A thesis entitled

Mechanisms of RNA polymerase III transcriptional activation by c-Myc

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for my Mum, Nancy Kenneth

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Summary

The Myc family of proto-oncogenes encodes transcription factors that play a pivotal role in regulating cellular proliferation, cellular growth, differentiation and apoptosis. To regulate cellular growth, it can activate a number of RNA polymerase II-transcribed genes which encode ribosomal proteins, translation factors and other components of the biosynthetic apparatus. c-Myc can also directly activate transcription by RNA polymerases I and III, thereby stimulating the production of ribosomal (r)RNA and transfer (t)RNA. As such, c-Myc may possess the capacity to induce the expression of all the ribosomal components. The work in this project aimed to investigate the mechanisms behind the c-Myc-dependent activation of RNA polymerase III transcription.

One mechanism by which activators of pol III transcription can stimulate the expression of class III genes is by promoting transcription complex formation. It had been previously demonstrated that c-Myc can interact with the pol III-specific transcription factor TFIIIB. Work in this thesis has further defined this interaction and demonstrated that activation of transcription by c-Myc can recruit this complex along with pol III to 5S rRNA and tRNA genes in vivo. Furthermore, the recruitment of TFIIIB and polymerase by c-Myc are distinct events, with a significant delay between TFIIIB and pol III binding, arguing against a pol III holoenzyme being recruited to the genes.

Most recent work on the mechanisms of transcriptional activation by c-Myc has focussed on its ability to influence chromatin structure. Transcriptional activation of

target genes by c-Myc may involve the remodelling of nucleosomes, since c-Myc has been shown to bind to the Snf5 subunit of the SWI/SNF complex, as well as the ATPase/helicases TIP48 and TIP49. In the present study, Snf5 and Brg1, both components of SWI/SNF, have been found at the promoters of pol III-transcribed genes. These may have a role in the regulation of pol III transcriptional activity.

c-Myc can also recruit a variety of histone modifying enzymes to the promoters of its target genes. It can bind to the co-factor TRRAP, a 440 kDa protein that forms the scaffold of a variety of histone acetyltransferase complexes. It has been demonstrated that c-Myc can recruit these complexes to certain target genes, and the increase in histone acetylation correlates with gene expression. The TRRAP co-factor along with an associated HAT was found to be present in a c-Myc-sensitive manner on pol III-transcribed genes, and their presence correlated with histone acetylation and gene expression. In addition to these findings, depletion of endogenous TRRAP by RNAi in cultured cells resulted in a specific down-regulation of pol III transcription in vivo.

In summary, this thesis has identified previously undescribed mechanisms by which c-Myc can activate transcription by pol III, and has identified novel co-activator proteins involved in the regulation of class III gene expression. This work has important implications in understanding the molecular basis of how activators can stimulate the expression of pol III-transcribed genes.

Contents

List of Figures

Chapter 1

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 1

Chapter 2

List of Abbreviations

4-OHT	4-hydroxytamoxifen
ARPP P0	acidic ribosomal phospoprotein P0
arg	arginine
bp	base pairs
bHLHZ	basic helix-loop-helix leucine zipper
Brf1	TFIIB-related factor 1
Brf2	TFIIB-related factor 2
BSA	bovine serum albumin
CDK	cyclin dependent kinase
ChIP	chromatin immunoprecipitation
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DSE	distal sequence element
DTT	dithiothreitol
eIF2Bε	eukaryotic initiation factor 2Bε
eIF4E	eukaryotic initiation factor 4E
ERK	extracellular signal-regulated kinase
F	forward primer
FCS	foetal calf serum
GA	glutathione agarose
GST	glutathione-S-transferase

H3	histone H3
H4	histone H4
HA	haemagglutinin
HA-Brf1	HA-tagged Brf1
HAT	histone acetyl transferase
HDAC	histone deacetylase
ICR	internal control region
IE	intermediate elements
KO	knockout
leu	leucine
MAPK	mitogen activated protein kinase
MBI	Myc box I
MBII	Myc box II
MBIII	Myc box III
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MycER	c-Myc-oestrogen receptor fusion protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pol I	RNA polymerase I
pol II	RNA polymerase II
pol III	RNA polymerase III
pol IV	single peptide RNA polymerase IV
PSE	proximal sequence element
R	reverse primer

RB	retinoblastoma protein
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electophoresis
SID	Sin3 interaction domain
SINEs	short interspersed elements
snRNA	small nuclear RNA
TAD	transactivation domain
TATA	TATA box
TBP	TATA box-binding protein
TRRAP	transactivation/transformation-domain associated protein
tyr	tyrosine
tRNA	transfer RNA
wt	wildtype

Chapter 1 Introduction

1.1 Eukaryotic Transcription

The initial stage of gene expression is the production of RNA molecules from the template DNA by the process of transcription. The process of transcription is performed by the nuclear RNA polymerases, which are large, complex multi-subunit enzymes (White, 2001). Mammalian cells contain four nuclear RNA polymerases each responsible for the transcription of a distinct set of genes, RNA polymerases I, II, III and IV (White, 2001; Kravchenco et al, 2005). RNA polymerase I (pol I) is responsible for the production of the 45S large ribosomal RNA (rRNA) pre-cursor, which is subsequently processed into the 5.8S, 18S and 28S rRNAs (Grummt, 2003). Although pol I is only responsible for the production of a single RNA species, it is responsible for approximately 60-70% of all nuclear transcription (Moss and Stefanovsky, 2002). RNA polymerase II (pol II) is responsible for the transcription of a vast array of genes, it produces the bulk of messenger RNAs (mRNAs), which are subsequently translated into proteins, and also synthesises the majority of small nuclear RNAs (snRNAs) (White, 2001). RNA polymerase III (pol III) transcribes the genes encoding the 5S rRNA and the transfer RNAs (tRNAs) as well as the production of a host of other essential short untranslated RNAs (Paule and White, 2000). Depending on cellular conditions, pol III is responsible for approximately 10% of all nuclear transcription (Moss and Stefanovsky, 2002). RNA polymerase IV has recently been identified and is expressed from an alternative transcript of the mitochondrial RNA polymerase gene (Kravchenko et al, 2005). Pol IV is responsible

for the synthesis of some mRNAs (Kravchenko et al, 2005). These RNA polymerases are subject to tight regulation, and this co-ordination of gene expression influences the fate of mammalian cells (White, 2001). The untranslated products produced by pols I and III are essential for sustained protein synthesis and therefore a fundamental determinant of the capacity of a cell to grow (White, 2005). The work performed in this thesis is involved in further understanding the regulation of transcription by pol III, and therefore furthering understanding of the control of cell growth.

1.1.2 Transcription and Cell Growth

The progression of the cell cycle and cellular proliferation cannot occur without cells first attaining a critical mass. As 80-90% of the dry mass of a cell is protein, the rate of protein synthesis is critical to both growth and therefore proliferation (Zetterberg and Killander, 1965; Baxter and Stanners, 1978). In most cells the number of mRNA molecules is greater than the number of ribosomes, therefore ribosome biogenesis is rate limiting for protein synthesis (Kief and Warner, 1981; Zetterberg and Killander, 1965). Each ribosome is composed of four RNA species and over eighty proteins. The synthesis of the rRNA is the rate limiting step in ribosome biogenesis as almost all rRNA is incorporated into ribosomes (Liebhaber et al, 1978). The four rRNAs that are incorporated into each ribosome are the 5.8S, 18S and 28S rRNAs, produced by pol I and the 5S rRNA, produced by pol III (White, 2005). These RNA species are all essential and required in equimolar amounts, therefore the study of the co-ordination of both pol I and pol III transcription is required for an understanding of cell growth. Levels of the pol III-transcribed tRNAs have also been demonstrated to be critical for the rate of protein synthesis and cell growth. Reducing levels of the initiator tRNA in

yeast can influence cell growth and prolong cell doubling time (Francis and Rajbhandary, 1990). This is supported by the fact that there is a general increase in tRNA synthesis in the growth response in all organisms examined (White, 2002). As well as the synthesis of both tRNAs and the 5SrRNA, pol III is also responsible for the production of a variety of other short untranslated RNAs critical to the biosynthetic capacity of the cell, which are discussed later in this introduction, and an understanding of protein synthesis and cell growth is not complete without considering transcription of by pol III (White, 2002).

1.1.3 Regulation of pol III transcription

Due to the essential function of pol III products for protein synthesis and cell growth, their transcription is highly regulated. A variety of cellular products involved in the regulation of pol III transcription will be described, as well as the mechanisms behind their control. A recent addition to the direct regulators of pol III transcription is the product of the c-Myc proto-oncogene (Gomez-Roman et al, 2003). c-Myc has been demonstrated to potently and directly activate the transcription of genes transcribed by pol III (Gomez-Roman et al, 2003). The work in this thesis will attempt to uncover the mechanisms behind this activation, and to identify any other co-activator proteins involved in the process.

1.2 Class III genes

Products of the genes transcribed by pol III are short RNAs, typically between 100 and 300 nucleotides in length, which remain untranslated. These short RNA molecules are involved in various cellular processes and are listed, along with their function, in the table below.

Table 1.1 – (Adapted from White, 2002)

Product	Function	Size
5S rRNA	Ribosomal component	120 nt
tRNAs	Translational Adaptors	70-90 nt
U6 snRNA	mRNA splicing	106 nt
7SK RNA	Transcriptional elongation by pol II	330 nt
7SL RNA	SRP component	300 nt
MRP RNA	rRNA splicing	265 nt
HI RNA	tRNA processing	369nt
Alu RNA	General pol II inhibition?	300nt
B2 RNA	General pol II inhibition?	180nt
VA RNA	Translational control	160 nt
EBER RNA	Translational control	165 nt

The majority of pol III-transcribed genes are involved primarily in protein synthesis from processing the mRNA, to being directly involved in the translation and

appropriately targeting the newly synthesised polypeptide. These functions of pol III-transcribed genes will be described in this introduction.

1.2.2 5S rRNA genes

Ribosomes consist of approximately 85 proteins, as well as four species of rRNA, the 5S, 5.8S, 18S and 28S rRNA (Doudna and Rath, 2002, Wool, 1979). The pol III transcribed 5S rRNA is an essential component of the ribosome, and is therefore essential in eukaryotic organisms for protein synthesis (Wool, 1979). The four rRNAs are required in equal stoichiometry, each being present in one copy per ribosome (White, 2001). Unlike the pol I-transcribed rRNAs, the 5S rRNA is transcribed in the nucleoplasm, but is transported to the nucleolus for processing and incorporation into the ribosome (Lafontaine and Tollervey, 2001). The human genome contains 300-400 5S rRNA genes and many of these genes exist in clusters or tandem repeats (International Human Genome Consortium, 2004).

1.2.2 tRNA genes

Along with the 5S rRNA, the tRNAs are essential in the translation of mRNAs. The tRNA molecules serve as adaptors that interpret the sequence of mRNA to specify the order of amino acid residues in a protein. tRNA molecules, when processed, are between 70 to 90 nucleotides in length and adopt an L shaped secondary structure (Draper, 1996; White, 2002). Although the tRNAs have similar structures they can only recognise a specific amino acid, the amino acid is covalently linked to the 3' end of the tRNA, and matches this to a specific codon in the message (White, 2002).

Each eukaryotic cell contains 50 to 100 tRNA species, and because of this number there is a great deal of redundancy among the tRNA genes (Sharp et al, 1984). The human genome contains approximately 500 tRNA genes and related pseudogenes, and these encode 60-90 tRNAs (International Human Genome Consortium, 2004). The tRNA genes in man are generally scattered throughout the genome, although some clustering is observed (International Human Genome Consortium, 2004).

1.2.3 U6 snRNA genes

An essential step in the production of mature mRNA molecules is the splicing of the pre-mRNA. Many protein-encoding genes transcribed by pol II are interrupted by non-coding regions called introns that must be removed by splicing to create a functional mRNA (Maniatis et al, 1987; Mattaj et al, 1993). Spliceosomes are small nuclear ribonucleoproteins (snRNPs) which are involved in pre-mRNA splicing within the nucleus (Maniatis and Reed, 1987; Mattaj et al, 1993). In addition to their protein component, spliceosomes contain five snRNAs (Maniatis and Reed, 1987; Mattaj et al, 1993). Four of the snRNAs are transcribed by pol II, but the U6 snRNA is transcribed by pol III (Kunkel et al, 1986; Reddy et al, 1987). The U6 snRNA is a 106 nucleotide transcript, and is the most conserved amongst the splicosome snRNAs (Reddy et al, 1987; Brow and Guthrie, 1988). It appears that the U6 snRNA is in relative excess in comparison to the other snRNAs in yeast and in higher eukaryotes, indicating that it is not rate-limiting in spliceosome production (Kaiser et al, 1995; White, 2002).

1.2.4 7SL RNA gene

The 7SL RNA is another product of pol III and this RNA forms the nucleic acid scaffold of the signal recognition particle (SRP), along with six different polypeptides (Walter and Blobel, 1982). The SRP is involved in protein trafficking through the insertion of nascent polypeptides into the endoplasmic reticulum (Walter and Blobel, 1982). The trafficking of proteins through this organelle is critical for post-translational modifications, appropriate folding and delivery to their appropriate sub-cellular locations (Keenan et al, 2001). The 300 nucleotide 7SL is extremely conserved throughout evolution, with the human genome containing four copies (International Human Genome Consortium, 2004).

1.2.5 Other Class III Genes encoding RNP Components

The 330 nucleotide 7SK RNA is a relatively abundant RNA present in the nucleus of eukaryotic cells (Mattaj et al, 1993; Murphy et al, 1986). Until relatively recently the function of 7SK remained somewhat of an enigma. Several studies have now demonstrated that 7SK is a negative regulator of pol II elongation (Nguyen at al, 2001; Yang et al, 2001). 7SK binds to a pol II elongation factor, PTEFb, and this interaction has inhibitory effects on this complex and pol III elongation (Nguyen at al, 2001; Yang et al, 2001).

The H1 RNA is the 369 nucleotide component of Rnase P, a complex involved in the 5' processing of pre-tRNA molecules (Bartkiewicz et al, 1989). The primary sequence of the H1 RNA is not homologous between organisms, but the tertiary

structure is thought to be highly conserved to retain its function (Morrissey and Tollervey, 1995).

The 265 nucleotide MRP RNA is a component of an endoribonuclease originally identified from its ability to cleave the mitochondrial transcript to generate a primer for mitochondrial DNA replication (Chang and Clayton, 1987; Chang and Clayton, 1989; Topper and Clayton, 1990). Although identified in the mitochondria the RNase MRP is predominantly in the nucleolus where it plays an important role in pre-rRNA processing (Morrissey and Tollervey, 1995).

1.2.6 Viral Genes transcribed by pol III

There are several viruses that encode class III genes, these viruse can use the pol III transcription machinery of host cells to express these genes (White, 2002). The adenoviral VA_I and VA_{II} genes are both transcribed by pol III in the late stages of adenoviral infection (Soderlund et al, 1976; Weinmann et al, 1974). The VA RNAs act by stimulating the translation of adenoviral mRNA at late times after infection (Thimmappaya et al, 1982). TheVA_I transcript is essential for this process, but deletion of the VA_{II} transcript has no effect on the viral life-cycle (Thimmappaya et al, 1982).

The Epstein-Barr virus (EBV) also contains two pol III-transcribed genes that are highly expressed in viral infections (Rosa et al, 1981). The two genes are 165 nucleotides long and are called EBER1 and EBER2 (Rosa et al, 1981). Although they are highly expressed in EBV infected cells, EBER1 and EBER2 are not necessary for

the normal viral repliction cycle (White, 2002). The EBER genes are able to substitute for the VA genes in adenoviral infection, so a similar role in stimulating translation is likely (White, 2002). Furthermore, EBERs are sufficient to induce growth in soft agar and tumours in mice, thus providing the first example of an oncogenic RNA (Kitagawa et al, 2000; Komano et al, 1999; Ruf et al, 2000).

1.2.7 SINEs

A variety of short interspersed elements (SINEs) in higher organisms are pol III templates (White,2002). The predominant SINEs in rodents are the B1 and B2 genes. The B1 gene family is dervived from the 7SL gene and is homologous to the Alu SINEs present in primate genomes (Ullu and Tschudi, 1984). The function of the B1 RNA remains elusive (Daniels and Deininger, 1985). The B2 family of genes are rodent specific SINEs and appear to have been derived from a tRNA gene, and are essentially tRNA pseudogenes (Daniels and Deininger, 1985). A function of the B2 SINE has recently been proposed in the heat shock response. B2 has been shown to bind to pol II and generally repress mRNA production in response to heat shock (Allen et al, 2004). The B1 and B2 genes are present in high copy numbers in the rat genome with 384 000 copies of B1 and 328 000 copies of the B2 gene (Rat Genome Sequencing Project Consortium, 2004)

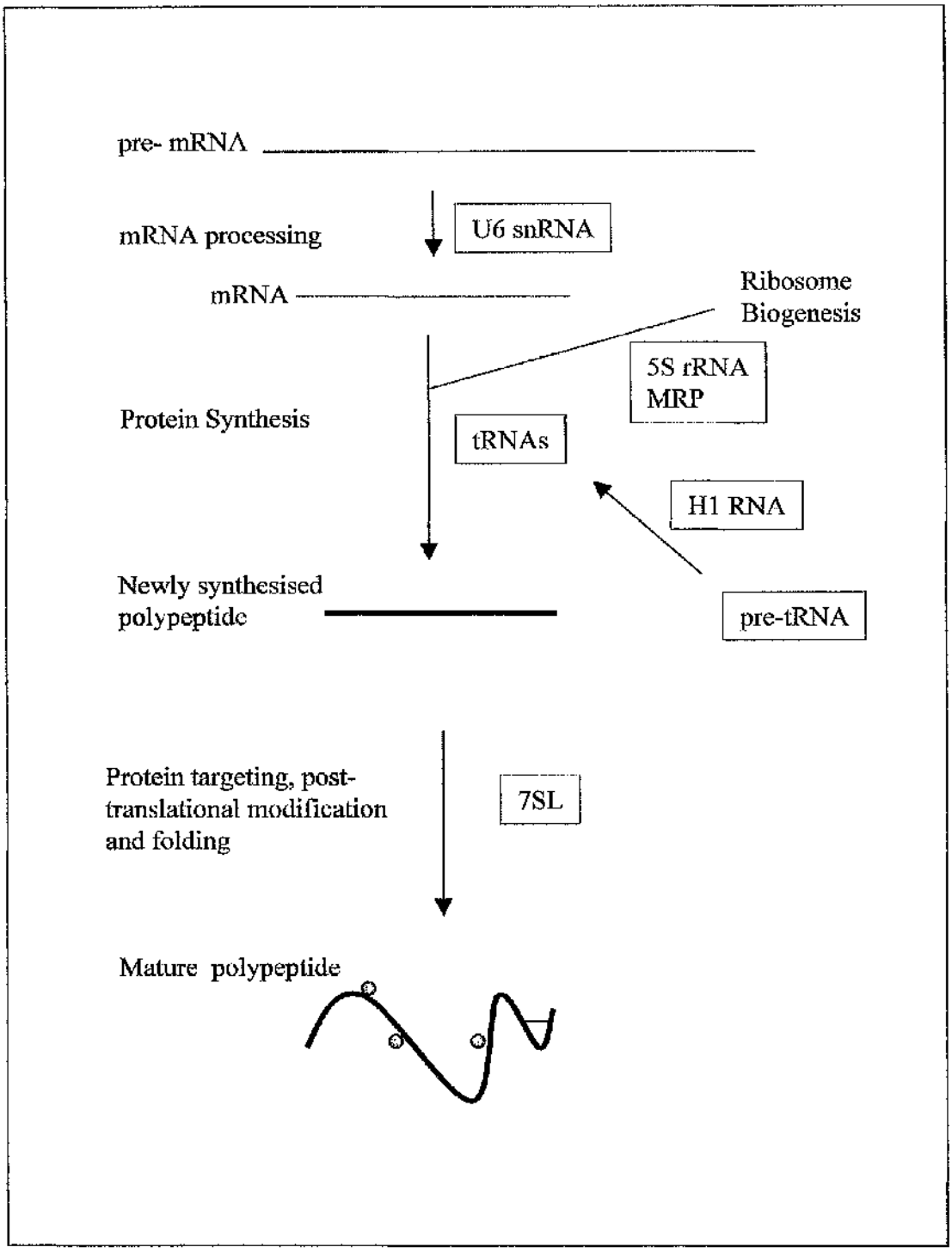
The major SINE in humans and other primates is the Alu family. The Alu family of genes represents approximately 10% of the human genome, with over one million copies (International Human Genome Consortium, 2001). Alu genes consist of two imperfect repeats derived from the 7SL gene separated by a 18bp spacer (Ullu and

Tschudi, 1984). The upstream repeat contains a functional promoter sequence, but Alu genes are generally expressed at very low levels in human cells (Liu and Schmid, 1993). Alu gene products may perform a similar role to the B2 RNA in the heat shock response in human and primate cells. Expression of Alu transcripts is increased in heat shock conditions and these RNAs can bind to and inhibit pol II (Mariner et al, 2005). The recent data proposing functions of SINEs is challenging the concept that these elements are merely 'junk' DNA.

The functions of genes transcribed by pol III are critical in the process of protein synthesis and their functions are summarised in figure 1.1.

Figure 1.1 Pol III transcripts are involved in the protein synthetic pathway

Several pol III transcripts are involved in the regulation of the protein synthetic machinery. The newly synthesised mRNA is processed by the spliceosome, which includes the U6 snRNA. The MRP RNA and the H1 RNA are involved in rRNA and tRNA processing, respectively. The tRNA and 5S rRNA genes are involved in the translation of the mature mRNA molecule, with the 7SL genes involved in the appropriate targeting and delivery of the newly synthesised peptide to the correct cellular location. Boxes indicate a pol III product.


pre- mRNA
mRNA processing
U6 snRNA
mRNA
Ribosome
Biogenesis
5S rRNA
MRP
Protein Synthesis
tRNAs
H1 RNA
Newly synthesised
polypeptide
pre-tRNA
Protein targeting, post-
translational modification
and folding
7SL
Mature polypeptide

1.3 Promoter Structures of Class III Genes

The promoter structures of genes transcribed by pol III are often markedly different from the promoters utilised by the other polymerases. The most unusual feature is that the vast majority require sequence elements downstream of the start site of transcription within the transcribed region, termed internal control regions (ICR). The structure of these promoters is discussed below and displayed in figure 1.2.

1.3.1 Type I promoter

Type I promoters are found only within the 5S rRNA genes. Much of the work done on the promoter structure of 5S rRNA genes has been performed using *Xenopus laevis*, as a model system. The *Xenopus laevis* somatic 5S gene ICR is made up from three conserved sequence elements: the A-block located between + 50 and +64, the intermediate element (IE) located between +67 and +72 and the C-block at +80 to +97; these elements are required for efficient transcription of the gene (Figure 1.2, Bogenhagen et al, 1985; Pieler et al, 1985 a,b; Pieler et al, 1987). The bases between the conserved elements appear to act merely as spacers, and mutation do not influence transcriptional efficiency, but short deletions or additions of bases in these regions reduces transcription and the stability of a transcription complex (Pieler et al, 1987). The 5S rRNA genes studied in other eukaryotic organisms have similar promoter structures and the spacing between the promoter elements is critical (White, 2002).

1.3.2 Type II promoter

The type II promoter is the most common promoter arrangement utilised by pol III-transcribed genes. Genes with type II promoters include the tRNA genes, the major SINEs and the VA genes (White, 2002). These genes are characterised by two conserved sequence elements, the A and B blocks, within the transcribed region (Figure 1.2; Galli et al, 1981). These sequence elements are about 10 base pairs in length, and are essential for the transcription of the gene (Galli et al, 1981). The A-block is homologous to the A-Block contained within Type 1 promoters, and these elements can be interchangeable, although the A- block is much closer to the start site of transcription in type II promoters (Ciliberto et al, 1983). The B-block is further downstream of the A-block, and these blocks are generally 30-40bp apart, but this spacing can be increased due to short introns in the coding regions of certain genes (Sharp et al, 1984; Geiduschek et al, 1988). Although the A and B blocks are necessary and sufficient for efficient transcription, flanking sequences are likely to have an influence upon the transcription of most if not all tRNA genes (White, 2002).

1.3.3 Type III promoters

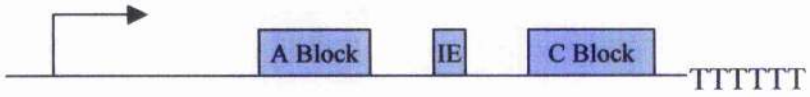
A few class III gene promoters lack any requirement for internal promoter elements; these are referred to as type III promoters. Examples of genes with a type III promoter are the U6 snRNA, MRP and 7SK genes (White, 2002). The best-characterised example of a type III promoter is that of the U6 snRNA gene. The sequences required for expression of the human and mouse U6 snRNA gene are the TATA box, a proximal sequence element (PSE) and a distal sequence element (DSE);

all of which lie upstream of the transcription start site (Figure 1.2; Das et al, 1988; Kunkel at al, 1989; Lobo et al, 1989). As well as the upstream sequences described above, the yeast U6 snRNA genes have functional A and B blocks with homology to those utilised in type II promoters that are not present in the U6 snRNA genes of higher eukaryotes (White, 2002).

