb. One argument against the importance of pRb up-regulation in the decrease of pol III transcription in PE cells comes from early mixing experiments, where it was shown that addition of increasing amounts of PE extract to EC extract resulted in stimulation of transcription, rather than its inhibition (White *et al.*, 1989). This suggests that the reduced transcriptional capacity of PE extracts is due to the absence of a positive activity, rather than the presence of a negative activity. A possible explanation for these observations, however, could be the presence of active Cdk complexes in the EC extracts, able to phosphorylate and inactivate pRb (and p107/p130) from PE extracts.

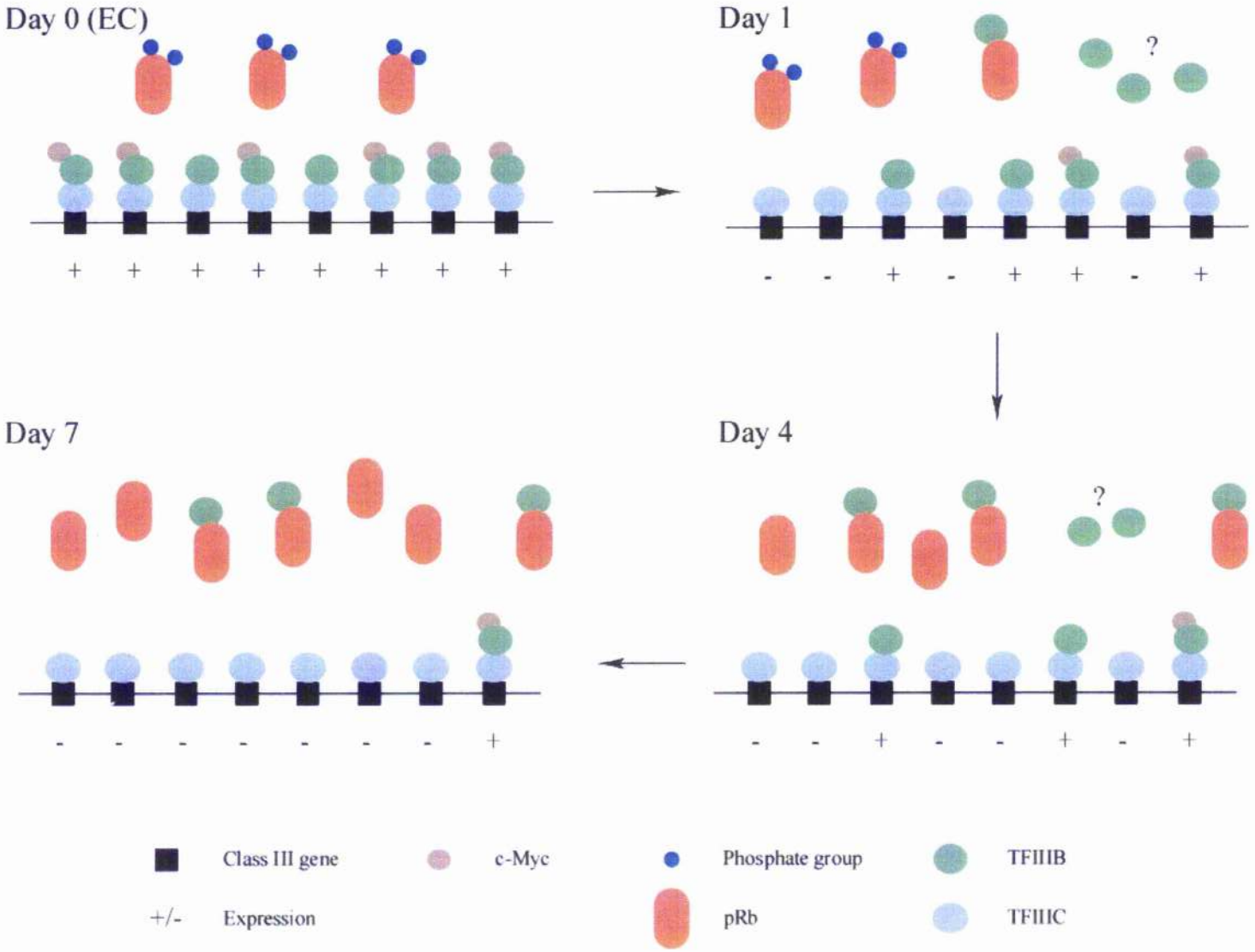
The reduction in the protein levels of Brf1 and Bdp1, observed between days 4 and 5 do not appear to be directly involved in the down-regulation of pol III transcription, but may instead be a consequence of reduced growth and proliferation rates. A summary of the model proposed here can be seen in Figure 7.1.