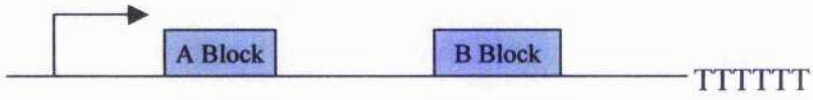
Figure 1.2 Structure of class III genes

The three types of promoter arrangement that are utilised by pol III are displayed in this figure. The start site of transcription is indicated with an arrow and the termination signal indicated by a run of T residues.

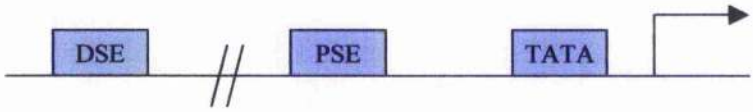
Type I e.g. 5S rRNA



Type II e.g. tRNA



Type III e.g. human U6 snRNA



1.4 Transcription Complex Assembly on Class III genes

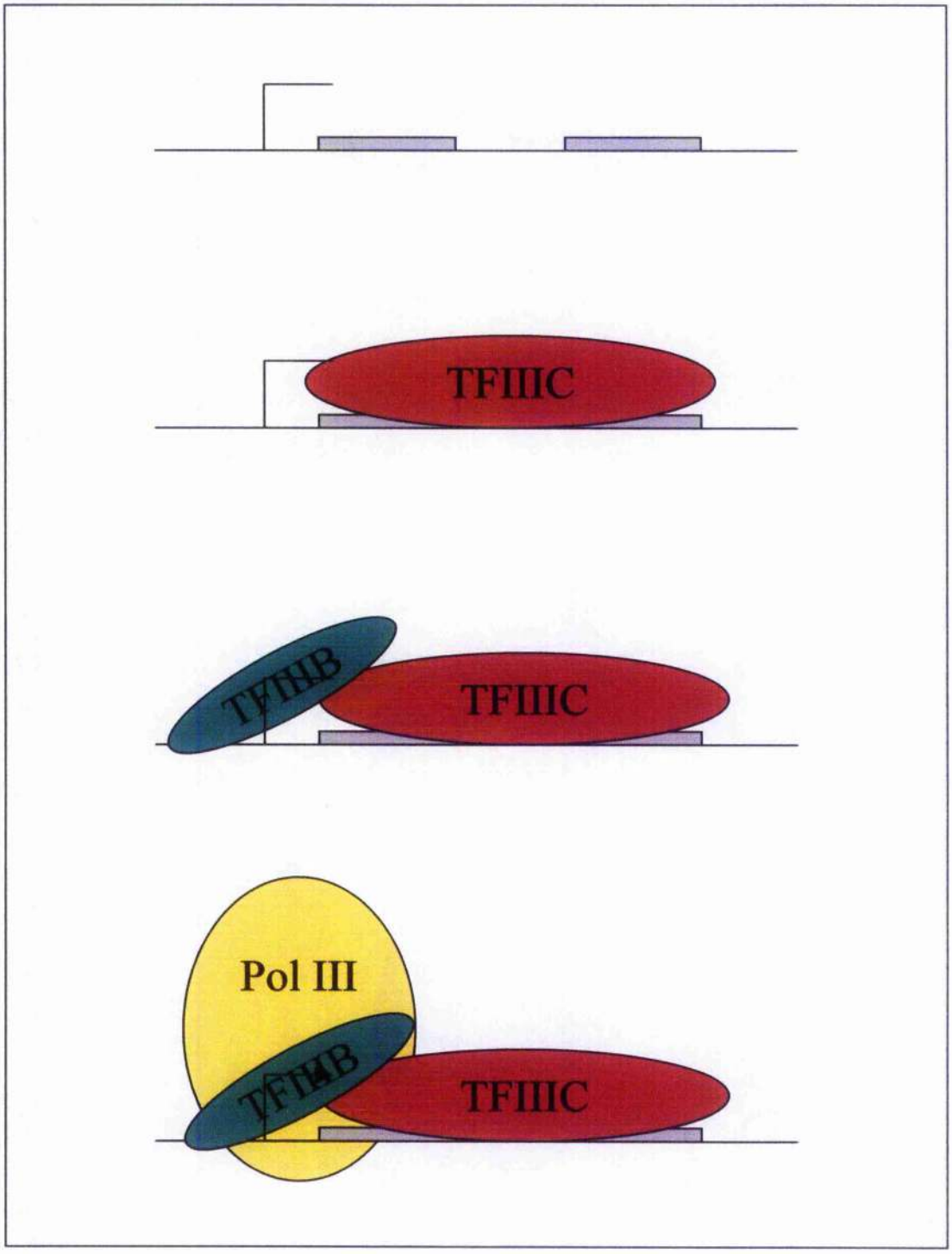
The nuclear RNA polymerases alone have little specificity for particular DNA sequences. In the case of pol III, recruitment to specific genes requires the prior assembly of pol III-specific general transcription factors. These factors vary depending on the nature of the promoter structure and the assembly of these transcription complexes will be discussed below.

1.4.1 Complex Assembly on a Type II promoter

The initial step in the formation of the transcription complex on type II promoters is the binding of the pol III-specific transcription factor TFIIIC (Paule and White, 2000; Schramm and Hernandez, 2002). This complex has been well defined in yeast and is a large and complex multi-subunit transcription factor. Yeast TFIIIC is made up of six subunits, which have an aggregate mass of over 500kDa (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). The subunits of TFIIIC, individually, have no DNA binding activity, but the formation of the entire complex creates two globular domains capable of binding to the A and B blocks of a type II promoter (Baker at al, 1986). Although TFIIIC binds to both the A and the B block of a type II promoter it is the B block that appears to be the major determinant of binding affinity (Baker et al, 1986). The tolerance of variable spacing between the A and B blocks is surprising as they are bound simultaneously by a single transcription factor complex, and explained by the fact that TFIIIC can be stretched to achieve this remarkable flexibility (Schultz et al, 1989).

Figure 1.3 Transcription complex assembly on a type II promoter

The pol III-specific transcription factor TFIIIC binds to the A and B blocks on the promoters of genes with a type II promoter. TFIIIC then recruits TFIIIB, which can then recruit the polymerase.


TFIIIC
TFIIIB
TFIIIC
Pol III
TFIIIB
TFIIIC

Human TFIIIC appears to be more complex than its yeast homologue. The biochemical fraction containing TFIIIC activity can be split into two functional components termed TFIIIC1 and TFIIIC2, with both required for transcription on type I and type II promoters, but only TFIIIC1 being required for transcription of genes containing type III promoters (Yoshinaga at al, 1987; Dean and Berk, 1987; Yoon et al, 1995; Lagna et al, 1994; Oettel et al, 1997). The identity of TFIIIC1 activity remains relatively ill defined and its polypeptide composition has not been unequivocally demonstrated (White, 2002). The TFIIIC1 activity stabilises the interaction between TFIIIC2 and the promoters of class III genes (Wang and Roeder, 1996). Human TFIIIC2 is relatively well defined, it is composed of 5 subunits, which have been identified and cloned, it has a mass similar to that of yeast TFIIIC and performs a similar function, but the subunits have weak or no homology with subunits of the yeast factor (Yoshinaga at al, 1989; Sinn et al, 1995; Wang and Roeder, 1996). The five subunits are TFIIIC 220, TFIIIC 110, TFIIIC 102, TFIIIC 90 and TFIIIC 63, each named after their mass in kDa (White, 2002; Schramm and Hernandez, 2002). The two largest subunits of TFIIIC2, 220 and 110, form the binding interface between TFIIIC and the B-block of type II promoters and TFIIIC 102 and 63 are associated with the A-block, with TFIIIC 90 bridging the two DNA binding domains (Schramm and Hernandez, 2002). Human TFIIIC2 plays additional roles to its yeast counterpart highlighted by the fact that two of its subunits TFIIIC 110 and 90 possess histone acetyl transferase activity (HAT) and that human TFIIIC2 can relieve chromatin-mediated repression of a tRNA gene (Hsieh et al, 1999; Kundu et al, 1999).

The main function of TFIIIC is to recruit another complex, TFIIIB, to the promoters of type I and II genes (Schramm and Hernandez, 2002; White, 2002). TFIIIB is the central class III initiation factor since it alone is sufficient to recruit the polymerase and specify the site at which transcription begins (Kassavetis et al, 1990). TFIIIB is another multi-subunit pol III specific transcription factor and it is a complex of three polypeptides in yeast and humans, one of which being the TATA-binding protein (TBP). TBP is essential in pol III transcription and is also involved in the transcription of genes transcribed by pol I and pol II (Burley and Roeder, 1996). Two pol III-specific TBP-associated factors are also required for TFIIIB activity. The first of these is a factor with homology to the pol II basal transcription factor TFIIB, and for this reason it is referred to as the TFIIB-related factor 1 (Brf1), and has a relative molecular mass of 90kDa in humans (Roberts et al, 1996). The other human TFIIIB associated TAF is Bdp1, with the major form being a 160 kDa polypeptide (Schramm et al, 2000). Several alternatively spliced forms of Bdp1 have been identified in human cells (Schramm et al, 2000). The roles of these variants remains unclear. A 250kDa variety of Bdp1 may play additional roles in pol III-transcription with it recently being proposed to be the unidentified TFIIIC1 activity (Weser et al, 2004).

Type I and II promoters of pol III-transcribed genes have no TATA element and the TBP-containing TFIIIB complex does not bind to DNA directly and is recruited via interactions with TFIIIC (Bieker et al, 1985; Setzer and Brown, 1985).

All three subunits of TFIIIB are required for polymerase recruitment, but only TBP and Brf1 have been shown to make direct interactions with the polymerase (Schramm and Hernandez, 2002). Of these interactions, the binding of Brf1 to the yeast C34 pol

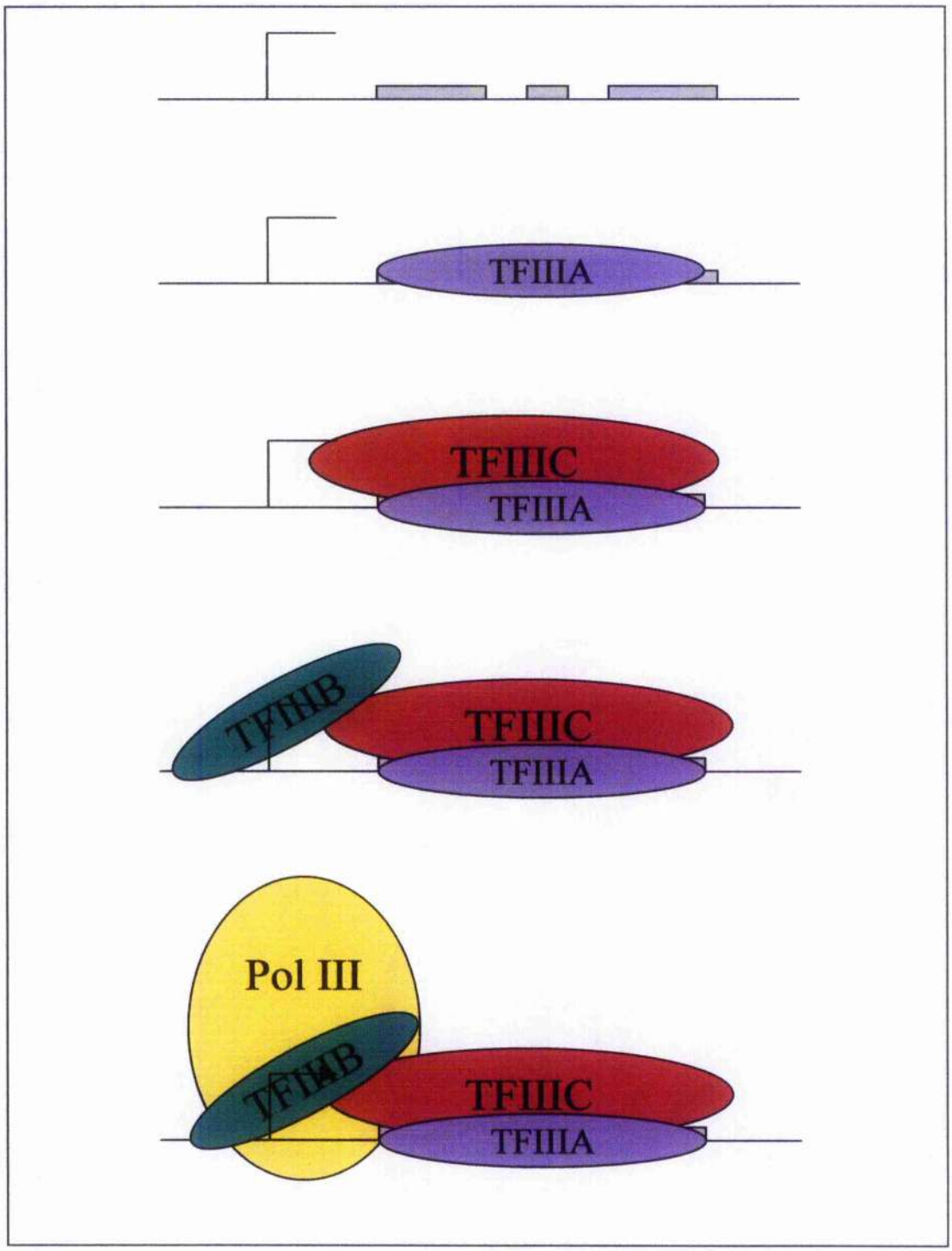
III subunit (RPC39 in humans) appears to be critical (Schramm and Hernandez, 2002). Additional contacts between TFIIIC and pol III may stabilise the transcription complex (Flores et al, 1999; Dumay et al, 1999; Hsieh et al, 1999).

1.4.2 Complex assembly on a type I promoter

The action of TFIIIC to recruit TFIIIB, and the subsequent TFIIIB-dependent recruitment of pol III is similar on type I and II promoters (Schramm and Hernandez, 2002; White, 2002). The differences in these promoters is that the type I promoter of the 5S rRNA genes lacks a functional B block and cannot directly recruit the TFIIIC complex. On these promoters TFIIIC is recruited through protein-protein interactions with another pol III-specific transcription factor TFIIIA (Figure 1.4; Schramm and Hernandez, 2002; White, 2002). TFIIIA was the first eukaryotic transcription factor to be purified to homogeneity and the first eukaryotic transcription factor to have its gene cloned (Engelke et al, 1980; Ginsberg et al, 1984). TFIIIA is composed of a single polypeptide, of a relative molecular mass of 42 kDa in humans, and is the founding member of the C_2H_2 zinc finger family of DNA-binding transcription factors, consisting of nine tandem, zinc-dependent DNA binding motifs (Miller at al, 1985, Moorfield and Roeder, 1994). The N-terminal three zinc fingers recognise the type I promoter-specific C-block in 5S rRNA promoters, and the interaction of these fingers contributes to 95% of total binding of TFIIIA to type I promoters (Foster et al, 1997; Nolte et al, 1998; Clemens et al, 1992). The precise interactions of TFIIIC with TFIIIA still remain unclear (Schramm and Hernandez, 2002).

Figure 1.4 Transcription complex assembly on a type I promoter

The type I promoter-specific transcription factor TFIIIA binds the internal control region of the gene. TFIIIC is then recruited to the promoter via protein-protein interactions with TFIIIA. This is then followed by the recruitment of TFIIIB and subsequent recruitment of the polymerase.

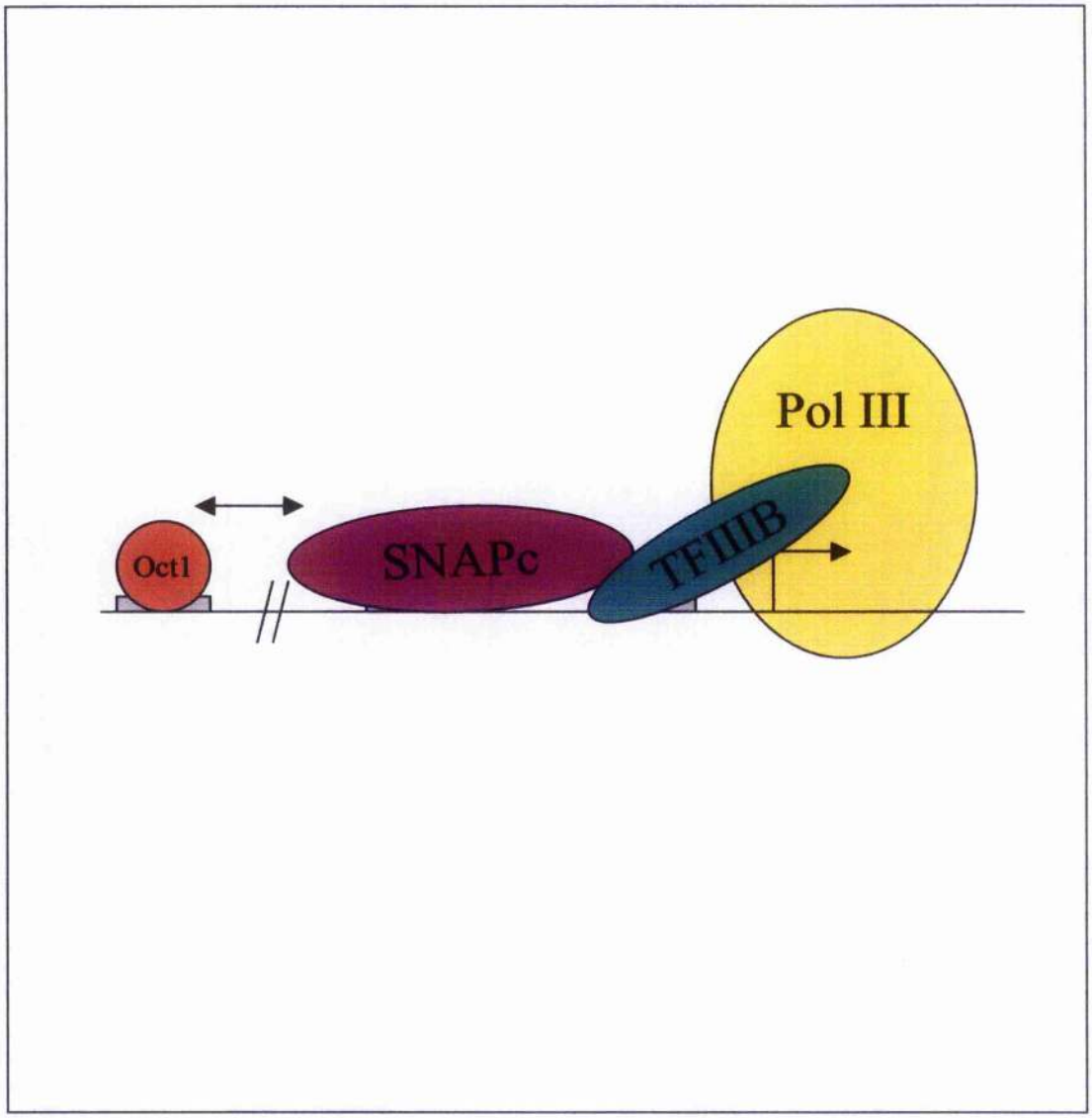

TFIIIA
TFIIIC
TFIIIA
TFIIB
TFIIIC
TFIIIA
Pol III
TFIIB
TFIIIC
TFIIIA

1.4.3 Complex Assembly on Type III promoters

The type III promoters associated with vertebrate U6 snRNA, 7SK and MRP genes have distinct factor requirements from pol III-transcribed genes with type I and II promoters. The complex assembly on these genes is outlined in figure 1.5. Type III promoters contain a TATA box and a PSE, which are recognised by the TBP component of a TFIIIB complex and a multi subunit complex termed $SNAP_C$ respectively (Schramm and Hernandez, 2002). The TFIIIB complex utilised in the transcription of genes with a type III promoter differs in subunit composition to that used by type I and type II promoters. The TFIIIB activity is made up from TBP, Bdp1 and a Brf1 homologue, Brf2 (Schramm et al, 2002). Protein-protein interactions between $SNAP_C$ and the TBP component of TFIIIB enhance their interaction with the promoters of type III genes, and this may explain the strict spacing between the PSE and the TATA box (Mittal and Hernandez, 1997; Schramm and Hernandez, 2002). The binding of these factors then leads to the subsequent recruitment of the polymerase by the TFIIIB complex (White, 2002). Although basal transcription only requires the presence of the PSE and the TATA box, the binding of another transcription factor, Oct1, to the upstream DSE element of type III gene promoters can enhance levels of transcription (Schramm and Hernandez, 2002).

Figure 1.5 Transcription complex assembly on type III promoters

$SNAP_C$ and TFIIIB bind co-operatively to the PSE and TATA box of type III promoters. Binding of another factor, Oct-1, to the upstream DSE enhances transcription of genes with a type III promoter. This is through interactions between $SNAP_C$ and Oct-1, which enhances $SNAP_C$/TFIIIB recruitment to the promoter. Polymerase is then recruited via interactions with $SNAP_C$/TFIIIB.


Pol III
Oct1
SNAPc
TFIIIB

1.4.4 Pol III

As previously mentioned, pol III is the largest and most complex of the nuclear RNA polymerases. As all three nuclear RNA polymerases perform essentially the same task, faithful transcription of a DNA template to produce a complementary RNA strand, it is of no surprise there are common subunits between polymerases and significant homology between some of the unique subunits (White, 2002). Pol III is well defined in budding yeast; it consists of 17 subunits, ten being unique to pol III, two being shared between pols I and III and five common between pols I, II and III (Schramm and Hernandez, 2002; White, 2002). Of these 17 subunits identified in yeast, 16 have been disrupted and demonstrated to be essential for function (Chedin et al, 1998). Human pol III has been purified by chromatography and from cell lines expressing tagged pol III subunits, and orthologues of the yeast subunits have been identified (Wang and Roeder, 1996; Wang and Roeder, 1997; Hu et al, 2002).

The two largest subunits of pol III are homologous to the two largest polypeptides of pol I and pol II, as well as having homology to the β' and β subunits of bacterial RNA polymerase (White, 2001). These conserved subunits, along with subunits shared between the eukaryotic pols, are thought to form the RNA polymerase catalytic core, with the unique subunits performing other essential roles such as template selection and transcription factor interaction sites (White, 2001).

1.4.5 Transcription Initiation and Elongation

Once the polymerase is recruited to the promoter it melts the double-helix around the start site of transcription. There is evidence that along with the polymerase, components of TFIIIB may be involved in his event (Kassevetis et al, 1998). In contrast to transcription by pols I and II, there are no elongation factors associated with the pol III transcription apparatus, this may be due to the extremely short length of class III genes, or unique subunits of the polymerase may perform this role (White, 2001). One of the mysteries associated with pol III transcription is how the polymerase transcribes through the pol III transcription apparatus, which covers the entire gene, without removing it or being significantly obstructed (Bogenhagen et al, 1982; Jahn et al, 1987). It may be that protein-DNA interactions between the transcription complex are disrupted during elongation, but protein-protein interactions between transcription factors keep the complex intact (White, 2002).

1.4.6 Transcription termination and reinitiation

Pol III requires a variety of accessory factors for accurate initiation of transcription, but can terminate transcription accurately and efficiently in the absence of accessory factors (Cozzarelli, et al, 1983; Hammond and Holland, 1983; Watson et al, 1984. The termination signals on class III genes are simple clusters of four or more T residues that are recognised by the polymerase and are sufficient for the accurate cessation of transcription by pol III (Bogenhagen and Brown, 1981).

The formation of a transcription complex and the initiation of transcription is a relatively slow process (Dieci and Sentenac, 1996). This slow step is avoided because pol III can be recycled without being released from the template, leading to a subsequent round of transcription being five to ten-fold faster than that of the initial transcript (Dieci and Sentenac, 1996). The bends in the DNA duplex caused by the binding of the pol III transcription factors may facilitate the reinitiation of transcription by bringing the start site into close spatial orientation with the end of the gene, thus when transcription complexes are assembled they are capable of multiple rounds of transcription (White, 2002).

1.5 Regulation of Pol III Transcription

1.5.1 Activators of pol III transcription

It has been previously demonstrated that serum stimulation of resting culture cells can activate pol III transcription (Johnston et al, 1974; Mauck et al, 1974). Mitogenic stimulation of quiescent cells results in the activation of several signalling cascades that can directly activate pol III transcription.

One example is the protein kinase, CK2. CK2 is a highly expressed and constitutively active Ser/Thr protein kinase with a large number of substrates and has been implicated in neoplasia and cell survival (Meggio and Pinna, 2003). The activity and/or levels of CK2 are elevated in response to mitogenic stimulation, and associated with both cell growth and proliferation (Meggio and Pinna, 2003). CK2 can bind directly to and phosphorylate the pol III transcription factor TFIIIB in both yeast and mammalian cells (Hockman and Schultz, 1996; Johnston et al, 2002). In mammalian cells this is postulated to increase the affinity between TFIIIB and TFIIIC2, thus facilitating the formation of the transcription complex (Johnston et al, 2002).

Another cellular signalling pathway stimulated upon addition of growth factors is the mitogen activated protein kinase (MAPK) cascade. Induction of this pathway leads to activation of the extracellular signal-related kinase (ERK), through a signalling cascade that involved a receptor tyrosine kinase, the GTPase Ras, the kinase Raf and the ERK kinase MEK (Downward, 2002). This pathway can promote cell growth in several ways including increasing translational capacity and initiating transcription by

pol I (Whitmarch and Davies, 2000; Stefanovsky, 2001). The MAPK kinase cascade can also stimulate transcription of the 5S rRNA and tRNA genes through ERK binding and phosphorylation of TFIIIB (Felton-Edkins et al, 2003a). Phosphorylation of TFIIIB enhances its ability to interact with TFIIIC and pol III, and promotes the formation of the transcription complex to directly stimulate pol III transcription (Felton-Edkins et al, 2003).

1.5.2 Repressors of pol III transcription

The product of the retinoblastoma susceptibility gene is an important tumour suppressor protein that prevents cell proliferation and growth in the absence of appropriate mitogenic signals (Kaelin, 1999; Weinberg, 1995). The Rb gene encodes a 110 kDa phosho-protein that can regulate the activity of several key transcription factors involved in the transcription of genes critical to cell growth and cell cycle progression (White, 2005). Transcription of pol III-transcribed genes can be efficiently repressed by addition of RB in vitro, or transfection of vectors encoding RB into cells (White et al, 1996; Chu et al, 1997; Hirsch et al , 2000). Conversely, in a RB-knockout cell line, transcription of poll III-transcribed genes is markedly increased (White et al, 1996). In resting cells underphosphorylated RB can inhibit pol III transcription by binding to and sequestering TFIIIB in the nucleoplasm in the G_0 and G_1 stages of the cell cycle (Scott et al, 2001). RB is inactivated by phosphorylation in the G_1-S transition by cyclin-dependent kinases and this inactivation of RB correlates well with an increase in pol III activity, suggesting an important role for RB in the regulation of pol III activity (Brown et al, 1998; Scott et al, 2001).

The tumour suppressor protein p53 is another inhibitor of pol III transcription. p53 is induced in response to a wide array of cellular insults, including hypoxia, ribonucleotide depletion and exposure to genotoxins, which leads to a complex program of events leading to either growth arrest or programmed cell death (Gomez-Lazaro et al, 2004). p53 regulates the transcription of a number of cellular targets including genes transcribed by pol III (Chesnokov et al, 1996; Cairns and White, 1998). TFIIIB, the target of the RB tumour suppressor, is also bound and repressed by p53 (Chesnokov et al, 1996, Cairns and White, 1998; Crighton et al, 2003). The binding of p53 to TFIIIB inhibits the formation of the transcription complex in vivo by blocking the interaction between TFIIIB and TFIIIC (Crighton et al, 2003). This is highlighted by the increased levels of TFIIIB and pol III present on the promoters of pol III-transcribed genes, coupled with the higher levels of pol III-transcribed products, in p53 knockout cells (Crighton et al, 2003).

Another negative regulator of pol III transcription is the Maf1 protein. Studies in yeast have identified that Maf1 can interact with both TFIIIB and pol III (Pluta et al, 2001; Upadhya et al, 2002). This protein is involved in the repression of pol III transcription in response to a variety of stresses including nutrient deprivation and DNA damage (Upadhya et al, 2002). Recent data have suggested that not only does Maf1 repress pol III transcription in yeast, but is involved in the regulation of 5S rRNA and tRNA genes in mammalian cells (Graham and White, unpublished observations).

1.6 Activation of pol III transcription by c-Myc

Pol III transcription is also directly and potently activated by the product of the c-Myc proto-oncogene (Gomez-Roman et al, 2003). The broad aim of this thesis is to develop an understanding of the mechanisms behind this activation to further expand our knowledge of the control and regulation of transcription by pol III

1.6.1 The c-Myc transcription factor

The c-Myc proto-oncogene has been the subject of intense study over the past two decades. The interest in this gene comes from the fact that c-Myc is involved in a variety of essential processes and that its deregulation often leads to tumour formation. The most striking example of this is observed in Burkitt's lymphoma. In Burkitt's, a chromosomal translocation results in the c-Myc gene being juxtaposed with Ig loci, leading to constitutive activation of the c-Myc gene (Nesbit et al, 1999). c-Myc deregulation has been observed in many tumour cells, and is often associated with poorly differentiated aggressive cancers. Examples include breast, colon, cervical, small cell lung carcinomas, glioblastomas, melanoma, myeloid and lymphoblastic leukemias and lymphomas (Dang et al, 1999, Nesbit et al, 1999). The mechanisms behind c-Myc activation and the nature of its target genes is becoming clearer and will be discussed in this chapter.

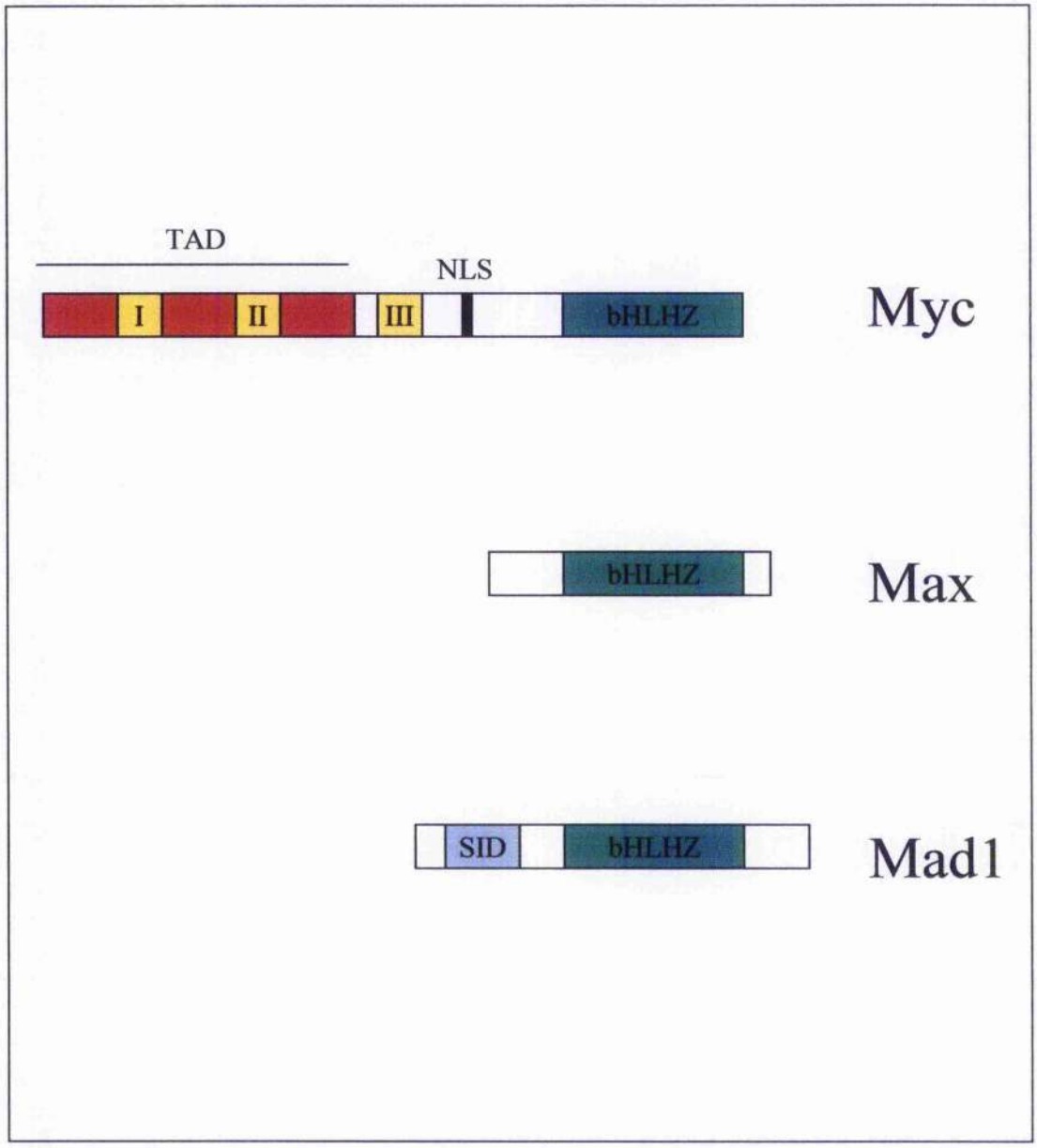
1.6.2 c-Myc structure and the Myc/Max/Mad Network

Myc was originally identified as the viral oncogene (v-myc) of the avian myelocytomatosis retrovirus, and was only the second oncogene identified (Sheiness et al, 1978). A cellular homologue of Myc (c-Myc) was identified in chickens through homology with its viral counterpart (Vennstrom et al, 1982). There is, in fact, a family of Myc proteins which have been identified in the mammalian genome, c-Myc, N-Myc, L-Myc and MycS (Grandori et al, 2000).

The product of the c-Myc proto-oncogene is a transcription factor, containing a transcription activation domain at its amino-terminus, and a carboxy-terminal region with homology to the bHLHZ family of transcription factors (Figure 1.6; Kato et al, 1990; Murre et al, 1989). The Myc family of proteins cannot form homodimers, except at high protein concentration in vitro, instead heterodimers are formed with another bHLHZ protein Max (Figure 1.6; Blackwood and Eisenman, 1991; Prendergast et al, 1991). Myc/Max heterodimerise to form a sequence-specific DNA binding complex. Myc/Max heterodimers recognise the enhancer-box (E-box) sequence CACGTG (as well as related non-canonical sites) and activate transcription from synthetic reporter genes containing this element within their promoters (Blackwood and Eisenman, 1991; Amati et al, 1992; Krezner et al, 1992). In contrast to Myc, Max proteins can homodimerise and recognise E-box sequences, but Max dimers appear to be transcriptionally inert (Amati et al, 1992; Krezner et al, 1992)

Figure 1.6 The Myc/Max/Mad network

Schematics of the Myc, Max and Mad proteins are displayed below. The known functional domains are indicated. bHLHZ: basic helix-loop-helix leucine zipper domain; TAD: transactivation domain; MBI: Myc Box I; MBII: Myc Box II; MBIII: Myc Box III; NLS: nuclear localisation signal; SID: Sin3 interaction domain.


TAD
NLS
I
II
III
bHLHZ
Myc
bHLHZ
Max
SID
bHLHZ
Mad1

Max is expressed in the absence of Myc; this led to the search for other Max interacting proteins. The Mad family of transcription factors represent four bHLHZ containing proteins, Mads 1-4, that can specifically bind with Max (Figure 1.6; Ayer et al, 1993; Zervos et al, 1993; Hurlin et al, 1995). Mad proteins are similar to the Myc family members as they homodimerise poorly, but form heterodimers with Max in vivo (Ayer et al, 1993; Zervos et al, 1993; Hurlin et al, 1995). Mad/Max dimers bind to the same E-box sequences that Myc/Max dimers recognise (Ayer et al, 1993; Zervos et al, 1993; Hurlin et al, 1995). However, in contrast to Myc, transcription assays demonstrate that Mad binding represses E-box dependent activation of a reporter gene (Ayer et al, 1993; Hurlin et al, 1995).

In general Max-interacting proteins are unstable and their expression tightly regulated (Grandori et al, 2000). Max itself is relatively stable and is constitutively expressed; control of the Myc/Max/Mad network is thought to be mediated by changes in the abundance of Max-interacting factors (Blackwood et al, 1992).

1.6.3 c-Myc Function

Work in this thesis will concentrate on c-Myc, the first member of the Myc family of transcription factors identified in cells. c-Myc has been demonstrated to alter the expression, both positively and negatively, of a wide variety of genes involved in many cellular processes (Grandori et al, 2000). As previously mentioned, c-Myc is a member of the bHLHZ family of transcription factors. The bHLHZ domain is present at the carboxy-terminal of the c-Myc protein and is essential for c-Myc function and binding to Max (Grandori et al, 2000). The amino terminal 143 amino acids of c-Myc can act as a transactivation domain (TAD) when fused to the DNA binding domain of

the yeast Gal4 protein (Kato et al, 1990). The amino terminus contains three highly conserved elements, referred to as Myc boxes (figure 1.6). Of these conserved element Myc box I (MBI) contains phosphorylation sites involved in targeting Myc's proteolytic destruction, and mutations in this region are observed in lymphomas (Dang et al, 1999; Bahram et al, 2000). The deletion of MBI results in reduced transformation activity of Myc in vivo (Pelangaris and Khan, 2003) The Myc Box II region (MBII) is dispensable to activate reporter genes in vitro, but it absolutely required for Myc-mediated transformation of cell in vivo (Stone et al, 1987). This activity of MBII in vivo could be attributed to the transactivation of target genes in a chromatin context requiring co-activator proteins that require the integrity of this region for binding (Eisenman, 2001). The final Myc Box, Myc Box III (MBIII), is also involved in the regulation of protein stability and is absolutely required for cellular transformation and full activation or repression of target genes (Herbst et al, 2004; Herbst et al, 2005).

c-Myc is broadly expressed during embryogenesis and in tissue that has a high rate of proliferation (Lüscher, 2001; Eisenman, 2001; Pelengaris and Khan, 2003). c-Myc expression correlates well with a high rate of proliferation, and in cell culture c-Myc expression is induced in response to a wide range of mitogenic stimuli. Other functions of the c-Myc protein include inhibition of terminal differentiation and induction of apoptosis (Lüscher, 2001; Eisenman, 2001; Pelengaris and Khan, 2003).

Targeted homozygous deletion of the c-Myc gene leads to embryonic lethality before 10.5 days gestation in mouse models, suggesting a critical role in development (Davis et al, 1993). Inactivation of the c-Myc gene in rat fibroblasts causes a prolonged cell

doubling time, indication a role for c-Myc in cell growth and proliferation (Mateyak et al, 1997).

Bona fide targets of the c-Myc transcription factor have been difficult to identify, due to relatively weak and variable transcriptional activation of synthetic targets or putative target genes (Grandori and Eisenman, 1997).

1.6.4 Linking c-Myc Target Genes to c-Myc Function

Much of the research in the c-Myc field has focussed on the influence of Myc proteins on the cell cycle. The deregulated expression of Myc has been shown to allow cells to enter S-phase and undergo mitosis in the absence of external growth factors, which is also a feature of transformed and tumour cell types (Eilers et al, 1991; Hannahan and Weinberg, 2000). Several studies have provided insights into how c-Myc promotes cell proliferation by identifying genes either activated or repressed by c-Myc that are involved in cell cycle progression. The G1 to S progression of the cell cycle is controlled by the activity of the cyclin-dependent kinase (CDK) complexes (Kohn, 1999). Components of the cyclin/CDK complexes are direct targets of the Myc/Max/Mad network including activation of the Cyclin D2 and CDK4 genes to promote cell cycle progression, as well as repression of cyclin-dependent kinase inhibitors $p15^{INK4b}$ and p21 (Bouchard et al, 1999; Hermeking et al, 2000; Herold et al, 2002; Feng et al, 2002).

The inability to find target genes that can substitute for c-Myc function, in the way that has been achieved for other transcription factors, has made it difficult to link c-

Myc action to specific pathways involved in cell cycle progression and transformation (Grandori et al, 2000; Patel et al, 2004). The difficulty in doing this may result from the broad range of target genes that c-Myc can affect. Indeed a large scale screen in Drosophilia identified that members of the Myc/Max/Mad network are associated with a large number of loci examined, this approximated to 15% of the class II genes examined (Orian et al, 2003).

1.6.5 c-Myc and Cell Growth

Much effort has gone into defining the role of c-Myc in cellular proliferation, but it is becoming clear that c-Myc has a major role in cell growth. Activation of Myc drives a rapid increase in translation and growth, which precedes cell proliferation (Iritani and Eisenman, 1999; Kim et al, 2000; Schuhmacher et al, 2001). In certain conditions c-Myc can lead to growth in the absence of proliferation (Beier et al, 2000). As well as the described role in the activation of pol III transcription, c-Myc has been shown to control many genes which can ultimately affect cell mass. These include the gene products of eIF4E and eIF2α, which are rate-limiting translation initiation factors (Rosenwald et al, 1993; Jones et al, 1996). When examining fibroblasts in which c-Myc has been deleted lower rates of both protein synthesis and ribosome biogenesis are observed (Mateyak et al, 1997). Myc can activate genes encoding protein components of the ribosome, as well as directly activating the transcription of pol I specific transcription factors (Kim et al, 2000; Boon et al, 2001; Poortiga et al, 2004). There is also a direct effect on pol I transcription with Myc potently and directly activating the transcription of the pre-rRNA, and it is present on the rDNA in vivo (Grandori et al, 2005; Arabi et al, 2005) The finding that transcripts synthesised by

pol III, such as the tRNA and 5S rRNA, are induced by c-Myc further indicates a major role for c-Myc in cell growth (Gomez-Roman et al, 2003, Felton-Edkins et al, 2003b).

1.7 Pol III trancription and cancer

1.7.1 Derepression of pol III transcription

Precise regulation of transcription by pol III is required for the normal growth and proliferation of cells. Transformed and tumour cells lose this tight regulation. Early evidence for such deregulation came from the finding that pol III is hyperactive in mice with myelomas, whereas the overall activity of pol II remains normal (Schwartz et al, 1974). Subsequent studies have revealed that a wide variety of transformed and tumour cell types have elevated levels of pol III transcription (White, 2004; White, 2005). The tumour suppressors p53 and RB, and their respective pathways, are found to be deregulated in most, if not all human cancers (Hollstein et al, 1991; Weinberg, 1995). The deregulation of these important proteins could contribute to aberrant activation of pol III transcription in tumour and transformed cell lines (White, 2004; White, 2005).

1.7.2 Activation of pol III transcription

For tumours to progress they need to have a raised cellular metabolism, and essential to this is an increase in protein biosynthesis. The oncoprotein Myc can not only drive proliferation by targeting pol II-transcribed genes involved in the cell cycle, but can also increase the biosynthetic capacity of cells by activation of transcription by pol I and pol III to promote cell growth (Dang et al, 1999; White, 2005). c-Myc is

deregulated in a wide range of cancers and this may contribute to the activation of pol III transcription in transformed and tumour cell types (Felton-Edkins et al, 2003b; White, 2004; White, 2005). Activators of pol III transcription, such as CK2 and ERK, have been found to be activated in several tumour models and the activation of these kinases could contribute to elevated levels of pol III transcription (White, 2004).

1.7.3 Overexpression of pol III transcription factors

The most obvious way to increase pol III output is by raising the level of one or more of the limiting transcription factors on which it depends. Levels of all five TFIIIC2 subunits are overexpressed at both the mRNA and protein levels in fibroblasts transformed by SV40 or polyomavirus (Felton-Edkins and White, 2002; Larminnie et al, 1999). These observations proved to be of clinical significance when elevated TFIIIC2 activity was found in each of nine human ovarian carcinomas relative to healthy adjacent tissue (Winter et al, 2000). The Brf1 and Bdp1 subunits of TFIIIB are also found to be overexpressed in a subset of cervical carcinomas (Daly et al, 2005). As these factors are dedicated exclusively to pol III transcription, these observation indicate a specific drive to elevate pol III transcription in cancer cells.

1.8 Aims of this Thesis

The work in this thesis aims to uncover the mechanisms by which c-Myc can activate transcription by pol III. This will include determining the precise interactions between c-Myc and the pol III transcription apparatus and how this acts to promote the activation of pol III-transcribed genes. c-Myc co-activator proteins are also necessary for the activation of certain target genes and for the ability of Myc proteins to transform cells. This thesis will address if there is a role for any co-activator proteins recruited by Myc in the activation of pol III transcription.

Only with a clear understanding of what genes c-Myc can act on, and what co-activators are involved in the process, can we start to realize and comprehend c-Myc's role in cancer and perhaps work to utilise it as a therapeutic target.

Chapter 2 - Materials and Methods

2.1 Plasmid Preparation

2.1.1 Transformation of competent cells

For plasmid propagation, E. coli XL-1 blue competent cells were transformed (Stratagene). Cells were stored at -80°C and were thawed on ice before transformation. 10-20ng of plasmid DNA was added to 50µl of thawed cells and mixed by gentle agitation. The cells were then incubated on ice for 20 minutes. Following this, the cells were heat-shocked at 42°C for 30 seconds. The cells were then recovered by adding 500 µl of SOC medium (LB Broth, 0.05% glucose, 10mM $MgSO_4$, 10mM $MgCl_2$), which had been pre-heated to 37°C, and then incubated at 37°C for 30 minutes. 100µl of the cells was then plated onto LB-agar plates (LB-broth, 2% agar, 100 µg/ml ampicillin) and then incubated at 37°C overnight.

2.1.2 Isolation and storage of plasmid DNA

A single colony was selected from the LB-agar plates and was used to inoculate 10 ml of LB-broth containing 100 µg/ml ampicillin. The culture was incubated at 37°C in an orbital shaker for 8 hours. The mini culture was then transferred into 500ml of LB-broth containing 100 µg/ml ampicillin, and incubated at 37°C in an orbital shaker

overnight. The following day the bacterial cells were harvested by centrifugation at 5100 rpm for 20 minutes at 4°C (in Sigma Laboratory Centrifuge 4K15). The plasmid DNA was isolated from the bacterial cells using the Qiagen Maxi-Prep Kit, according to the manufacturer's instructions.

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 mixed with 10 ml of buffer P2 (200 mM NaOH, 1% SDS) to lyse the cells. This reaction was allowed to proceed for 5 minutes at room temperature before neutralisation with 10 ml of buffer P3 (3 M potassium acetate, pH5.5), which results in the formation of a precipitate of potassium dodecyl sulphate. The bacterial proteins and chromosomal DNA were co-precipitated with the detergent whilst the plasmid DNA remained in solution due to a lack of close protein interactions. Precipitation was enhanced by a 20 minute incubation on ice, before being centrifuged at 20,000g for 30 minutes at 4°C (in Sigma Laboratory Centrifuge 4K15). The supernatant was then added to a Qiagen-tip 500, which had previously been equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100). The Qiagen-tip 500 contains an anion-exchange resin to which the plasmid binds tightly, allowing the supernatant to pass through. The resin was then washed twice with 30 ml of buffer QC (1M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol) and the 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 of room temperature isopropanol. This was immediately centrifuged at 15,000g at 4°C for 30 minutes. The plasmid DNA pellet was then washed with 70% ethanol, dried at room temperature for 10 minutes and resuspended in an appropriate volume of sterile dH_2O.

Plasmids were stored at -20°C. The concentration of the plasmid DNA was determined spectrophotometrically using a quartz cuvette, using the equation 1 A_{260} = 50 µg/µl dsDNA.