This study has focused on the Brf1-containing TFIIIB complex, necessary for transcription from type 1 and 2 promoters. That is not to say, however, that class III genes with type 3 promoters are not down-regulated during differentiation. Meissner *et al.* (1995) showed that U6 snRNA transcription is dramatically reduced following differentiation. The authors attributed this decrease to reduced levels of PBP (also called SNAPc), as well as one or more activities in the TFIIIC-containing fraction. In a later report, this was narrowed down to the poorly-characterised TFIIIC1 activity, whereas TFIIIC2 activity (see introduction) was unaffected (Meissner *et al.*, 2002). The latest report from the same group suggests that an essential component of this

TFIIIC1 activity is Bdp1 (Weser *et al.*, 2004), which is, in fact, a component of the TFIIIB complex, essential for transcription from all three promoter types. Furthermore, some regulators of pol III transcription, for example pRb and CK2, can affect genes with type 3 promoters in the same manner as genes with type 1 and 2 promoters.

Figure 7.1 Proposed model for the down-regulation of pol III transcription during differentiation of F9 cells

In undifferentiated F9 EC cells (day 0), TFIIIB is free to bind to TFIIIC on target genes and initiate their transcription. c-Myc has been shown to up-regulate pol III transcription, while pRb, a known repressor is hyperphosphorylated and therefore inactive. Within 24 hours of induction of differentiation (day 1), the levels of c-Myc fall dramatically, whereas pRb becomes underphosphorylated, allowing binding of TFIIIB. These changes result in a significant down-regulation of pol III activity, which may be compounded by other factors, like the ERK MAPK pathway (represented by the question mark). By day 4, pRb levels increase, enhancing the repressive effect on pol III transcription. Finally, TFIIIB levels decline (day 7). TFIIIC levels appear to stay the same throughout the differentiation.


Day 0 (EC)
+ + + + + + + +
Day 1
?
- - + - + + - +
Day 4
?
- - + - - + - +
Day 7
- - - - - - - +
Class III gene
c-Myc
Phosphate group
TFIIIB
+/- Expression
pRb
TFIIIC

It would be interesting to investigate further the regulation of Brf1 and Bdp1 at the mRNA level. The pattern of the abundance of the two mRNA species appears to be quite similar, with an overall down-regulation at the end of the analysed time period. It would be interesting to see if the loss of tumourigenicity exhibited by the F9 EC cells differentiating to PE-like cells correlates with reduced expression of the two genes. The mechanism by which this occurs, although maybe not as important for the F9 cell system, might provide some insight into the overexpression of Brf1 seen in some cancers (Daly *et al.*, 2005).

Overall, it appears that the reduction in pol III transcription during the differentiation of F9 cells is not as simple as was previously thought. Rather than one protein complex (TFIIIB) being responsible, it seems that various different pathways, most of which target TFIIIB, work together to accomplish this. It is also important to consider these mechanisms as a whole, rather than independently, as there is considerable cross-talk between them. For example, ERK, which activates pol III transcription by phosphorylating TFIIIB, can also stabilise c-Myc and induce cyclin D production, thus inactivating pRb (Cheng *et al.*, 1998; Sears *et al.*, 1999), while pRb can stabilise p53 by blocking the activity of Mdm2 (Hsieh *et al.*, 1999a). The result of this is that small perturbations in one pathway can lead to significant changes in other regulatory pathways, and thus, amongst other things, to repression of pol III transcription.

CHAPTER 8

References

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