Table 2.1 Description of plasmids

Plasmid	Description	Origin
pGex2T-N262	Encoding GST fusion protein containing the amino acids 1-262 of the c-Myc protein	Gift from Bob Eisenman (Gomez-Roman et al, 2003)
PGex2T RB (379-928)	Encoding GST fusion containing the amino acids 379-928 of the RB protein	Gift from Dennis McCance (Kaelin et al, 1991)
pGex2T	Encoding GST used as a control	Gift from Bob Eisenman (Gomez-Roman et al, 2003)
pcDNA3 HA Brf1	Encoding HA tagged wild-type Brf1 protein	Generated in house
pcDNA3 HA Brf1 (S250A)	Encoding HA tagged Brf1 protein with a serine>alanine mutation at residue 250	Generated in house
pcDNA3 HA Brf1 (L>A)	Encoding HA tagged Brf1 protein with leucine>alanine mutations introduced at positions 100 and 102	Generated in house
pcDNA3 FLAG Myc	Encoding FLAG tagged wild-type c-Myc protein	Gift from Lars-Gunner Larsson (von der Lehr et al, 2003)
pcDNA3 FLAG Myc ΔMBI	Encoding FLAG tagged c-Myc protein with amino acids 43-65 deleted	Gift from Lars-Gunner Larsson (von der Lehr et al, 2003)
pcDNA3 FLAG Myc ΔMBII	Encoding FLAG tagged c-Myc protein with amino acids 128-143 deleted	Gift from Lars-Gunner Larsson (von der Lehr et al, 2003)
pSuper TRRAP si	Encoding shRNAs against the TRRAP transcript	CRUK shRNA library
pSuper MyoD si	Encoding shRNAs against the MyoD transcript	CRUK shRNA library

2.2 GST pull-down assays

2.2.1 Preparation of recombinant GST fusion proteins

For plasmid propagation, E. coli BL21 Rosetta competent cells were transformed (Invitrogen). Cells were stored at -80°C and were thawed on ice before transformation. 10-20ng of plasmid DNA was added to 50µl of thawed cells and mixed by gentle agitation. The cells were then incubated on ice for 20 minutes. Following this the cells were heat-shocked at 42°C for 30 seconds. The cells were then recovered by adding 500 µl of SOC medium (LB Broth, 0.05% glucose, 10mM $MgSO_4$, 10mM $MgCl_2$), which had been pre-heated to 37°C, and then incubated at 37°C for 30 minutes. 100µl of the cells was then plated onto LB-agar plates (LB-broth, 2% agar, 100 µg/ml ampicillin) and then incubated at 37°C overnight. A single colony of transformed bacteria was grown overnight in 10ml of LB broth with 100µg/ml at 37°C in an orbital shaker. The 10ml of saturated culture was used to inoculate 500ml of LB Amp, and this culture was grown at 37°C in an orbital shaker until the culture reached an OD_{595} of 0.6-1.0. 1ml of sample was then removed, pelleted by centrifugation, for 30s at 13,000g, and resuspended in 50µl 2x SDS sample buffer (150 mM Tris-HCl pH 6.8, 1% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.5% bromophenol blue), as an uninduced control. The large culture was then induced with 0.1mM final concentration of IPTG and grown for 4 hours at 37°C in an orbital shaker. A 0.5ml sample of the induced culture was removed, pelleted by centrifugation for 30s at 13,000g, and resuspended in 50µl 2x SDS sample buffer. The remaining culture was harvested by centrifugation, 5100rpm for 10minutes at

4°C, and snap frozen on dry ice, the pellet was then stored at -80°C. The uninduced and the induced sample were resolved by SDS-PAGE, and the gel stained with coomassie brilliant blue stain, to check for induction of the protein. Once induction was confirmed, the cells were resuspended in 20ml of lysis buffer, and sonicated 2x for 30 seconds on ice. The crude lysate was then centrifuged at 10,000g for 10min at 4°C. The clarified extract was then aliquoted and snap frozen in a dry ice methanol bath.

2.2.2 In vitro transcription and translation of proteins in reticulocyte lysate

In vitro transcription and translation of proteins was performed using the Novagen single tube protein in vitro transcription and translation kit. Reactions were carried out using 1 µg of plasmid DNA or 2 µl of PCR product. PCR reactions were carried out using reagents described in section 2.7.3, without radio-labelled dCTP, using 0.25 µg of plasmid template (pcDNA3-HA-Brf1) in a total reaction volume of 50 µl (PCR conditions: denaturing - 95°C 5 min; cycling [x35] - 95°C 1min, 55°C 1min, 72°C 2 min; final extension 72°C 5 min). The template DNA was made up to 2 µl and added to 8 µl of transcription mixture and incubated at 30°C for 30 minutes. To the transcription mixture, 30 µl of translation mixture, 40 µCi ^{35}S Methionine and 6 µl dH_2O, was added. The mixture was then incubated at 30°C for 1 hour.

Table 2.2 Primers used to produce templates for in vitro transcription and translation of fragments of Brf1

Region of Brf1	Forward Primer	Reverse Primer
1-280	5' TAATACGACTCACTATA GGGAGAGCCACCATG 3'	5'- GCTCGTCTCGAGCTAGAA CTCATCAATGGTCAAC-3'
281-677	5' TAATACGACTCACTATA GGGAGAGCCACCATGAAG ATCGACGTGGAG 3'	5'-TTGCAACTCGAGTCAGTAG CCGTCGTCCTCATC-3'
1-94	5' TAATACGACTCACTATA GGGAGAGCCACCATG 3'	3'-CGTCGTCTCGAGCTAGAACT CATCAATGGTCAAC-5'
94-280	5' TAATACGACTCACTATA GGGAGAGCCACCATGCAC CACCTGGGGAACCAG 3'	5'- GCTCGTCTCGAGCTAGAACT CATCAATGGTCAAC-3'
94-200	5' TAATACGACTCACTATA GGGAGAGCCACCATGCAC CACCTGGGGAACCAG 3'	5'-TCTCCCTCGAGTCACAGGCAC GGGTCTATGGC-3'
200-281	5' TAATACGACTCACTATA GGGAGAGCCACCATGGCC ATAGACCCGTG3'	5'- GCTCGTCTCGAGCTAGAACT CATCAATGGTCAAC-3'

(T7 promoter underlined)

2.2.3 GST pull down assay

GST or GST fusion proteins were coupled to glutathione agarose beads (Sigma). 20 µl of packed volume of glutathione agarose beads were incubated with the GST or GST fusion protein containing clarified extracts for 1 hour at 4°C on a rotating wheel. During this incubation period, 10µl of reticulocyte lysate containing the in vitro translated protein was incubated with 20 µl packed volume of pre-washed glutathione agarose beads in a total volume of 600 µl made up with 1 x TBS, to pre-clear the in vitro translated protein. The beads bound to the recombinant GST proteins were washed three times to remove non-specific binding of bacterial proteins and to these beads 250 µl of pre-cleared reticulocyte lysate was added to each condition. The binding reaction was allowed to proceed for 30 minutes at 4°C on a rotating wheel. The beads were then washed three times with 500 µl of 1 x TBS to remove non-specific binding to the immobilised recombinant proteins before the bound material was released by the addition of an equal volume of 2 x protein sample buffer. Samples were then resolved by SDS-PAGE and the recombinant proteins were then visualised by coomassie staining (0.5% coomassie brilliant blue, 30% methanol, 10% acetic acid) and subsequent destaining (30% methanol, 10% acetic acid) to ensure equal loading of samples. The gels were then incubated with autofluor (National Diagnostics), to enhance the radio-labelled signal. The gels were then dried under a vacuum and exposed to autoradiographic film overnight at -80°C. Binding was quantified using the ImageJ programme (National Institute for Health, USA).

2.3 Cell Culture

Cell culture was performed in a class II hood using standard aseptic technique and sterile reagents and conditions.

2.3.1 HeLa cells and HEK 293 cells

HeLa and HEK 293 cells were maintained in DMEM (Cambrex) supplemented with 10% foetal bovine serum (Sigma), 2mM L-Glutamine (Sigma), 50 units/ml penicillin (Sigma) and 50µg/ml streptomycin (Sigma). Cells were passaged when 80-90% confluent (3-4 days between passages) using buffered trypsin (Sigma).

2.3.2 MycER and pBabe cells

MycER and pBabe cells were maintained in DMEM supplemented with 10% foetal bovine serum, 2mM L- Gluatmine, 50 units/ml penicillin, 50µg/ml streptomycin and 5µg/ml puromycin (Sigma). Cells were passaged when 80-90% confluent using buffered trypsin. To activate the MycER protein, MycER cells were grown to confluency, then serum starved (0.25% Serum) for 48 hours to lower levels of endogenous c-Myc, before addition of the oestrogen homologue, 4-hydroxytamoxifen (4-OHT). As a control for the addition of 4-OHT, pBabe cells were treated in exactly the same way.

2.3.3 Rat 1A wildtype and Rat 1A c-myc null cells

Rat 1A cells were maintained in DMEM supplemented with 10% foetal bovine serum, 2mM L-Glutamine, 50 units/ml penicillin and 50µg/ml streptomycin. Rat 1A c-Myc$^{-/-}$ cells were maintained in DMEM supplemented with 10% foetal bovine serum, 2mM

L-Glutamine, 50 units/ml penicillin, 50µg/ml streptomycin and 300 µg/ml G418 (Promega) (Matek et al, 1997).

2.3.4 Mouse erythroleukaemia (MEL) 585 cells

MEL 585 cells were maintained in RPMI 1640 (Cambrex) supplemented with 15 % foetal bovine serum, 2mM L-Glutamine, 50 units/ml penicillin and 50µg/ml streptomycin. To differentiate MEL cells N,N'-Hexamethylene-bisacetamide (HMBA) (Sigma) was added to the media to a final concentration of 5mM.

2.3.5 Cryo-storage of cells

Live cells were subjected to long-term storage by resuspending near subconfluent 75cm^2 flasks in 2ml of maintenance media containing 10% DMSO. This suspension was aliquoted into cryotubes and placed at -80°C overnight to ensure slow freezing, before being transferred to liquid nitrogen for long-term storage.

Cells were recovered by rapid thawing at 37°C and were then added to 9 ml of pre-warmed media. The resultant mixture was then centrifuged at 298g for 5 minutes at room temperature. After centrifugation, the supernatant was removed and the cells resuspended in 12 ml of fresh media before being transferred to a 75 cm^2 flask.

2.4 Preparation of Extracts

2.4.1 Preparation of extracts for western blots and co-immunoprecipitations

Cultured cells were placed on ice and the maintenance media aspirated. The cells were then washed twice in PBS. Cells were then scraped into PBS (2ml per 10cm dish: 0.5ml per 2cm well) and transferred to 50ml Falcon tubes. Cells were centrifuged at 500g for 5min at 4°C, and the PBS discarded. Cells were resuspended in approx 1ml of PBS and transferred to a microfuge tube. The tube was then centrifuged at 13,000g for 30 sec, and the PBS discarded. The cell pellet was then resuspended in freshly made microextraction buffer (450 mM NaCl, 50mM NaF, 20 mM HEPES pH 7.8, 25% glycerol, 1 mM DTT, 0.5mM phenylmethylsulphonyl fluoride (PMSF), 0.2 mM EDTA, 40µg/ml bestatin), the volume used being equivalent to the volume of the pellet. The resuspended sample was then snap frozen on dry ice, and immediately thawed in a 30°C water bath. This freeze thaw cycle was repeated three times to lyse the cells. Following the final thaw the tubes were then centrifuged at 13,000g at 4°C for 10 min to separate the cell debris. The supernatant was quickly removed and snap frozen on dry ice. Microextracts were stored at –80°C.

2.4.2 Preparation of nuclear-enriched extracts

Two 10cm dishes of cultured cells were placed on ice and the maintenance media aspirated. The cells were then washed twice in PBS. Cells were then scraped into

PBS (2ml per 10cm dish) and transferred to 15ml Falcon tubes. Cells were centrifuged at 500g for 5min at 4°C, and the PBS discarded. The pellet was resuspended in 2ml of PBS and split into 2 1.5ml microfuge tubes. The tubes were centrifuged for 5 sec, 13,000g at 4°C. The supernatant was removed and the cells in each tube were resuspended in 1ml of Buffer A (10mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.2mM PMSF). The tubes were centrifuged for 5 sec, 13,000rg at 4°C. The supernatant was removed and the cells in each tube were resuspended in 1ml of Buffer A. The cells were placed on ice for 15 minutes to swell and lyse the cells. The tubes were vortexed for 30 seconds and then centrifuged for 5 sec, 13,000g at 4°C. The supernatant was then discarded and each cell pellet was resuspended in an equal volume of buffer C (10mM HEPES pH 7.9, 25% (v/v) glycerol, 0.45M NaCl, 1.5mM $MgCl_2$, 0.125mM EDTA, 0.5mM DTT, 0.2mM PMSF). The cells were incubated on ice for 15 minutes. The tubes were centrifuged for 5 minutes, 13,000g at 4°C. The supernatant was removed, aliquoted and snap frozen on dry ice. The nuclear-enriched extracts were stored at -80°C.

2.4.3 Determination of protein concentrations

Protein concentrations were determined using Bradford's reagent (BioRad) diluted 1 in 5 with distilled H_2O. The colour change produced in this reagent in response to being mixed with protein can be quantified by absorbance at 595 nm, and these values are directly proportional to the concentration of protein in the sample. For each experiment, a standard curve was constructed by measuring absorbance of 0,2,4,6,8,10 and 12 μg of BSA in 1ml of Bradford's reagent. 1μl of each microextract was added to 1ml of reagent. Absorbance readings at 595nm were performed in

duplicate, and the protein concentration of each sample was determined from the standard curve.

2.5 Separation of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

2.5.1 SDS-PAGE

Protein extracts, as prepared in section 2.4, were resolved by denaturing SDS-PAGE on 10% (unless otherwise indicated) polyacrylamide gels (375mM Tris pH 8.8, 0.1% SDS), with 4% polyacrylamide stacking gels (125mM Tris pH 6.8, 0.1% SDS). Prior to loading, 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). Electrophoresis was performed in SDS running buffer (0.1% SDS, 76.8 mM glycine, 10mM Tris pH 8.3) at 200V. Electrophoresis was continued for approximately 60 minutes, until the bromophenol dye had moved to the bottom of the gel.

2.5.2 Western Blot Analysis

Followed SDS-PAGE, proteins were transferred to a nitrocellulose membrane (BioRad) using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system. Transfer was carried out in 1 X transfer buffer (76.8 mM glycine, 10mM Tris pH 8.3, 20% methanol) at 50V, overnight at 4°C. Membranes were then stained using 1 x Ponceau S to ensure efficient transfer of the protein to the membrane, and subsequently washed with PBS. Membranes were then blocked in milk buffer (32.5 mM Tris, 150 mM NaCl, 0.2% Tween-20, 5% skimmed milk powder (Marvel)) for 2 hours at room temperature. The membranes were then incubated in the presence of

the appropriate primary antibody diluted in milk buffer for 2 hours at room temperature. Primary antibodies and their appropriate concentrations are listed in table 2.3. Membranes were washed three times in milk buffer to remove excess primary antibody. Membranes were then incubated in the presence of the appropriate secondary antibody (Dako) at a dilution of 1:1000 in milk buffer. Subsequently, membranes were washed five times with western wash buffer (32.5 mM Tris, 150 mM NaCl, 0.2% Tween-20), to remove excess secondary antibody. The bound antibodies were then detected using enhanced chemiluminescence (ECL), as directed by the manufacturer (Amersham).

Table 2.3 Antibodies used in western blot analysis

Protein	Antibody	Type	Dilution	Source
Brf1	128	Serum	1:1000	In house
Bdp1	2663	Serum	1:1000	In house
TBP	SI-1	Polyclonal	1:1000	Santa Cruz
TFIIIC 110	4286	Serum	1:1000	In house
TFIIIC 220	Ab 7	Serum	1:1000	In house
RPC 155	1900	Serum	1:1000	In house
c-Myc	N-262	Polyclonal	1:500	Santa Cruz
Max	C-17	Polyclonal	1:1000	Santa Cruz
TRRAP	H-300	Polyclonal	1:500	Santa Cruz
HA	F-7	Monoclonal	1:2500	Santa Cruz
FLAG	M2	Monoclonal	1:2500	Sigma
Actin	C-11	Polyclonal	1:5000	Santa Cruz
SP-1	1C6	Monoclonal	1:1000	Santa Cruz

2.6 Co-immunoprecipitation

2.6.1 Transfection of HEK293 cells using Lipofectamine

HEK 293 cells were plated into 10 cm dishes and were transfected at a confluency of approximately 70-80%. One 10 cm dish was transfected per condition with a total of 4 μg of plasmid DNA per dish; this was comprised of 1μg of pcDNA3-Brf1 and 3 μg of a Myc expression vector (described in table 2.1). The appropriate mix of plasmid DNA for each condition was mixed with 0.5 ml of OptiMEM media (Gibco). In a separate tube, 20μl of Lipofectamine (Invitrogen) was mixed with 0.5ml of OptiMEM. This Lipofectamine mix was added to the tube containing the plasmid DNA and mixed by pipetting. The DNA/Lipofectamine complexes were allowed to form by incubation at room temperature for 45 minutes in the dark. During this incubation, the cells were washed with 5 ml of OptiMEM, and then 4 ml of OptiMEM added per plate. The plates were then incubated at 37°C for the remainder of the incubation time (approximately 30 minutes). After the incubation, 1ml of the Lipofectamine-DNA-OptiMEM mix was added to each dish and the plates left to incubate for 5 hours at 37°C in the incubator. After the incubation period, the Lipofectamine-DNA-OptiMEM mix was removed and replaced with 10 ml of fresh maintenance media. Cells were incubated for a further 48 hours to allow expression of the transfected DNA. Cells were harvested and protein extracts were prepared as described in section 2.4.2.

2.6.2 Co-immunoprecipitation

Anti-FLAG (Sigma) antibody was coupled to protein-G sepharose beads. 25 µl of packed beads was used per immunoprecipitation (IP); these beads were washed twice with 200 µl 1 x TBS, prior to incubation with 5 µl anti-FLAG antibody, made up to a total volume of 50 µl with TBS on a shaker for 1 hour at 4°C. Following antibody binding, beads were washed twice with 1 x TBS to remove excess antibody. For co-immunoprecipitation reactions, 250 µg of protein extract was added to the beads and incubated end over end for 2 hours at 4°C. The beads were then washed five times with 1 x TBS (25 mM Tris pH 7.6, 150mM NaCl) before the bound material was released by the addition of an equal volume of 2 x protein sample buffer. Samples were then analysed by SDS-PAGE and subsequent western blot analysis.

2.6.3 Co-immunoprecipitation using ^{35}S-labelled proteins

25 µl of packed beads was used per immunoprecipitation (IP); these beads were washed twice with 200 µl 1 x TBS, prior to incubation with 1µg of anti-TRRAP or anti-TFIIA antibody, made up to a total volume of 50 µl with TBS on a shaker for 1 hour at 4°C. Following antibody binding, beads were washed twice with 1 x TBS to remove excess antibody. For co-immunoprecipitation reactions, 150 µg of HeLa nuclear extract as well as 5 µl of in vitro transcribed and translated protein was added to the beads and incubated end over end for 2 hours at 4°C. The beads were then washed five times with 1 x TBS before the bound material was released by the addition of an equal volume of 2 x protein sample buffer. Samples were then analysed by SDS-PAGE and subsequent western blot analysis.

2.7 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

2.7.1 RNA Extraction

Total cellular RNA was extracted from tissue culture cells grown in 10cm dishes using TRI reagent (Sigma). Culture media was removed from the cells and the cells were scraped into 1ml of TRI and transferred into sterile microfuge tubes. The tubes were incubated for 1 min at room temperature before 200µl of chloroform was added to each sample, which was subsequently mixed by vortexing for 15 seconds. Samples were incubated for a further 5min at room temperature before being centrifuged at 13,000g for 15 minutes. The centrifugation step separated the samples in to 3 phases : the lower red organic phase containing the proteins, the intermediate phase which contained the DNA, and the upper clear aqueous phase containing the RNA. The RNA-containing aqueous phase was carefully removed and transferred to a clean microfuge tube, 0.5 ml of isopropanol was added to the sample to precipitate the RNA, the tubes were then mixed by vortexing, incubated for 10min at room temperature and centrifuged for 10min at 13,000rpm at 4°C. The pellet was washed with 75% ethanol, centrifuged again for 10min at 13,000 at 4°C. The wash was removed and the pellet dried at room temperature for approx 10min. The RNA was redissolved in approximately 20µl DEPC H_2O and samples were heated to 50°C to facilitate resuspension. All RNA samples were stored at -80°C. The concentration of the RNA was determined spectrophotometrically using a quartz cuvette using the equation 1 A_{260} = 40 µg/µl RNA.

2.7.2 cDNA production

3μg of RNA, prepared as outlined in section above, was added to 200ng of hexanucleotide primers (Roche) made up to a final volume of 24μl. Tubes were incubated at 80°C for 10 minutes for primer annealing. Tubes were transferred to ice then 8μl of 5 X First Strand Buffer (Invitrogen Life Technologies), 4μl of 0.1M dithiolthreitol (DTT) (Invitrogen Life Technologies), 2μl of a dNTP mix containing all four dNTPs at a concentration of 10mM each (Promega) and 1 μl of Superscript II reverse transcriptase (Invitrogen Life Technologies). Reverse transcription reaction was incubated at 42°C for 1 hour. Reaction was terminated by incubating tubes at 70°C for 15 min to destroy the enzyme.

2.7.3 Polymerase Chain Reaction (PCR)

Each PCR was performed using 2μl of cDNA (As described in 2.7.2) or 2μl of ChIP DNA (as described in section 2.8). Each PCR reaction had a total volume of 20μl containing 1x Mg^{2+} free taq DNA polymerase buffer (Promega), 1.5mM $MgCl_2$, 0.2mM of each of dATP, dCTP, dGTP and dTTP, and 1.8μCi of [α-^{32}P] dCTP (Amersham). Primer sequences and cycling parameters are described in table 2.4. Reaction products were diluted 1:1 with formamide loading buffer (98% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol, 5mM EDTA), and resolved on 7% polyacrylamide sequencing gels containing 7% urea and 1x TBE (45 mM Tris, 45 mM boric acid, 0.625 mM EDTA pH 8.0). Gels were pre-run at 40W for 30 minutes in 1 x TBE prior to loading 2 μl of samples. Before loading, samples were heated at

95°C for 2 minutes. Electrophoresis was carried out for 1 hour at 40W, and then gels were vacuum dried for 1 hour at 80°C. PCR products were visualised by autoradiography.

Table 2.4 Primers for RT-PCR

Transcript	Primer	Cycle Number	Product Length	PCR Conditions (denaturing; cycling; final elongation)
5S rRNA	5' GGCCATACCACCCTGAACGC 3' 5' CAGCACCCGGTATTCCCAGG 3'	18-20	107 bp	95°C for 3 min; 95°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 5min
ARPPP0	5' GCACTGGAAGTCCAACTACTTC 3' 5' TGAGGTCCTCCTTGGTGAACAC 3'	18-20	266 bp	95°C for 2 min; 95°C for 1 min, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min
$tRNA^{Leu}$	5' GAGGACAACGGGGACAGTAA 3' 5' TCCACCAGAAAAACTCCAGC 3'	25-27	88 bp	95°C for 3 min; 95°C for 30 s, 68°C for 30 s, 72°C for 30 s; 72°C for 5 min
$tRNA^{Tyr}$	5' AGGACTTGGCTTCCTCCATT 3' 5' GACCTAAGGATGTCCGCAAA 3'	25-27	84 bp	95°C for 3 min; 95°C for 1 min, 65°C for 30 s, 72°C for 15 s; 72°C for 1min
TRRAP	5' CAGGAAGTGAAACGCTTAGG 3' 5' GTCTTCAGAAGGTTCTGAAGAC 3'	26-28	252 bp	95°C for 3 min; 95°C for 1 min, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min

2.8 Chromatin immunoprecipitation (ChIP) assay

Cells were grown in 10cm tissue culture dishes until approaching confluency for ChIP assays. One 10cm dish of cells was used per IP. Formaldehyde was added to the culture medium to a final concentration of 1% to cross-link the protein DNA complexes. Crosslinking was allowed to proceed for 10 minutes at 37°C. Excess glycine was then added at a final concentration of 0.125M, to stop the crosslinking, and the plates transferred to ice for harvesting. The cells were harvested in the plating media/formaldehyde/glycine mix and transferred to 50ml Falcon tubes. The pellets were then harvested by centrifugation at 500g for 5 minutes at 4°C. The cell pellets were washed in ice cold PBS, followed by centrifugation at 500g for 5min at 4°C (The pellets could then be snap frozen at this stage on dry ice, and stored at -80°C, for analysis at a later date).

The cell pellets were washed with ice cold PBS/0.5% NP-40, then centrifuged at 500g for 5 minutes at 4°C. Following removal of the supernatant from this wash, cells were then resuspended in 40ml of high salt buffer (0.5% NP-40, PBS, 1M NaCl), and incubated on ice for 30 minutes. Following the incubation the cells were centrifuged at 1500rpm for 5 minutes at 4°C and washed with 40ml PBS/1% NP-40. The cell pellets were then resuspended in 40ml of low salt buffer (0.5% NP-40, 10mM Tris HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl) and incubated on ice for 30 minutes. Following the incubation, the tubes were centrifuged at 500g for 5 minutes at 4°C. Pellets were then resuspended in 1ml of low salt buffer and passed through a 26g needle three times. Low salt buffer was then added to the suspension to a final volume of 2.7ml. 300µl of 20% sarcosyl was then added to the cell suspension to lyse

the nuclei. The lysed nuclei were then transferred to a sucrose cushion and centrifuged at 4000g for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 3ml TE. The suspension was then transferred to a second sucrose cushion, and centrifuged at 4000g for 10 minutes at 4°C. The final pellet containing the genomic DNA and cross-linked proteins was resuspended in 2ml TE (10 mM Tris pH 8.0, 1mM EDTA), and the DNA then sheared into smaller fragments by sonication (Branson sonifier 250, 10 x 10 s, duty cycle 30%). 0.2 ml of 11x NET Buffer (1.56 M NaCl, 5.5 mM EDTA, 5.5% NP-40, 550 mM TrisCl, pH 7.4) was added to the 2ml sample, which was then transferred to microfuge tubes and centrifuged at 13,000g for 5 minutes. The supernatant was then aliquoted evenly in microfuge tubes. The indicated amount of antibody was added per aliquot (Table 2.6), and these were incubated, end over end, overnight at 4°C.

The following day, 50µl of protein G sepharose beads was added to each tube, and these were left to incubate for a further 2 hours. The beads were then recovered on polypropylene columns (Pierce), and washed twice with 10ml RIPA (50 mM TrisHCl, pH 8.0, 150mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40) buffer, twice with 10ml LiCl (10 mM Tris HCl, 250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, pH 8.0) and finally twice with TE. The beads were then transferred to microfuge tubes and the protein DNA complexes eluted with 200µl TE/1%SDS by incubating end over end for 10 minutes at room temperature. This elution was repeated and the supernatants pooled. The pooled supernatants along with the inputs were incubated overnight at 42°C in the presence of proteinase K to degrade the antibodies and proteins. The DNA was then extracted twice using 400µl phenol/ chloroform/ isoamylalchohol (25:24:1) and once using chloroform alone. 1 ml of

ethanol (2.5x volume) and 40µl of 3M sodium acetate was added and the tubes were thoroughly mixed by inversion. The DNA was precipitated at -20°C overnight. Samples were centrifuged at 13,000g for 20 minutes to pellet the precipitated DNA. The supernatant was removed and the pellets were washed with 150µl 70% ethanol and re-centrifuged at 13,000g for 5 minutes. The supernatant was removed and the pellets were air dried before adding 50µl of TE to resuspend the DNA. The samples were then analysed by PCR, primer sequences and conditions are displayed in the table below (Table 2.5)

Table 2.5 Primers used in PCR analysis of ChIP samples

Transcript	Primers	Cycle Nunber	Product Size	PCR Conditions (denaturing; cycling; final elongation)
5S rRNA	5' GGCCATACCACCCTGAACGC 3' 5' CAGCACCCGGTATTCCCAGG 3'	20-25	107 bp	95°C for 3 min; 95°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 5min
ARPPP0	5' GCACTGGAAGTCCAACTACTTC 3' 5' TGAGGTCCTCCTTGGTGAACAC 3'	20-25	266 bp	95°C for 2 min; 95°C for 1 min, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min
Cyclin D2 Promoter	5' GGCATAACCTTTATCCCTGGTT 3' 5' AACCCCATGGATTCCTATTGATT 3'	25-30	252 bp	95°C for 3 min; 95°C for 1 min, 60°C for 30 s, 72°C for 1 min; 72°C for 5 min
p21 Coding Region	5' CTCTGGGAAGCCAGAAGTTGTT 3' 5' GGTCCAGTCCCTGCATCTAAGT 3'	25-30	257bp	95°C for 3 min; 95°C for 1 min, 55°C for 30 s, 72°C for 1 min; 72°C for 5 min
$tRNA^{Leu}$	5' GAGGACAACGGGGACAGTAA 3' 5' TCCACCAGAAAAACTCCAGC 3'	25-30	88 bp	95°C for 3 min; 95°C for 30 s, 68°C for 30 s, 72°C for 30 s; 72°C for 5 min
$tRNA^{Tyr}$	5' AGGACTTGGCTTCCTCCATT 3' 5' GACCTAAGGATGTCCGCAAA 3'	25-30	84 bp	95°C for 3 min; 95°C for 1 min, 65°C for 30 s, 72°C for 15 s; 72°C for 1min

Table 2.6 Antibodies for ChIP analysis

Protein	Antibody	Type	Source	Quantity used per IP
Brf1	128	Serum	In house	20 µl
Bdp1	2663	Serum	In house	20 µl
TBP	mTBP-6	Monoclonal	In house	200 µl
TFIIIC 110	4286	Serum	In house	20 µl
TFIIIC 220	Ab 7	Serum	In house	20 µl
RPC 155	1900	Serum	In house	20 µl
c-Myc	N-262	Polyclonal	Santa Cruz	4 µg
Max	C-17	Polyclonal	Santa Cruz	4 µg
TRRAP	H-300	Polyclonal	Santa Cruz	4 µg
Acetyl Histone H4	06-866	Polyclonal	Upstate	4 µg
Acetyl Histone H3	06-599	Polyclonal	Upstate	4 µg
Gcn5	H-75	Polyclonal	Santa Cruz	4 µg
Tip60	H-93	Polyclonal	Santa Cruz	4 µg
TAF_I48	M19	Polyclonal	Santa Cruz	4 µg
TFIIA	FL-109	Polyclonal	Santa Cruz	4 µg
TFIIB	C18	Polyclonal	Santa Cruz	4 µg

2.9 Transient transfection of shRNA s to knockdown endogenous protein levels

Hela cells were plated into 6 well dishes at a confluency of 4 $x10^5$ cells per well. The cells were left to grow overnight, resulting in a confluency of approximately 80-90% of cells per well. For each well to be treated, 2μg of the pSuper RNAi plasmid was added to 250 μl of OptiMEM and mixed. In a separate tube, 7.5μl of Lipofectamine 2000 (Invitrogen Life Technologies) was mixed with 250 μl of OptiMEM and incubated at room temperature for 5 minutes. This Lipofectamine 2000 mix was added to the tube containing the plasmid DNA and mixed by pipetting. The DNA/Lipofectamine 2000 complexes were allowed to form by incubation at room temperature for 20 minutes in the dark. During this incubation the cells were washed with 3 ml of DMEM, and then 2 ml of DMEM added per well. The plates were then incubated at 37°C for the remainder of the incubation time (approximately 15 minutes). After the incubation, 0.5 ml of the Lipofectamine 2000-DNA-OptiMEM mix was added to each dish and the plates left to incubate for 5 hours at 37°C in the incubator. After the incubation period, 2.5 ml of DMEM containing 20% FBS was added to the cells. Cells were incubated for a further 48 hours to allow expression of the transfected DNA. Cells were harvested and protein extracts and RNA were prepared as described previously.

Chapter 3: Investigating the interaction between TFIIIB and c-Myc

3.1 Introduction

c-Myc has been demonstrated to activate genes transcribed by pols I, II and III (Chapter 1; Oskarsson and Trumpp, 2005). Unlike the promoters of pol I and pol II-transcribed genes activated by c-Myc, pol III-transcribed genes do not contain good matches to the E-Box DNA sequence that is recognised by c-Myc/Max heterodimers (E-boxes). Instead the interaction between c-Myc and class III genes appears to be through protein-protein interactions with the pol III-specific transcription factor TFIIIB (Gomez-Roman et al, 2003; Felton-Edkins et al, 2003b). It is believed that it is TFIIIB, rather than DNA sequence, that is responsible for the presence of c-Myc on pol III-transcribed genes (Gomez-Roman et al, 2003; Felton-Edkins et al, 2003b).

3.1.1 Regulation of pol III transcription by altering levels and activity of TFIIIB

TFIIIB is a complex composed of three subunits TBP, Brf1 and Bdp1 (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). As discussed in chapter 1, the function of TFIIIB is to bind to class III genes, in a TFIIIC-dependent manner, and subsequently recruit pol III to the start site of transcription, where it also plays a role in promoter opening (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). The interaction between TFIIIB and promoter-bound TFIIIC is, at least in

yeast, the rate-limiting step in the formation of an active transcription complex (Moir et al, 1997; Ramcau et al, 1994). Given the pivotal role of TFIIIB in the formation of the transcription complex, it is of no surprise that pol III transcription can be regulated through modulation of its activity.

In both yeast and mammalian cells the levels of pol III transcription can be controlled by altering the levels of subunits of TFIIIB. In S. cerevisiae pol III transcription declines during the transition from logarithmic to stationary phase growth (Sethy at al, 1995). This is attributed to lower levels of the Brf1 protein (Sethy et al, 1995). In mammalian cells, the differentiation of F9 embryonal carcinoma cells into parietal endoderm is accompanied by a decrease in the rate of pol III transcription (White et al, 1989). This decrease is due to specific down-regulation of TFIIIB, specifically of Brf1 and Bdp1 (White et al, 1989; Alzuherri and White, 1998; Athineos and White, unpublished observations).

Levels of pol III transcription fluctuate during the cell cycle. Pol III transcription increases during late G1 and declines during mitosis (White et al, 1995). Add-back experiments demonstrated that TFIIIB activity is limiting during early G1 (White et al, 1995). The levels of TFIIIB subunits do not appear to change during the cell cycle, but their action is controlled by regulation of their activity (Scott et al, 2001; Fairley et al, 2003). The tumour suppressor proteins p53 and RB can inhibit pol III transcription through interactions with TFIIIB (Chu et al, 1997; Larminie et al, 1997). In resting cells, TFIIIB is inactivated through an interaction with RB, which prevents its interaction with both TFIIIC and pol III, therefore preventing transcription complex formation on pol III-transcribed genes (Sutcliffe at al, 2000). The binding of

RB to the TFIIIB is proposed to be dependent on the TFIIIB specific TAFs, as addition of TBP cannot rescue RB mediated inhibition of pol III transcription in vitro (Larminie et al, 1997). Inactivation of RB through phosphorylation results in the release of TFIIIB and subsequent activation of pol III transcription (Scott et al, 2001).

Another repressor of pol III transcription that acts on TFIIIB is the p53 tumour suppressor protein (Cairns and White, 1998). The expression of p53 is induced in response to a variety of cellular insults, and its induction leads to an inhibition of pol III transcription (Gomez-Lazoro et al, 2004; Hofseth at al, 2004). This inhibition is through direct binding to TFIIIB, through the TBP subunit (Chesnokov et al, 1996; Cairns and White, 1998; Crighton et al, 2003). Once bound by p53, TFIIIB loses its ability to interact with TFIIIC and this results in a marked decrease of TFIIIB and pol III occupancy on pol III-transcribed genes (Crighton et al, 2003)

TFIIIB is also regulated by phosphorylation of its Brf1 subunit (Johnston et al, 2002; Felton-Edkins et al, 2003a; Fairley et al, 2003). The oncogenic kinase CK2 can bind to TFIIIB and phosphorylate Brf1 (Johnston et al, 2002). The phosphorylation of TFIIIB by CK2 facilitates its recruitment to TFIIIC (Johnston et al, 2002). TFIIIB is also phosphorylated by the MAP kinase Erk (Felton-Edkins et al, 2003a). Erk binds to and phosphorylates Brf1, and this leads to an increase in the interaction between TFIIIB and TFIIIC and consequent increase in pol III transcription (Felton-Edkins et al, 2003). Brf1 is also phosphorylated during mitosis, but this phosphorylation event is involved in the repression, rather than the activation of pol III transcription (Fairley et al, 2003).

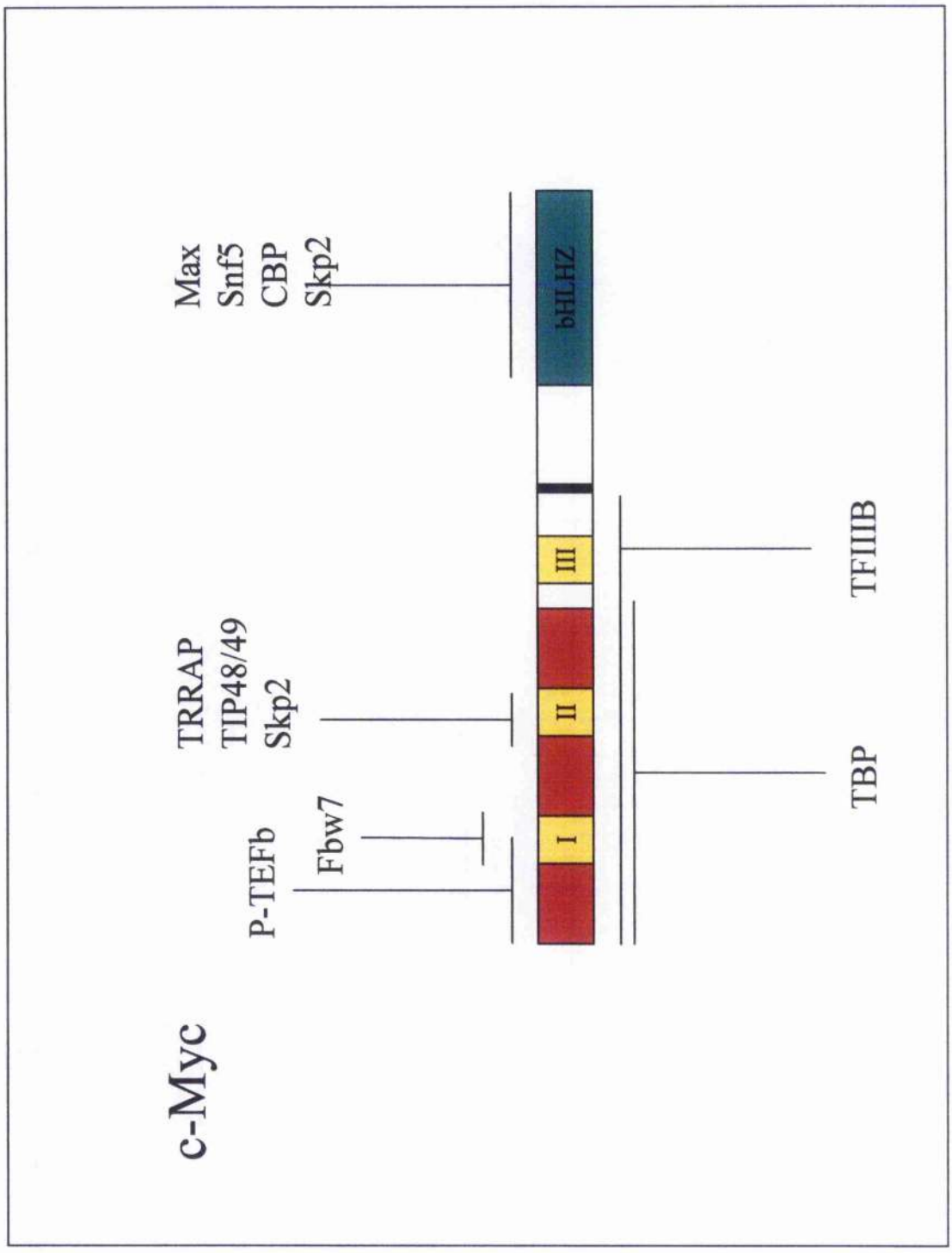
3.1.2 c-Myc interacting proteins involved in transcriptional activation

Like TFIIIB, c-Myc can interact with a variety of proteins to both positively and negatively alter its activity. c-Myc can interact with proteins that can influence its action in a number of ways: recruitment of chromatin remodelling activities (SWI/SNF and relatives) (Cheng et al, 1999; Wood et al, 2000), recruitment of modifying complexes (CBP, TRRAP and associated complexes) (McMahon et al, 1998; Bouchard et al, 2001; Frank et al, 2001; Vervoorts et al, 2003), interaction with ubiquitin ligases (Fbw7 and Skp2) (Welcker et al, 2004, Yada et al, 2004; Kim et al, 2003; von der Lehr et al, 2003) and a host of other factors (Reviewed in Sakamuro and Prendergast 1999) (Figure 3.1).

As well as these co-activator proteins c-Myc has also been shown to bind to the general transcription factors required for transcription by pol I and pol II. It has been demonstrated that c-Myc can bind to the TFIID complex via interactions with TBP and to the RAP74 subunit of TFIIF (Maheswaran et al, 1994; McEwan et al, 1996). TFIIF and TFIID are associated with the c-Myc TAD and it is proposed that these interactions are associated with c-Myc transcriptional activation (Maheswaran et al, 1994; McEwan et al, 1996). c-Myc can also prevent the formation of the pol II transcription complex through association with the pol II-specific transcription factor TFII-I, to repress target genes (Roy et al, 1993) The interaction with TFII-I is proposed to prevent the interaction between TFIID, TFII-I and the promoter (Roy et al, 1993).

Figure 3.1 Schematic of c-Myc protein indicating interaction domains for a variety of co-activator protein

Schematic of c-Myc indicating the conserved regions indicating the points of interaction with co-activator proteins, Snf5, TRRAP, TIP48/49, Skp2, Fbw7, P-TEfb, TBP, TFIIIB and Max. The regions of the c-Myc protein are described in figure 1.5.


c-Myc
I
II
III
bHLHZ
P-TEFb
Fbw7
TRRAP
TIP48/49
Skp2
Max
Snf5
CBP
Skp2
TBP
TFIIIB

It was recently demonstrated that c-Myc can also directly activate pol I transcription (Grandori et al, 2005; Arabi et al, 2005). c-Myc is present on the rDNA repeats and its occupancy correlates with the occupancy of the pol I-specific transcription factor SL1 (Grandori et al, 2005). SL1 is an essential complex composed of TBP and three pol I-specific TAFs; TAF_I110, TAF_I63 and TAF_I48 (Grummt, 2003). The SL1-c-Myc interaction is through two conserved regions of the c-Myc protein; the bHLHZ and the MBII domains (Grandori et al, 2005) (Figure 3.1).

Promoters of the c-Myc target genes transcribed by pol I and pol II contain the E-box sites necessary for c-Myc/Max binding, and c-Myc may use these to recruit general transcription factors to the promoters of these genes (Grandori et al, 2000; Grandori et al, 2005). As previously discussed, it is through TFIIIB rather than DNA binding that c-Myc interacts with pol III-transcribed genes, therefore this simple model of c-Myc binding to DNA and recruiting the general transcription factors, is unlikely to be the case.

In this chapter the interaction between TFIIIB and c-Myc will be examined. As TFIIIB is subject to regulation by multiple factors, the interplay between these regulators needs to be studied fully to understand how they exert their influence on pol III transcription in vivo. To do this, the interactions between subunits of TFIIIB and c-Myc will be determined and further defined. These interactions will be compared to how other regulators of pol III transcription bind to this complex and to determine if they share any common binding domains. Also in this chapter, the TFIIIB interaction domain of c-Myc will be further defined and mutant c-Myc

proteins will be tested for their ability to bind to TFIIIB and to stimulate pol III transcription.

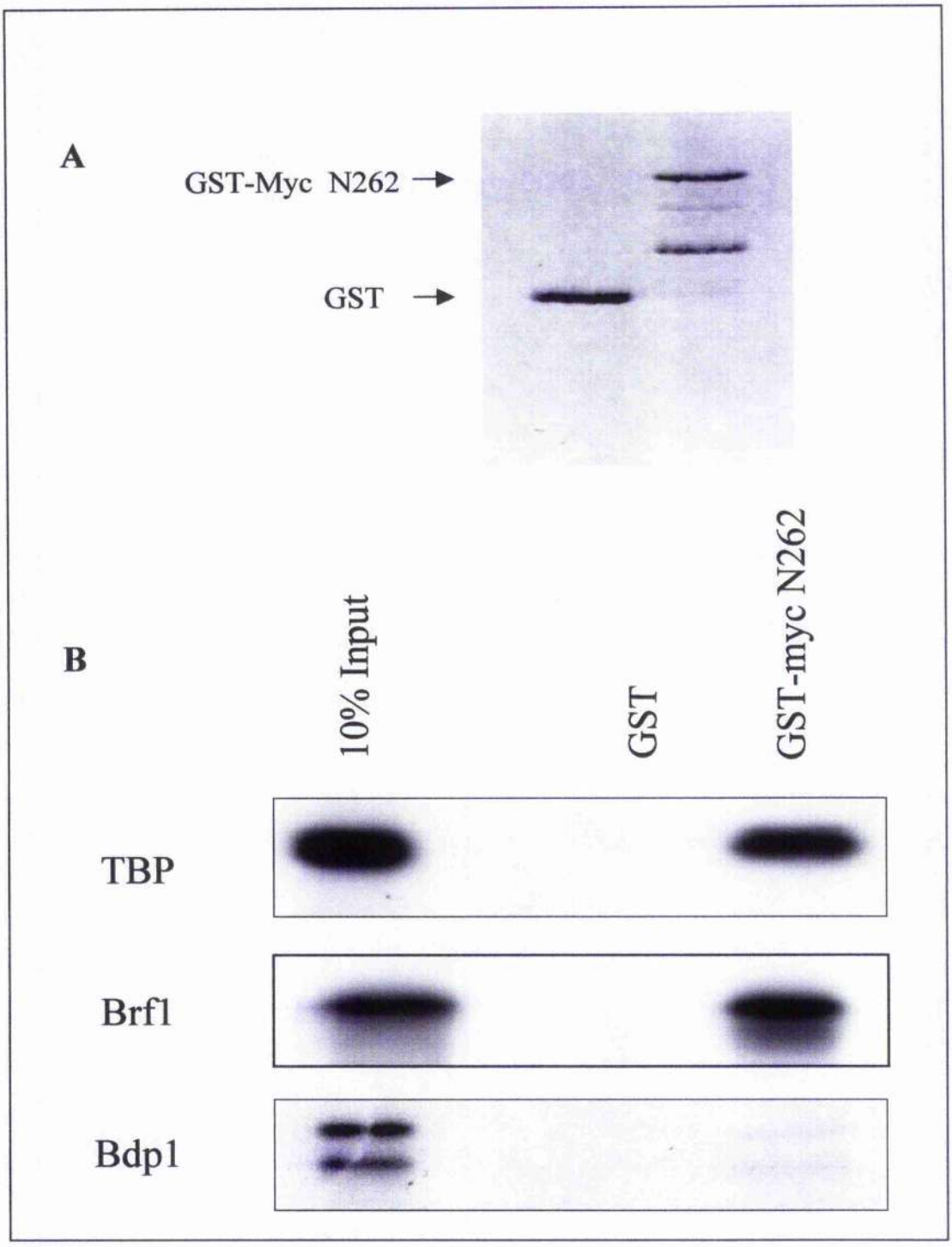
3.2 Results

3.2.1 Mapping the TFIIIB interaction with c-Myc

As summarised in the introduction to this chapter, c-Myc can bind to the pol III-specific transcription factor TFIIIB (Gomez-Roman et al, 2003). The interaction between c-Myc and TFIIIB was confirmed by co-immunoprecipitation and co-fractionation experiments (Gomez-Roman et al, 2003), but the subunit(s) of TFIIIB that c-Myc interacts with are unknown. It was also demonstrated by pull-down experiments that TFIIIB interacts with the N-terminal 262 amino acids of c-Myc, which includes part of the central domain of c-Myc and the minimal TAD, but does not include the C-terminal basic helix loop helix zipper domain (Gomez Roman et al, 2003). The binding of c-Myc to TBP has been previously reported and this interaction is dependent on amino acids 1-143 of c-Myc, which is the minimal TAD (McEwan et al, 1996). To investigate whether c-Myc could bind to any of the other components of TFIIIB individually, pull-down assays were performed using GST fused to the N-terminal 262 amino acids of c-Myc and GST alone. Recombinant proteins were expressed in bacteria and immobilised on glutathione agarose beads. The proteins were then resolved by SDS-PAGE and the gels stained to check for equivalent protein levels (Figure 3.2a). In vitro transcribed and translated Brf1, Bdp1 and TBP, radio-labelled using ^{35}S methionine, were then incubated with the immobilised proteins to test for binding. The TBP component of TFIIIB binds specifically to GST-Myc N262 (Figure 3.2b). This interaction between TBP and c-Myc has been previously reported by several groups (Maheswaran et al, 1994; McEwan et al, 1996). Also seen in this experiment is a strong interaction between

Figure 3.2 c-Myc interacts with the TBP and Brf1 components of TFIIIB

GST-Pull down analysis of c-Myc binding to subunits of TFIIIB. A. Coomassie staining of SDS-PAGE gel to demonstrate levels of GST and GST-Myc N262 used in the pull-down. B. ^{35}S-labelled in vitro transcribed and translated Brf1, Bdp1 and TBP were tested for their ability to interact with immobilised GST or GST-Myc N262.


A
GST-Myc N262
GST
B
10% Input
GST
GST-myc N262
TBP
Brf1
Bdp1

GST-Myc N262 and Brf1 (Figure 3.2b). The binding of c-Myc to TBP and Brf1 is specific, as the other pol III-specific TAF Bdp1, does not bind to the c-Myc fusion protein (Figure 3.2b).

The Brf1 protein is critical in the control of pol III transcription through interaction with both activators and repressors. To further investigate the Brf1-c-Myc interaction, we tested the ability of Brf1 fragments to bind to the recombinant c-Myc protein, to further define the c-Myc binding domain of Brf1.

The structure of the Brf1 protein can be split into two domains, the N-terminal TFIIB-related domain and a C-terminal domain, which is unique to Brf1 (Figure 3.3; Mital et al, 1996). The N-terminal 280 amino acids of hBrf1 are 24 % identical to hTFIIB, sharing similar domains including a Zn ribbon domain and two imperfect direct repeats (Mital et al, 1996). The direct repeats of hBrf1 are homologous to those found in cyclins and to the pocket domain of RB and its relatives (Figure 3.3; Mital et al, 1996; Hisatake et al, 1993). It is through this N-terminal domain that Brf1 can interact with some of the activators and repressors of pol III transcription. The C-terminal domain of hBrf1, which shares no homology with TFIIB, contains the high affinity TBP binding site and is critical for the formation and function of the TFIIIB complex (Figure 3.3; Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Khoo et al, 1994). The C-terminal region of Brf1 is homologous with Brf proteins from other organisms, but has no obvious similarity to other known proteins (Mital et al, 1996). To investigate the interaction between Brf1 and c-Myc, smaller regions of the Brf1 coding sequence were amplified by PCR to be used as templates to produce the smaller peptides (figure 3.4a). To do this, the forward primers contained a T7

Figure 3.3 Schematic of hBrf1

Schematic diagram of Brf1 indicating the conserved domains; Zn ribbon, direct repeats and the Brf1 homology regions, H1 H2 and H3, and their relative positions in the polypeptide. Also indicated is the region of Brf1 that is homologous with TFIIB.

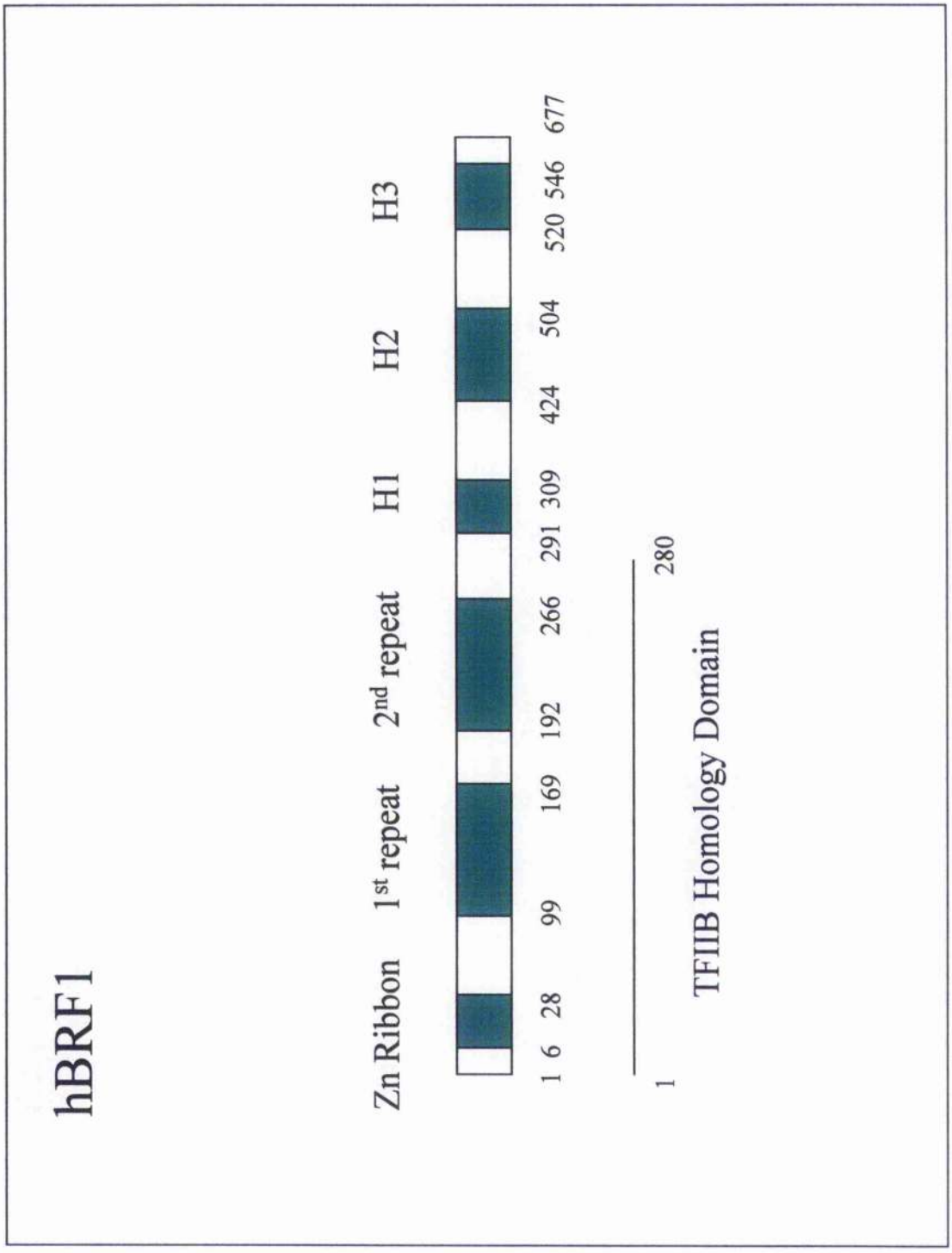
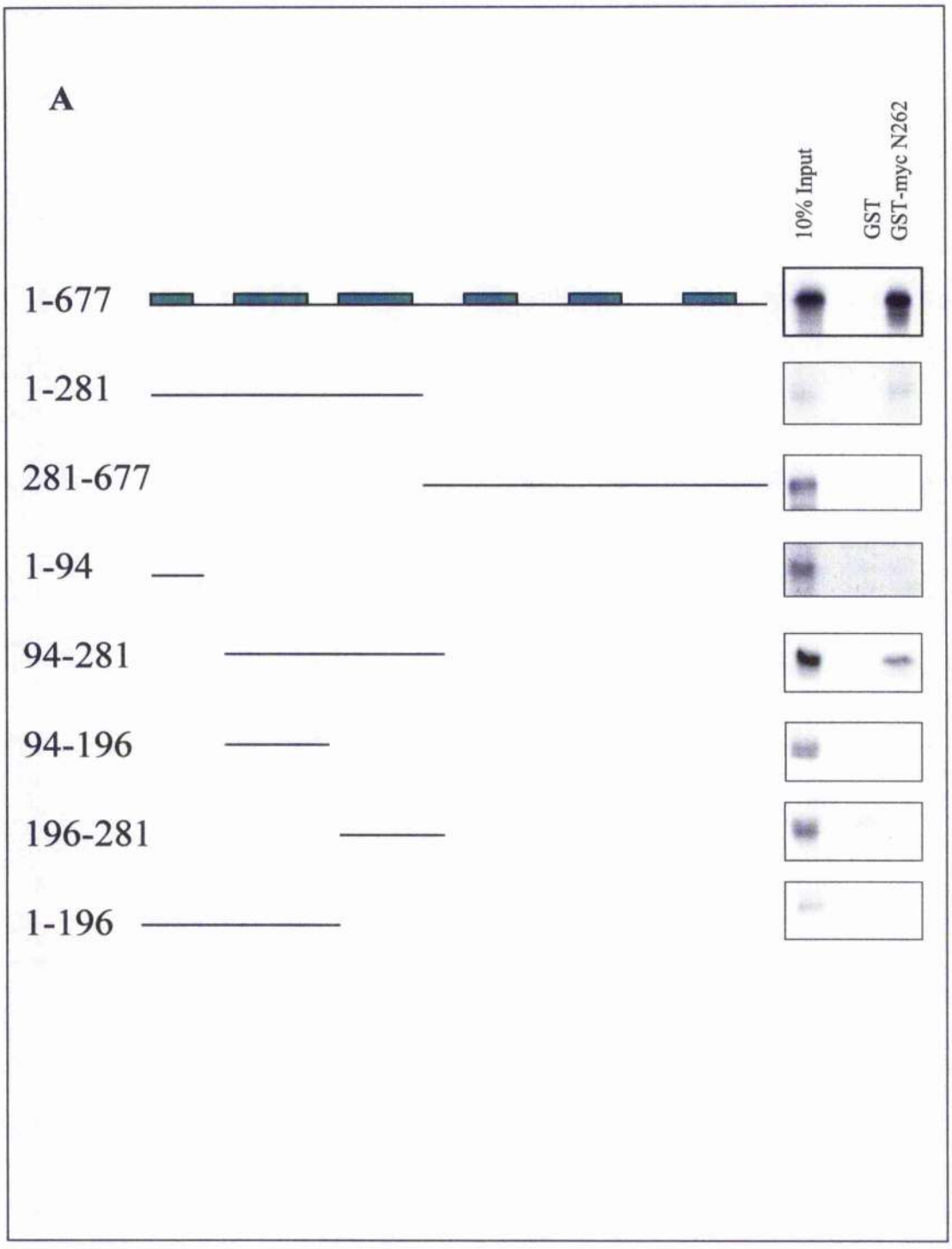
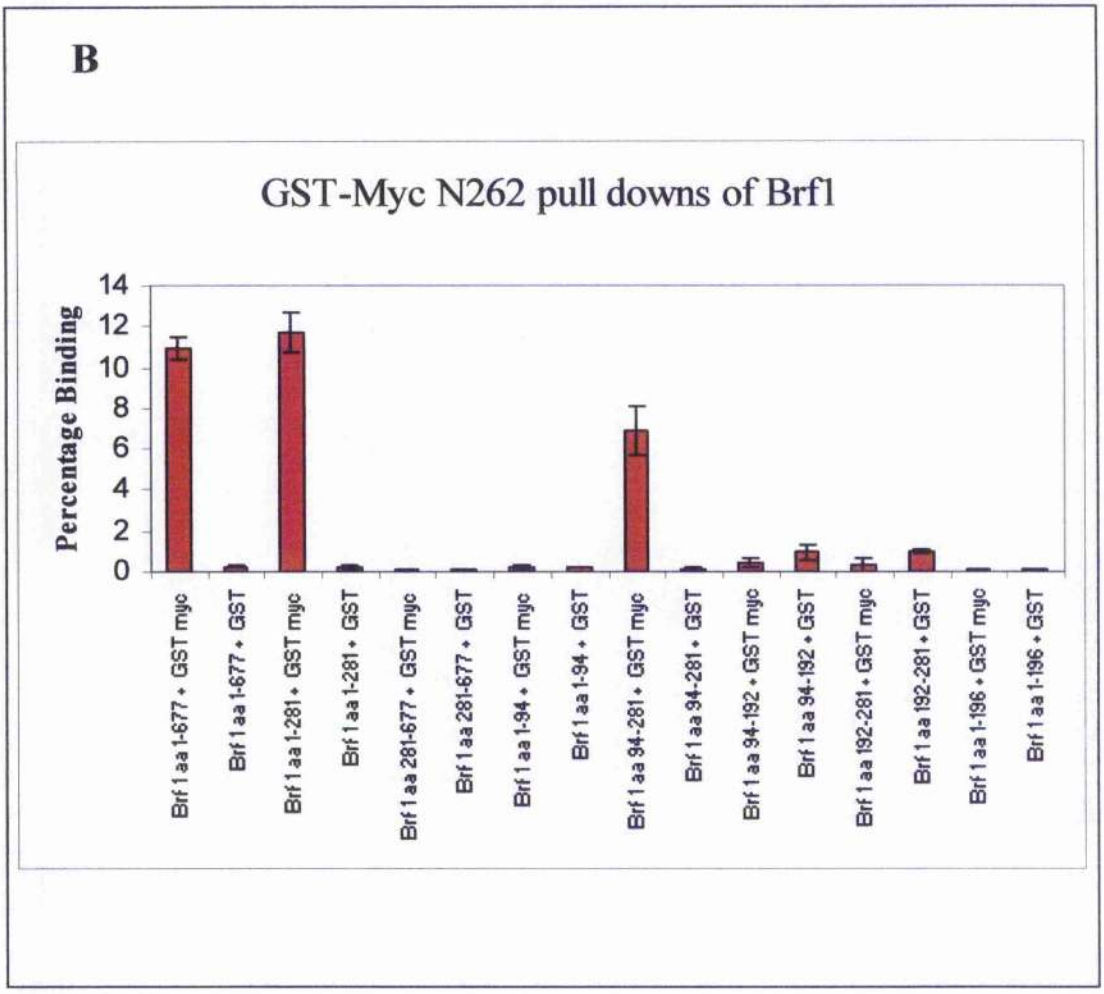

hBRF1
Zn Ribbon
1st repeat
2nd repeat
H1
H2
H3
1 6 28 99 169 192 266 291 309 424 504 520 546 677
1
280
TFIIB Homology Domain

Figure 3.4 c-Myc interacts with the direct repeats of Brf1

A. Immobilised GST and GST-Myc N262 were used to pull down a range of ^{35}S labelled in vitro transcribed and translated fragments of Brf1 (indicated in figure) to test for specific binding to c-Myc (Fragments under 30 kDa were run on 15% SDS-PAGE gels). B. Graphs quantifying the binding of these fragments to GST and GST-Myc N262 as a percentage of the input (Quantification done using ImageJ).


A
10% Input
GST
GST-myc N262
1-677
1-281
281-677
1-94
94-281
94-196
196-281
1-196


B
GST-Myc N262 pull downs of Brf1
Percentage Binding
14
12
10
8
6
4
2
0
Brf 1 aa 1-677 + GST myc
Brf 1 aa 1-677 + GST
Brf 1 aa 1-281 + GST myc
Brf 1 aa 1-281 + GST
Brf 1 aa 281-677 + GST myc
Brf 1 aa 281-677 + GST
Brf 1 aa 1-94 + GST myc
Brf 1 aa 1-94 + GST
Brf 1 aa 94-281 + GST myc
Brf 1 aa 94-281 + GST
Brf 1 aa 94-192 + GST myc
Brf 1 aa 94-192 + GST
Brf 1 aa 192-281 + GST myc
Brf 1 aa 192-281 + GST
Brf 1 aa 1-196 + GST myc
Brf 1 aa 1-196 + GST

promoter, and the PCR products were in vitro transcribed and translated in the presence of ^{35}S-labelled methionine. The in vitro transcribed and translated Brf1 fragments were used in GST pull-down assays to test for an interaction with the N-terminal 262 amino acids of c-Myc (Figure 3.4a). The C-terminal region of Brf1, encoded by amino acids 281-677, which is conserved within Brf1 homologues, but has no similarity to TFIIB, does not interact with the c-Myc fusion protein (Figure 3.4a). There is an interaction observed between the N-terminal region (amino acids 1-280) and GST-Myc N262 (Figure 3.4a). When smaller fragments of the N-terminal region were assayed it was observed that the Zn-finger region (amino acids 1-93) does not interact with GST-Myc N262. When the direct repeats of Brf1 (amino acids 94-280) were tested, it was found that they can bind to the c-Myc fusion protein (Figure 3.4a). The interaction between c-Myc and the direct repeats is dependent on the integrity of both repeats, as neither can bind when tested individually (amino acids 94-200 and 200 to 280) (Figure 3.4a). Quantification of several of these experiments indicates that the direct repeats alone do not bind as efficiently to c-Myc as the entire TFIIB-related region, indicating some function of the Zn ribbon domain in the interaction (Figure 3.4b).

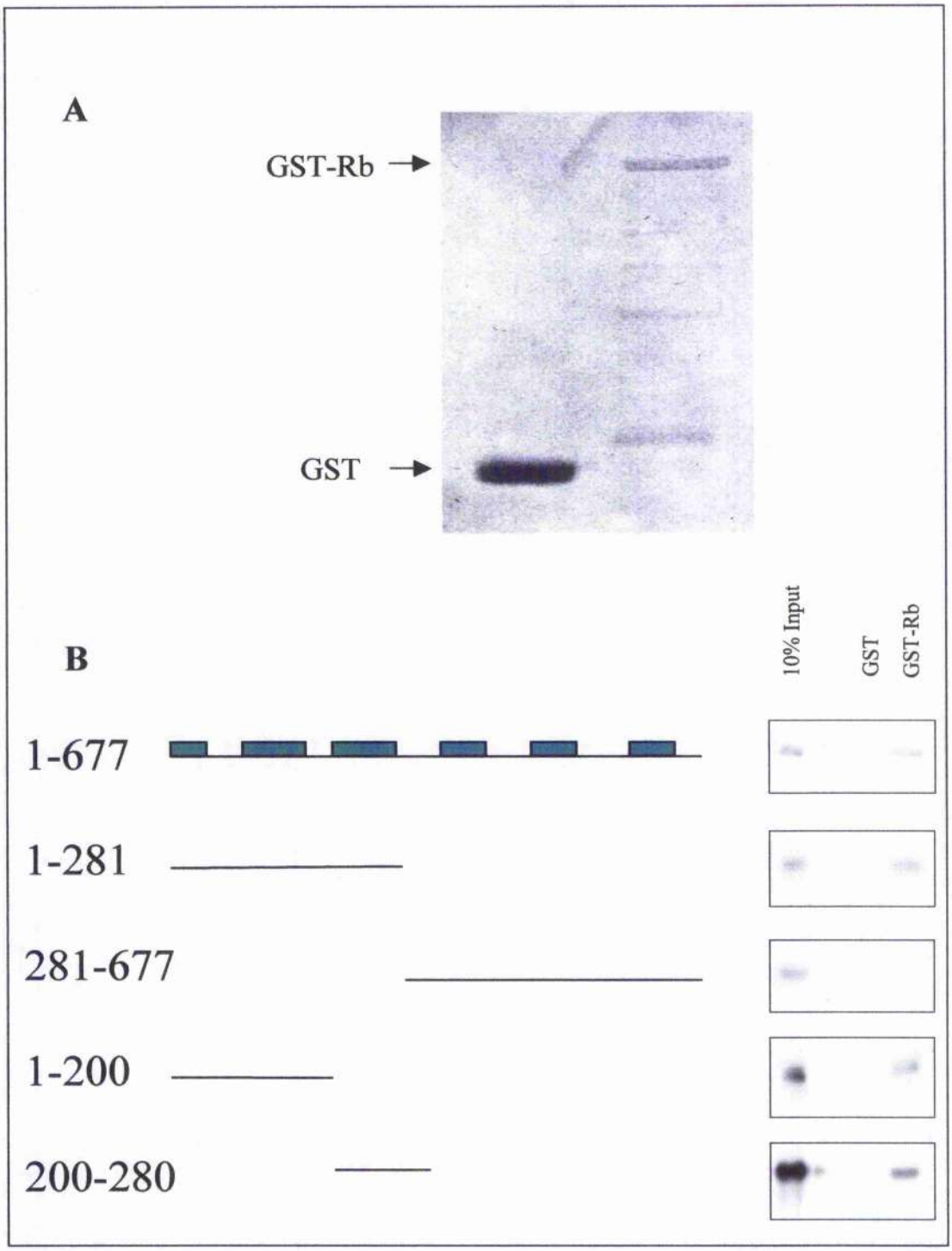
The tumour suppressor RB has been demonstrated to interact with the Brf1 subunit of TFIIIB, and it is through an interaction with TFIIIB that RB functions to repress pol III transcription (Sutcliffe and White, unpublished observations; Larminie et al, 1997; Chu et al, 1997). To compare what regions of Brf1 are required for RB binding to those required for c-Myc binding a similar pull-down approach was performed with in vitro transcribed and translated fragments of the Brf1 protein and testing their ability to interact with immobilised recombinant RB. The GST-RB fusion protein used

contains residues 379-928 of RB; this region is sufficient to inhibit pol III transcription and also to bind TFIIIB (Larminie et al, 1997). This fragment of RB includes the pocket domain and is the minimal region necessary to regulate cell growth and proliferation (Weinberg, 1995; Whyte, 1995). Recombinant GST and GST-RB (379-928) proteins were expressed in bacteria and immobilised on glutathione agarose beads and proteins were resolved by SDS-PAGE and the gels stained to check for input protein levels (figure 3.5a). When the smaller fragments of Brf1 were tested for an interaction with GST-RB (379-928) the N-terminal region of Brf1 (amino acids 1-280) bound efficiently to the RB protein (figure 3.5b). When the C-terminal region of Brf1 (amino acids 281-677) was tested for binding to RB no interaction was observed (figure 3.5b). Smaller fragments of the N-terminal region of Brf1 were tested for an interaction with GST-RB (379-928). GST-RB (379-928) can interact specifically with a fragment of Brf1 containing the Zn ribbon motif and the 1st direct repeat (amino acids 1-200) and an interaction is also observed between the 2nd repeat of Brf1 in isolation (amino acid 200-280) (Figure 3.5b). This indicates that two separate regions of Brf1 are involved in the interaction with RB (Figure 3.5b). It is also interesting to note that the RB interaction with Brf1 is different to the c-Myc interaction with Brf1.

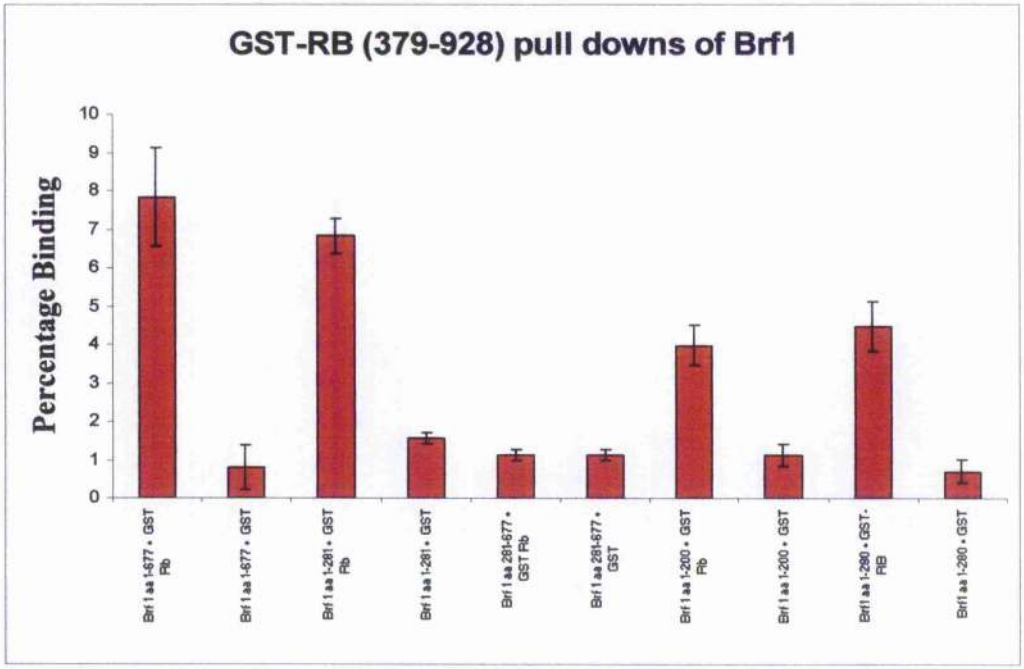
The direct repeats of Brf1 are involved in the interaction between TFIIIB and another activator of pol III transcription, the MAP kinase Erk (Felton-Edkins et al, 2003a). A mutation of Brf1 in the ERK docking domain can prevent pol III activation by mitogenic stimuli. It had previously been found that members of the MAP kinase family interact with their substrates via specific docking sites, known as D-domains (Sharrocks et al, 2000). Mutation in such a domain of Brf1, through substitution of

Figure 3.5 The RB interaction with Brf1 is distinct from the c-Myc interaction

A. Coomassie stained gel, demonstrating levels of GST and GST –RB typically used in these experiments. B. Immobilised GST and GST-RB (379-928) were used to pull down a range of ^{35}S-labelled in vitro transcribed and translated fragments of Brf1 (indicated in figure) to test for specific binding to RB (Fragments under 30 kDa were run on 15% SDS-PAGE gels). B. Graphs quantifying the binding of these fragments to GST and GST-RB (379-928) as a percentage of the input (Quantification done using ImageJ).


A
GST-Rb
GST
B
10% Input
GST
GST-Rb
1-677
1-281
281-677
1-200
200-280

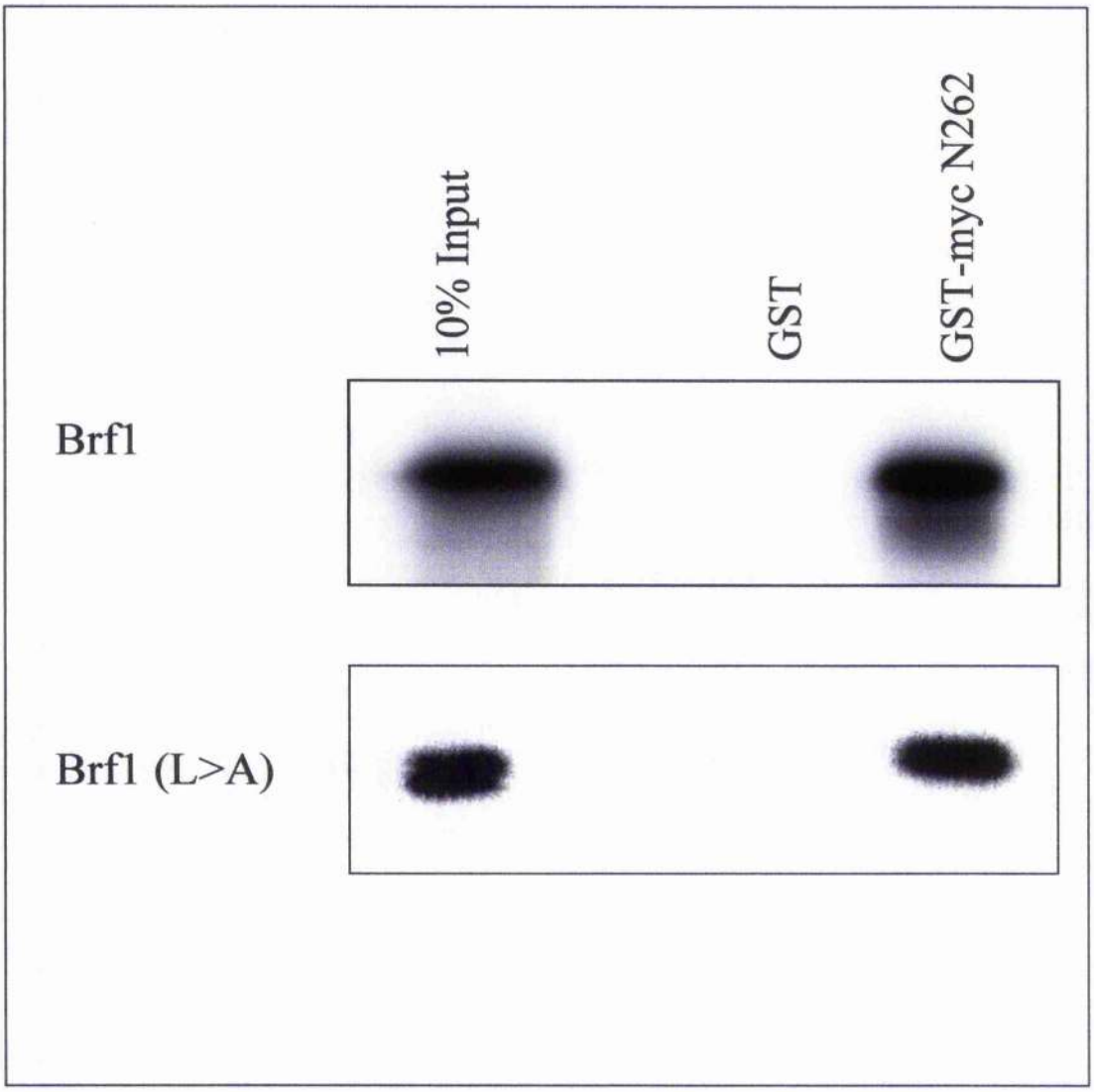
C


GST-RB (379-928) pull downs of Brf1
Percentage Binding
10
9
8
7
6
5
4
3
2
1
0
Brf1 aa 1-677 + GST Pb
Brf1 aa 1-677 + GST
Brf1 aa 1-281 + GST Pb
Brf1 aa 1-281 + GST
Brf1 aa 281-677 + GST Pb
Brf1 aa 281-677 + GST
Brf1 aa 1-200 + GST Pb
Brf1 aa 1-200 + GST
Brf1 aa 1-280 + GST-RB
Brf1 aa 1-280 + GST

leucine residues at position 100 and 102 in the Brf1 sequence to alanines (Brf1 L>A), results in a reduced interaction between Brf1 and ERK (Felton-Edkins et al, 2003a). To examine any possible effects on c-Myc binding to this mutated form of the protein, its interaction was examined in a pull-down assay using recombinant GST-MycN262 (Figure 3.6). The results of this experiment indicate that mutation of the D-domain of Brf1 did not have any effect on the ability of the protein to bind to c-Myc (Figure 3.6).

Figure 3.6 A mutation in Brf1 that disrupts ERK binding has no effect on the c-Myc interaction

Immobilised GST and GST-Myc N262 were used to pull down either wildtype ^{35}S labelled in vitro transcribed and translated Brf1 or Brf1 (L>A) to test for any difference in binding to c-Myc.


10% Input
GST
GST-myc N262
Brf1
Brf1 (L>A)

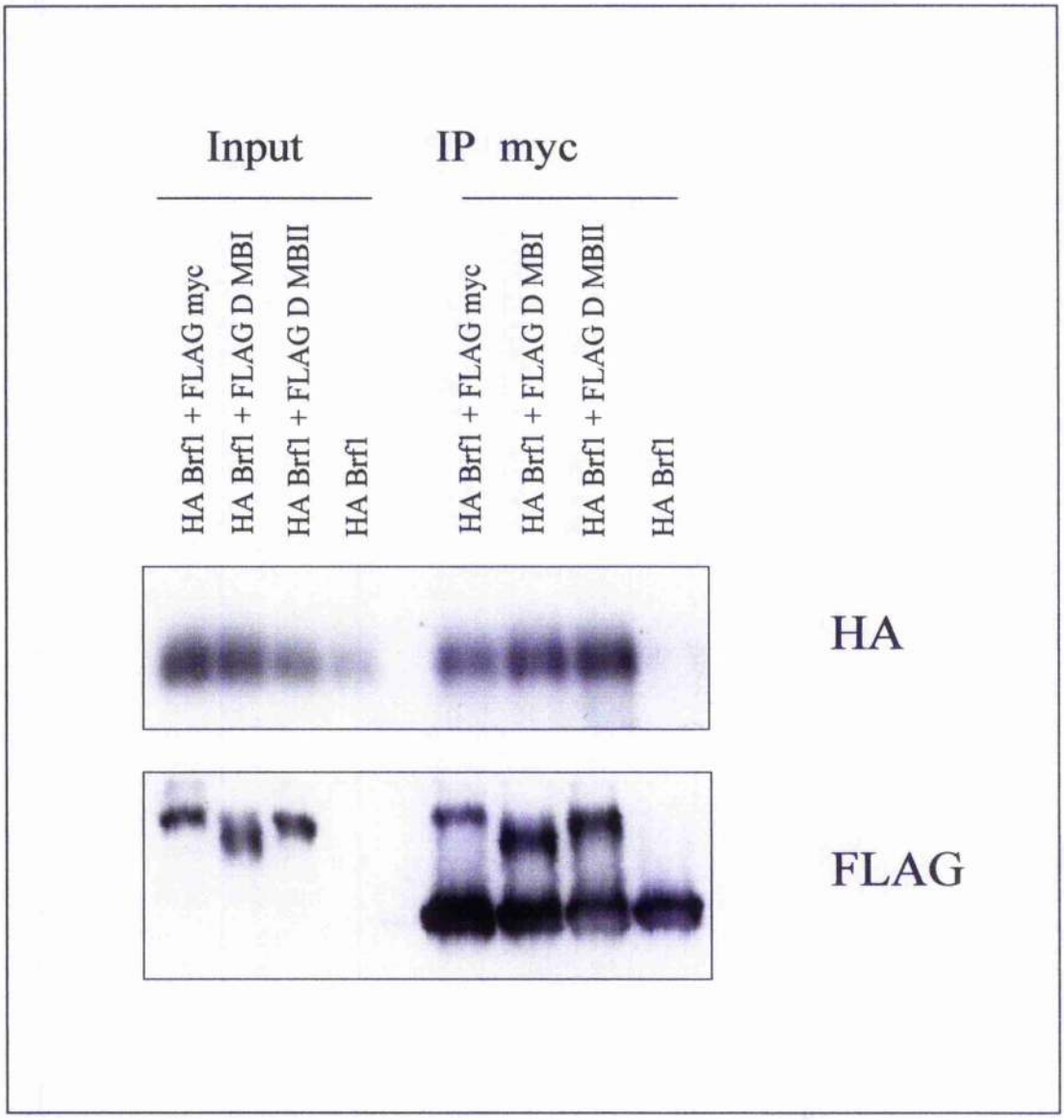
3.2.2 Conserved regions of c-Myc are not required for binding to TFIIIB

A host of studies have been performed to identify regions of c-Myc that are involved in protein/protein interactions and the biological activity of the protein. Several studies have identified two regions of the c-Myc protein that are highly conserved between members of the c-Myc family, and are critical for the biological activity of c-Myc, termed Myc Box I (MBI) and Myc Box II (MBII) (Grandori et al, 2000; Patel et al, 2004). MBI and MBII are located between amino acids 45-63 and 128-143 in the c-Myc protein respectively (Grandori et al, 2000). Deletion or mutation of these conserved elements has profound effects on the biological activities of c-Myc (Stone et al, 1987; Sarid et al, 1987; Freytag et al, 1990; Goruppi et al, 1994). As discussed in the introduction to this chapter, several proteins have been shown to interact with these regions of c-Myc and play critical roles in Myc biology.

To examine the potential roles of these conserved regions of c-Myc in the interaction with TFIIIB, co-immunoprecipitation experiments were performed. Vectors encoding FLAG-tagged c-Myc proteins were transfected into HEK 293 cells coding for the wild-type protein, a mutant lacking MBI (amino acids 45-63 deleted), a mutant lacking MBII (amino acids 128-143 deleted) and an empty vector. Co-transfected with the Myc constructs was a vector encoding HA-tagged Brf1. Co-immunoprecipitations were performed by immunoprecipitating the extracts using an anti-FLAG antibody followed by western blotting with an anti-HA antibody (Figure 3.7). As expected, the wildtype c-Myc protein interacts specifically with TFIIIB

Figure 3.7 Deletion of MBI or MBII of the c-Myc protein does not effect binding to TFIIIB

HEK 293 cells were transiently transfected with a vector encoding HA-Brf1 along with vectors encoding FLAG tagged wildtype c-Myc, c-Myc ΔMBI, c-Myc ΔMBII and empty vector. Extracts from these cells were immunoprecipitated using an anti-FLAG antibody, and the resolved by SDS-PAGE and then analysed by western blotting with anti-FLAG and anti-HA antibodies.


Input
IP myc
HA Brf1 + FLAG myc
HA Brf1 + FLAG D MBI
HA Brf1 + FLAG D MBII
HA Brf1
HA Brf1 + FLAG myc
HA Brf1 + FLAG D MBI
HA Brf1 + FLAG D MBII
HA Brf1
HA
FLAG

(Figure 3.7; Gomez-Roman et al, 2003). Deletion of the conserved Myc boxes does not interfere with the interaction between c-Myc and TFIIIB, with equivalent amounts of Brf1 co-immunoprecipitating with the mutant Myc proteins (Figure 3.7).

3.3 Discussion

As previously discussed, c-Myc binds to TFIIIB and it is this interaction which is thought to be responsible for the recruitment of c-Myc to the promoters of pol III-transcribed genes. It has been reported previously that c-Myc can bind to TBP, but I have observed a novel interaction with another TFIIIB subunit, Brf1 (Figure 3.2). To further investigate this interaction, fragments of Brf1 were tested for their ability to bind to c-Myc. The c-Myc binding site on Brf1 was a region spanning amino acids 94-281 (Figure 3.4a and 3.4b). This region contains the two imperfect direct repeats of Brf1 and the presence of both of these repeats appears to be necessary for c-Myc binding, perhaps involving regions not together in the sequence, but in close proximity within the tertiary structure of the protein. The zinc ribbon domain of Brf1, although not sufficient for binding to c-Myc, appears to be necessary for the full interaction, as deletion of this region results in lower affinity binding (Figure 3.4b).

TFIIIB and its Brf1 subunit are involved in multiple interactions with regulators of pol III transcription. The RB tumour suppressor binds to TFIIIB to inhibit pol III transcription complex formation (Larminie et al, 1997; Sutcliffe et al, 2000). RB also binds to Brf1, and this interaction is also in the direct repeats of Brf1 (Figure 3.5b and 3.5c). Unlike the binding of c-Myc, RB can bind to both of the direct repeats individually, suggesting a different binding motif in Brf1 (3.5b and 3.5c).

Although the binding of c-Myc and RB to Brf1 is clearly through different mechanisms, it would be interesting to know if these proteins can bind to TFIIIB simultaneously, as they have opposite roles in regulating pol III transcription (White,

2004; White 2005). It may be that these proteins can compete with each other in vivo to convert TFIIIB into active and inactive states. Although this interplay between the two regulators may happen, the role of c-Myc in activating pol III transcription is clearly more than a de-repressor of RB inhibition as it can activate pol III transcription in murine fibroblasts that are deficient for RB and the related pocket proteins (Gomez- Roman et al, 2003).

Another regulator of pol III transcription is the MAPK ERK. ERK can bind to and phosphorylate the Brf1 subunit of TFIIIB. This phosphorylation event can promote the formation of the pre-initiation complex on pol III-transcribed genes. A mutant form of Brf1, which has less affinity for ERK, can bind to c-Myc as efficiently as wild type Brf1 (Figure 3.6). As both ERK and c-Myc will be activated and will both exert their effect on pol III transcription in response to similar mitogenic stimuli, the use of this mutant will be help to elucidate how these separate proteins activate pol III transcription.

Further experiments need to be performed to further define the interaction between c-Myc and Brf1. If we can identify the interaction domain of Brf1 that can bind to c-Myc, we will be able to further understand the role of this activator in pol III transcription. A mutant Brf1 deficient in c-Myc binding, but capable of interaction with the pol III transcription apparatus and other regulators of transcription may help us answer the question of how important c-Myc is in the activation of pol III transcription. Also, perhaps more interestingly, a mutant form of Brf1, which cannot interact with c-Myc, may also let us investigate how important the activation of pol III transcription is for c-Myc-dependent transformation of cells.

To identify residues within the direct repeats of Brf1 that are involved in c-Myc binding, we can compare residues between yeast Brf (yBrf) and human Brf1. When used in pull down assays, yBrf1 binds to the recombinant c-Myc protein used in these assays (data not shown). If the sequences of yBrf and hBrf1 are compared and only regions that share high homology are targeted, it may be possible to develop a strategy to identify potential binding domains. It would be useful to also use the TFIIB sequence in this comparison, but as there are studies suggesting c-Myc and TFIIB cannot interact (McEwan et al, 1996), as well as studies suggesting that they do (Shrivasta et al, 1996; Stefan Roberts, personal communication), this controversy would have to be resolved before TFIIB could be used in the comparison.

As mentioned in the introduction, c-Myc contains two regions within the TAD that are extremely well conserved throughout the Myc family of proteins, MBI and MBII. These regions are critical to the ability of c-Myc to transform cells and are also important for interactions with co-activator proteins.

Co-immunoprecipitation assays demonstrate that mutant c-Myc proteins lacking these regions can bind to TFIIIB as efficiently as the wildtype protein (Figure 3.8). The region of c-Myc binding to TFIIIB is still unclear and needs to be investigated, but is narrowed down to the N-terminal 262 amino acids of c-Myc, not dependant on the conserved MBI and MBII regions (Figure 3.7). Further experiments need to be performed to narrow down this interaction domain, to define regions of c-Myc critical for TFIIIB binding.

Experiments have been performed to assess the ability of c-Myc proteins missing MBI or MBII to stimulate pol III transcription. Preliminary results suggest that both MBI and MBII are critical for c-Myc dependent activation of pol III transcription (data not shown). The lack of activation when using mutant c-Myc proteins, even though they are wildtype for TFIIIB binding, may suggest that co-activator proteins that bind to these regions of c-Myc, may be involved in the c-Myc dependent activation of pol III transcription.

In experiments later in this thesis, co-activator proteins that can bind to c-Myc will be investigated in their ability to contribute in the c-Myc dependent activation of pol III transcription.

Chapter 4 Activation of Poi III transcription by c-Myc results in sequential recruitment of transcription factors

4.1 Introduction

The data presented in Chapter 3 demonstrates that the product of the c-Myc oncogene binds to two subunits of the pol III-specific transcription factor TFIIIB. The functional consequences of this interaction are investigated in the remainder of this thesis. This chapter focuses on the potential ability of c-Myc to recruit pol III specific transcription factors to form an active transcription complex on the promoters of target genes. Determining the mechanisms behind the c-Myc-dependent activation of pol III transcription is crucial when attempting to understand c-Myc's contribution to tumourigenesis.

4.1.1 Pol III transcriptional regulaton

Transcription can be divided into three stages: initiation, elongation and termination. As discussed in Chapter 1 due to the short length of the transcripts and the ability of pol III to recognise a termination signal without the need of accessory factors, elongation and termination are not thought to be the main stages that pol III transcription is regulated (White, 2002). Therefore, the primary mechanism by which a pol III-transcribed gene is regulated is at the level of transcriptional initiation. To

initiate transcription of class III genes, the basal transcription factors and the polymerase need to be recruited to the promoter of the target gene. Activation of the vast majority of pol III-transcribed genes requires the recruitment of a multi-subunit, pol III-specific transcription factor, TFIIIC. This complex recognises and binds to the promoter in a sequence-specific manner. This is followed by the recruitment of another pol III-specific complex, TFIIIB, that is recruited by protein:protein interactions with TFIIIC (Schramm and Hernandez, 2002; White 2002; Chapter 1). The polymerase, which cannot recognise the start sites of transcription alone, is then recruited to the start sit of transcription mainly through protein:protein interactions with TFIIIB (Schramm and Hernandez, 2002; White 2002; Chapter 1). In studies completed to date the polymerase recruiting factor, TFIIIB, is targeted by activators and repressors of pol III transcription to either prevent or promote the formation of the pol III pre-initiation complex (White, 1998, White, 2004).

4.1.2 Regulators of Pol III transcriptional Initiation

TFIIIB is a multi-subunit complex containing the TATA-binding protein, TBP, and two pol III specific TAFs, Brf1 and Bdp1 (Chapter 1; Schramm and Hernandez, 2002). As previously discussed, both activators and repressors of pol III transcription have been shown to target TFIIIB to exert their action on pol III transcription (White, 1998, White, 2004). The oncogenic kinase CK2 has been demonstrated to be involved in the activation of pol III transcription in yeast and mammals (Hockman and Schultz, 1996, Johnston et al, 2002). CK2 can bind to and phosphorylate TFIIIB to activate transcription. The target subunit differs in higher eukaryotes, compared to lower eukaryotes, with the pol III specific TAF, Brf1, being the target in mammals,

and TBP being the target in yeast (Johnston et al, 2002; Ghavidel and Schultz, 1997). This binding results in the assembly of an initiation complex through a greater affinity of TFIIIB for TFIIIC (Johnston et al. 2002). Another activator of pol III transcription is the MAP kinase ERK. The ERK MAP kinase cascade has been demonstrated to promote cell growth by activating transcription by pol I, by directly phosphorylating and activating the pol I-specific transcription factor UBF (Stefanovsky et al, 2001). ERK induces Pol III transcription by binding to and phosphorylating Brf1 (Felton-Edkins et al, 2003a). This phosphorylation event leads to the formation of the basal transcription complex, by increasing the affinity of TFIIIB for both TFIIIC and pol III (Felton-Edkins et al, 2003a). It has been demonstrated using synthetic inhibitors of the MAP kinase pathway, that promoter occupancy of both TFIIIB and pol III is lower when Erk in inactive (Felton-Edkins et al, 2003a).

Pol III transcription can also be repressed by tumour suppressor proteins that exert their effect on TFIIIB. When in its active, hypo-phosphorylated form, the tumour suppressor Rb can inhibit pol III transcription by binding to TFIIIB (White et al, 1996; Larminie et al, 1997; Chu et al, 1997; Sutcliffe et al, 2000). This binding of Rb to TFIIIB inhibits the interaction between TFIIIB and TFIIIC, and the interaction of TFIIIB and the polymerase (Sutcliffe et al, 2000). The tumour suppressor p53 can also inhibit pol III transcription by targeting TFIIIB (Chesnokov et al, 1996; Cairns and White, 1998). p53 can bind to the TBP subunit of TFIIIB and this binding of p53 to TFIIIB compromises its ability to interact with TFIIIC (Cairns and White, 1998). Induction of p53 in cultured cells leads to TFIIIB occupancy on pol III-transcribed genes being reduced, while TFIIIC occupancy remains constant (Crighton et al, 2003). The sequestration of TFIIIB by tumour suppressors represents a mechanism by which normal cells can prevent inappropriate expression of pol III products, to

regulate the biosynthetic capacity of the cell. Experiments in this chapter will investigate whether c-myc can activate transcription by pol III by targeting pol III-specific transcription factors.

4.1.3 Activation of Transcription through increases in transcription factor expression

Another mechanism to regulate pol III output is by raising the levels of one or more of the limiting transcription factors on which it depends. Changes in pol III gene expression have been shown to coincide with changes in the expression of pol III specific transcription factors. The activity of the pol III promoter recognition complex, TFIIIC, is raised in response to adenoviral infection or SV40 transformation (Hoeffler and Roeder, 1985; White et al, 1990). Levels of all five subunits of TFIIIC are upregulated in fibroblasts transformed with SV40 or polyoma virus and, more clinically relevant, all five subunits are elevated at the protein and mRNA level in each of a panel of ovarian carcinoma biopsies as compared to normal tissue (Larminie et al, 1999; Felton-Edkins and White, 2002; Winter et al, 2000)

Specific subunits of the polymerase recruitment factor, TFIIIB, have also been found to be elevated in tumour cell lines to contribute to an increase in pol III-transcription. TBP is overexpressed as a consequence of activation of the Ras pathway in virus transformed cell culture lines, and this leads to an increase in pol III-transcription (Wang et al, 1997). Overexpression of TBP has also been found in a panel of colon carcinomas compared to normal tissue, and overexpression of TBP has been found to cause tumours in nude mice (Johnston et al, 2003). Levels of the pol III-specific

TAFs, Brf1 and Bdp1, have been demonstrated to be elevated in some transformed and tumour cell lines (Daly at al, 2005; Felton-Edkins et al, 2002).

Not only are subunits of the pol III transcription apparatus unregulated in response to transformation, elevating the protein levels of the pol III-specific TAF, Brf1, using an inducible cell line, is sufficient to activate pol III transcription and drive cellular growth and proliferation (Marshall and White, unpublished observations).

Although c-Myc can directly activate pol III transcription independently of de novo protein synthesis (Gomez-Roman et al, 2003), c-Myc may play an additional role in regulating expression of pol III-specific transcription factors.

4.1.4 Activation of transcription by c-Myc through interactions with basal transcription factors

The c-myc transcription factor can interact with components of the pol II basal transcription machinery. It has been shown that c-Myc is associated with TBP and TFIIF in vivo (Maheswaran et al, 1994; McEwan et al, 1996). The interaction between c-Myc and components of the basal transcription machinery may represent a mechanism through which c-Myc can contribute to the recruitment of the pol II pre-initiation complex to the promoters of target genes (Maheswaran et al, 1994). It has been demonstrated that the recruitment of TBP to the promoters of certain target genes efficiently activates transcription, suggesting recruitment of TBP can be rate-limiting in the activation of transcription of protein-encoding genes (Chetterjee and Struhl, 1995; Klages and Strubin, 1995). c-Myc may work as an activator in this way

by recruiting the TBP-containing TFIID complex to the promoters of target genes. c-myc has been shown to interact with the pol III-specific TBP containing TFIIIB complex. As genes transcribed by pol III do not contain well defined E-box sequences in their promoter regions, it is thought that the interaction with TFIIIB is responsible for the presence of c-myc at the promoters of pol III-transcribed genes (Gomez-Roman et al, 2003; Felton-Edkins et al, 2003b, Chapter 3). As pol III-transcribed genes do not contain E-box sequences, the simplistic model through which c-myc-max heterodimers bind to the promoters of target genes and contribute to the binding of a TBP-containing factor to the promoter, cannot be the case in transcription of pol III-transcribed genes. c-Myc may still contribute to the formation of a pol III pre-initiation complex by acting in concert with TFIIIB Experiments in this chapter focus on the formation of the pol III pre-initiation complex in response to activation of c-Myc.

4.2 Results

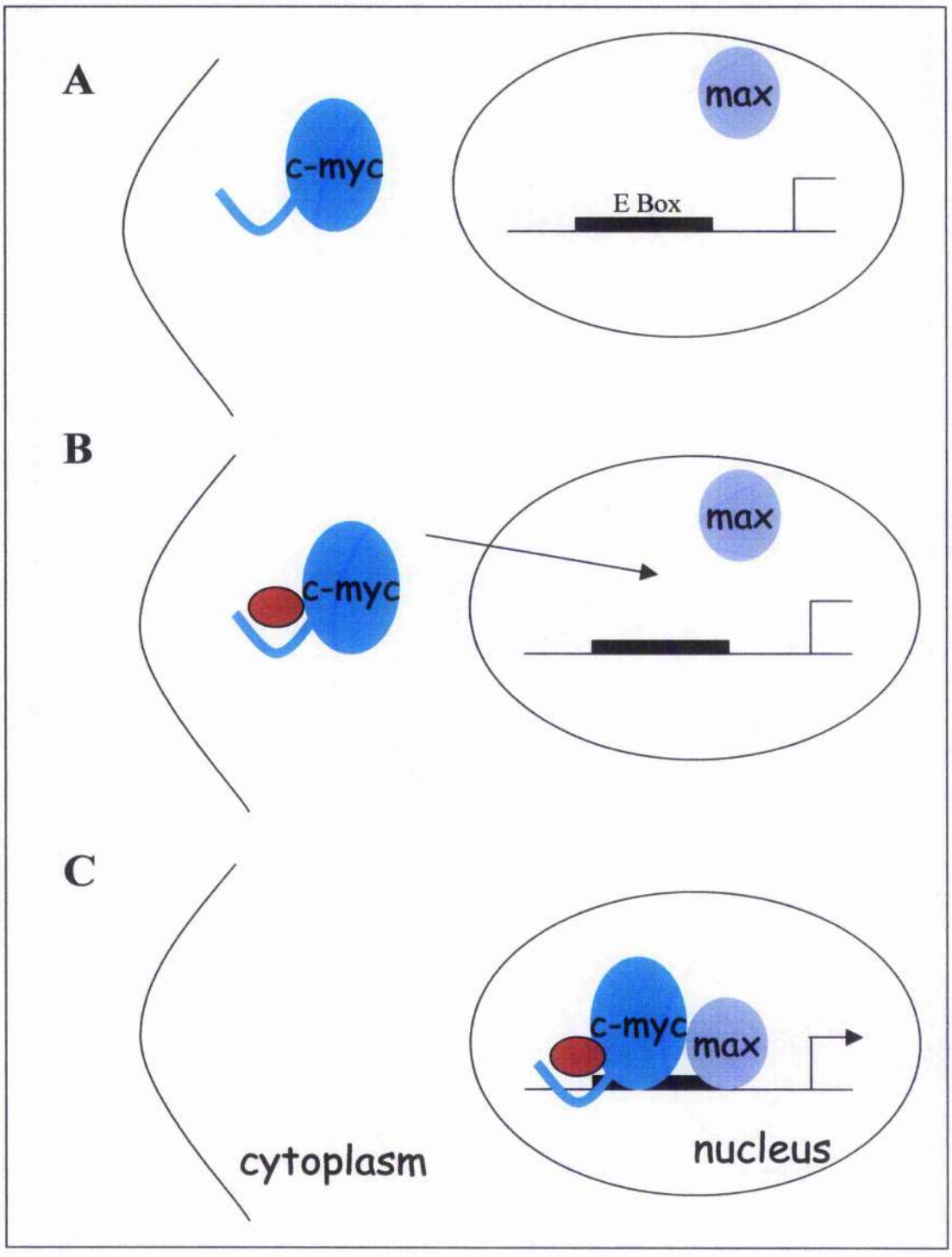
4.2.1 c-Myc is recruited to class III genes in vivo

As summarised in the introduction to this chapter, c-Myc is a potent and direct activator of pol III transcription (Gomez-Roman et al, 2003). To attempt to uncover the mechanisms by which c-Myc can achieve this, a c-Myc-inducible cell line was used. The cell lines were derived from A31 murine fibroblasts stably transfected with a vector encoding a c-Myc fusion protein (MycER) or vector alone (pBabe). The MycER vector encodes full length c-myc fused to a modified oestrogen receptor binding domain at its C-terminal bHLHZ DNA binding domain (Littlewood et al, 1995). The modified oestrogen receptor ligand-binding domain can no longer bind to oestrogen, but retains affinity for the synthetic ligand, 4-hydroxytamoxifen (4-OHT) (Littlewood et al, 1995, Danielian et al, 1993). In the absence of ligand the MycER fusion protein remains cytoplasmic, unable to enter the nucleus (Figure 4.1). Upon addition of the ligand, 4-OHT, the MycER protein can refold and translocate to the nucleus to interact with its co-activator protein Max, to then transactivate c-Myc target genes (Figure 4.1; Bouchard et al, 2001).

To determine that the cells were behaving as predicted, the cells were serum starved for 48 hours, to lower levels of endogenous c-Myc, and then treated with 4-OHT to activate the MycER protein. Nuclear enriched extracts were made from pBabe and MycER cells and nuclear levels of MycER were determined by western blot after the indicated time points (Figure 4.2). The MycER protein is translocated to the nucleus of MycER cells 2 hours after addition of 4-OHT, and remains high at the 4 hour time

Figure 4.1 Schematic of c-myc activation in the MycER cell line

A. MycER cells are serum starved to lower levels of endogenous c-myc. In the absence of ligand the MycER protein is excluded from the nucleus and remains cytoplasmic, unable to act on its target genes. B. Upon addition of 4-OHT the ligand binds to the hormone-binding domain of the oestrogen receptor protein, and this results in a conformational change of the fusion protein. C. This conformational change leads to nuclear translocation of the MycER protein, which allows it to dimerise occupancy with Max transactivate its target genes.


A
c-myc
max
E Box
B
c-myc
max
C
c-myc
max
cytoplasm
nucleus

point (Figure 4.2). A signal is detected for nuclear MycER at the 0 hr time point, which is prior to activation by the ligand (Figure 4.2). This is due to "leakiness" in the system, with a small pool of the MycER protein being present in the nucleus in the absence of ligand. As expected no signal was obtained from the nuclear extracts from the pBabe cells (Figure 4.2).

In order to assess c-Myc binding to class III genes in these cell lines, ChIP assays were performed. The protein-DNA complexes were immunoprecipitated using a c-Myc-specific polyclonal antibody. Recovered DNA was analysed by PCR using specific primers. In response to MycER activation, the chimeric protein is recruited to the promoter of the Cyclin D2 gene after 2hours and remains associated at 4hours (Figure 4.3a; Bouchard et al, 2001; Frank et al, 2001). In addition, the MycER protein is recruited to the pol III-transcribed 5S and $tRNA^{Leu}$ genes in response to addition of 4-OHT (Figure 4.3a). The recruitment of MycER to the promoters of class III genes and the cyclin D2 positive control is specific, as no recruitment is observed at the p21 promoter (Figure 4.3a). ChIP assays were performed to ensure the material immunoprecipitated using the anti c-Myc antibody was due to the activated MycER protein, and not due to an artefact caused by addition of 4-OHT. pBabe cells were treated with 4-OHT and cells harvested at the indicated time points. No increase in DNA corresponding to the $tRNA^{Leu}$ and the 5S rRNA gene was immunoprecipitated using a c-Myc antibody after 4-OHT treatment, as determined by PCR, indicating the effect was specific to the MycER cell line (Figure 4.3b).

Figure 4.2 MycER protein translocates to the nucleus in response to activation by 4-OHT

Nuclear enriched extracts were prepared form cells containing pBabe or MycER vectors, treated with 200nM 4-OHT to activate the MycER protein for the indicated times. Samples were resolved by SDS-PAGE and western blotting was performed using anti-c-Myc and anti-TAF_I48 antibodies.

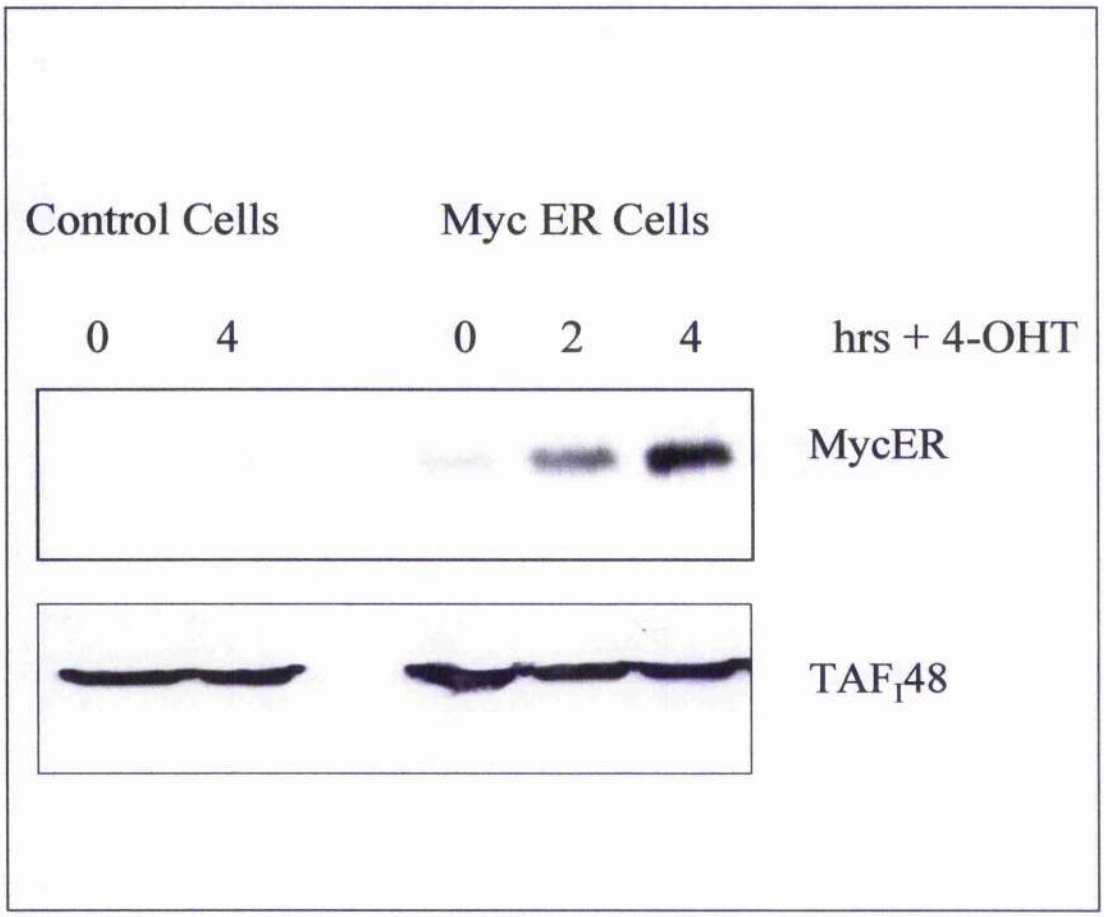
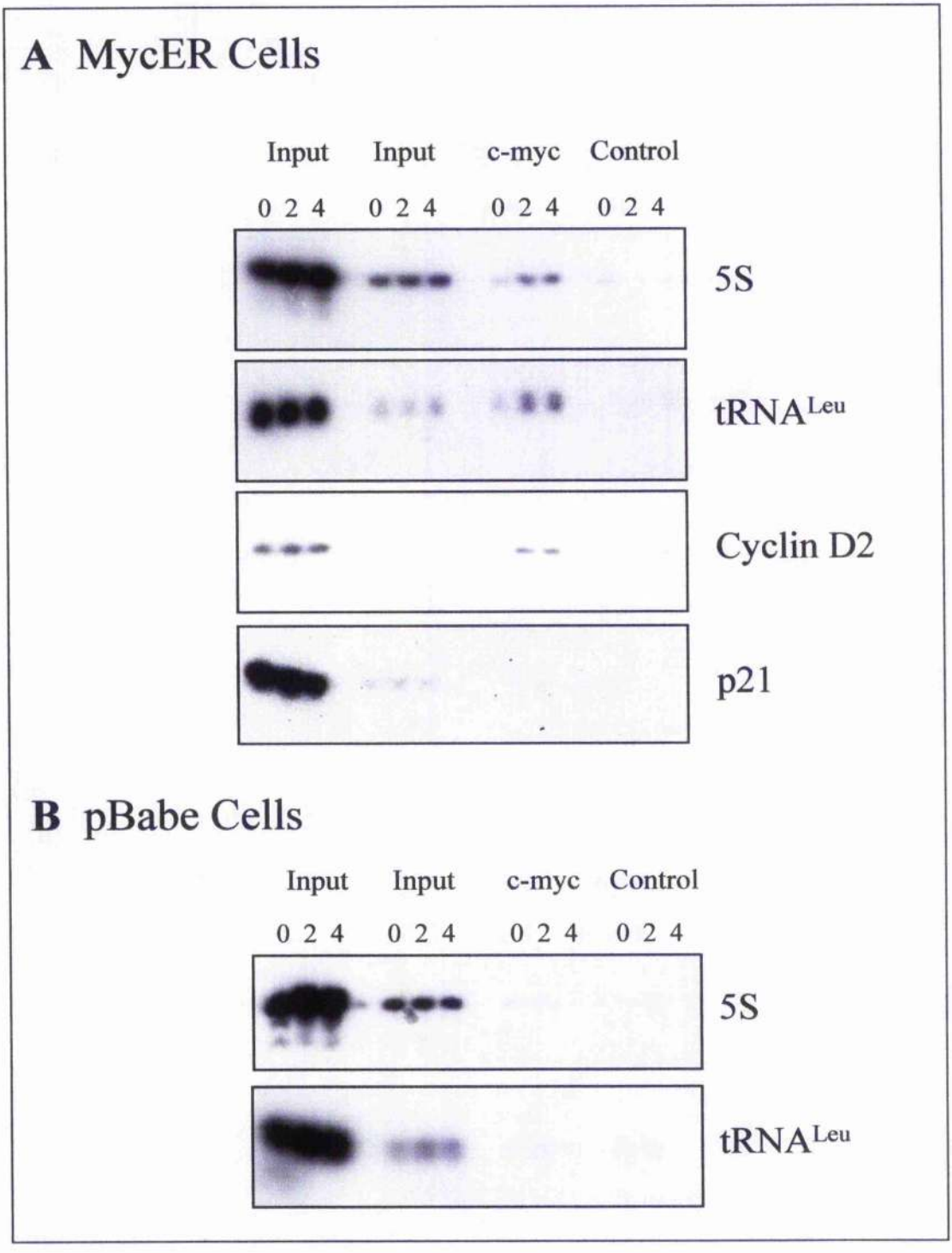

Control Cells
Myc ER Cells
0
4
0
2
4
hrs + 4-OHT
MycER
TAF$_I$48

Figure 4.3 MycER is recruited to pol III-transcribed genes

A. MycER was induced in fibroblasts by addition of 200nM 4-OHT. ChIP assays were performed at the indicated time points after MycER activation using antibodies against c-myc and TAF_I48 (control). The samples were normalised by comparing the genomic DNA inputs. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using primers specific to the promoters of 5S, $tRNA^{Leu}$, Cyclin D2 and p21 genes, as indicated. B. ChIPs were performed as above using the pBabe control cell line. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using primers specific to the promoters of 5S and $tRNA^{Leu}$ genes.


A MycER Cells
Input
Input
c-myc
Control
0 2 4
0 2 4
0 2 4
0 2 4
5S
tRNA Leu
Cyclin D2
p21
B pBabe Cells
Input
Input
c-myc
Control
0 2 4
0 2 4
0 2 4
0 2 4
5S
tRNA Leu

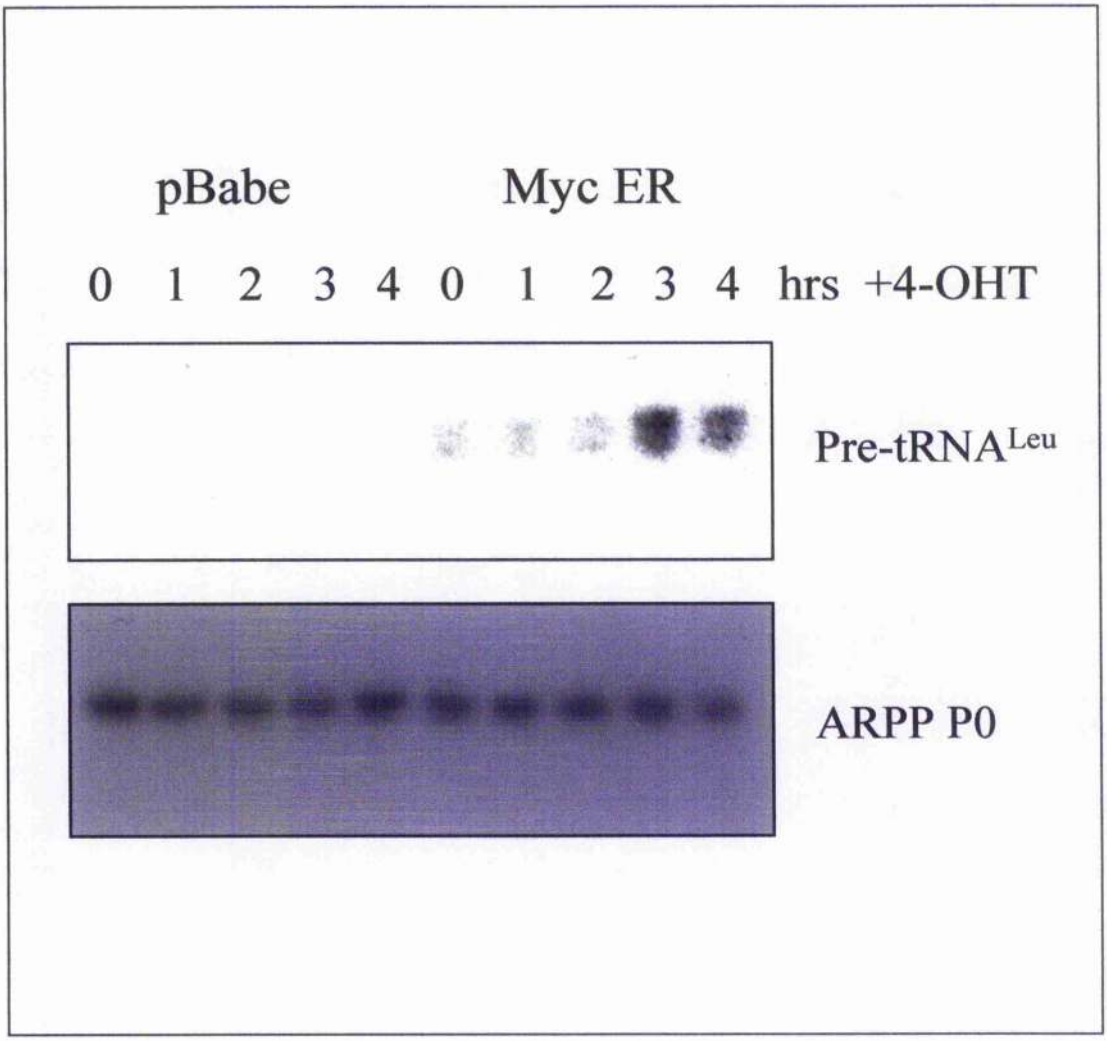
RT-PCR analysis of RNA harvested from Rat derived MycER cells activated with 4-OHT revealed a specific increase in the levels of pol III transcripts (Gomez-Roman et al, 2003). To determine the timing of the induction of pol III transcription in murine MycER cells, RT-PCR analysis of pol III transcripts was performed using RNA harvested from pBabe and MycER cells treated with 4-OHT. The levels of pol III transcripts were normalised to the pol II-transcribed ARPP P0 transcript. The levels of the pol III transcript pre-tRNA^{Leu} is not affected by the addition of 4-OHT in the pBabe cell line (Figure 4.4). In contrast, the addition of 4-OHT to the MycER cells caused an induction of the pol III-transcribed tRNA^{Leu} and 5S rRNA genes 3 hours after addition of the hormone to the cells (Figure 4.4). The timing of the induction pol III transcription in A31 derived MycER cells is similar to that observed in Rat derived MycER cells (Figure 4.4; Gomez-Roman et al, 2003). There was a higher level of transcription of the tRNA^{Leu} gene in the MycER cells compared to the pBabe cells before treatment with 4-OHT (Figure 4.4). The higher level of transcription of the tRNA gene in the MycER cells compared to the pBabe cells is likely to be due to the small amount of the MycER protein present in the nucleus of MycER in the absence of 4-OHT (Figure 4.2).

4.2.2 Activation of c-Myc results in a sequential recruitment of the Pol III transcription apparatus to class III genes

As outlined in the introduction to this chapter, and in chapter 1, activators of Pol III transcription can act by increasing levels of pol III transcription factors or can act at the level of transcriptional initiation to promote the formation of the pol III transcription complex. Using the MycER cell line the relative promoter occupancies

Figure 4.4 Pol III transcriptional activation by MycER activation

Cultured pBabe and MycER cells were treated with 200nM 4-OHT for the indicated time points and the cells were harvested for total RNA. RNA was analysed by RT-PCR for expression of 5S, pre-tRNA and ARPP P0 (control) using gene-specific primers.


pBabe
Myc ER
0 1 2 3 4 0 1 2 3 4 hrs +4-OHT
Pre-tRNA^{Leu}
ARPP P0

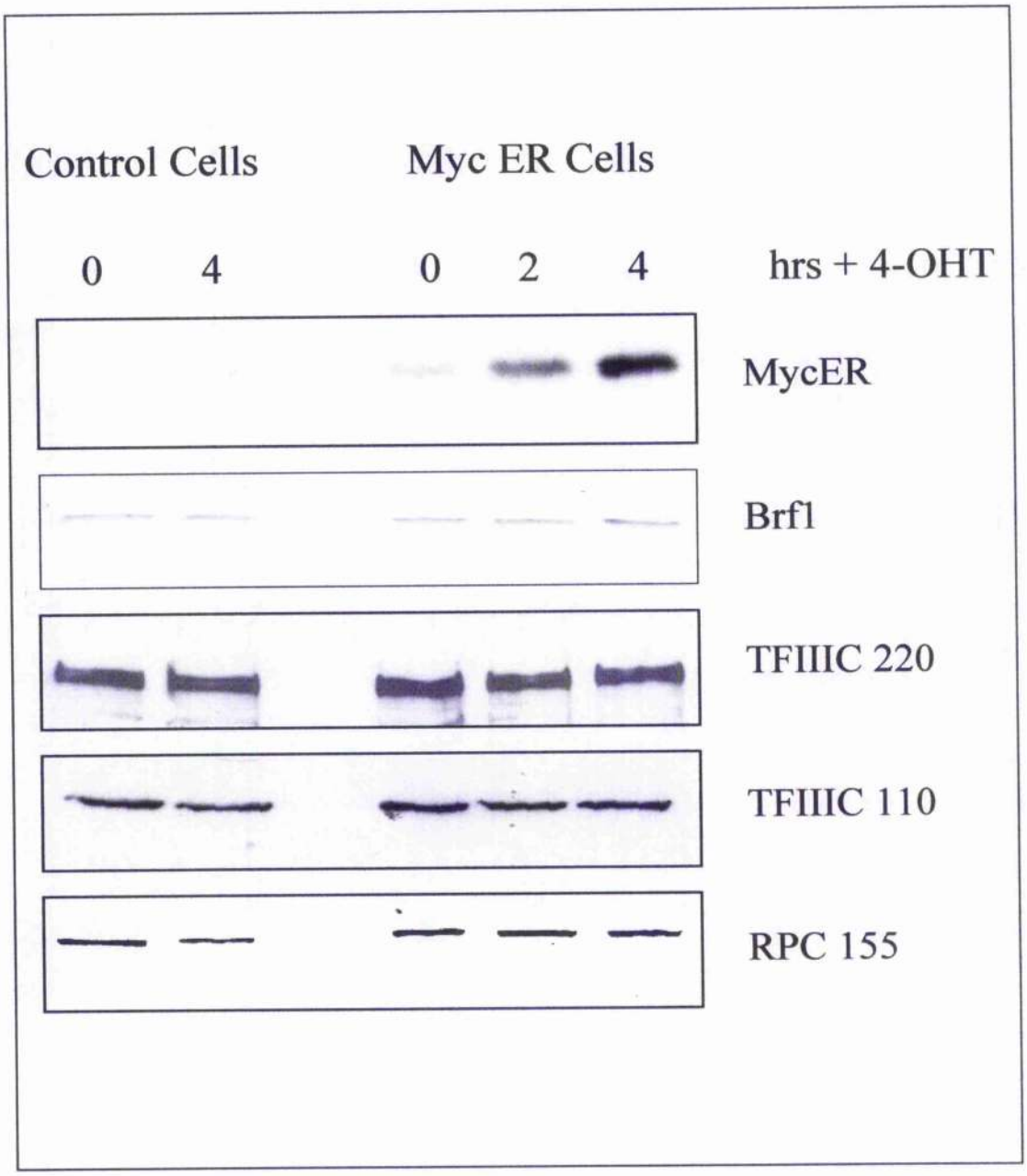
of the pol III transcription apparatus was measured in response to MycER activation, to assay any role of c-Myc in formation of this complex.

It has been previously determined that pol III transcription can be activated by c-Myc independently of any secondary events due to c-Myc mediated regulation of protein encoding genes (Gomez-Roman et al, 2003). To confirm pol III transcription factors were not being induced in these assays, western blots were performed on nuclear enriched extracts from pBabe and.MycER cells treated with 4-OHT. Western blotting revealed that there was no induction of the protein levels of Brf1, Bdp1, TFIIIC 110, TFIIIC 220 or the large pol III sub-unit RPC 155 in response to c-Myc activation (Figure 4.5). This result confirmed that c-Myc is activating pol III transcription, in these experimental conditions, by a mechanism other than the expression of pol III specific transcription factors.

When activating pol I transcription c-Myc can stimulate recruitment of the pol I-specific transcription factors UBF and SL1, as well as the polymerase (Grandori et al, 2005; Arabi et al, 2005). To determine if there are any changes in the promoter occupancy of the pol III transcription apparatus in response to the activation of c-myc, ChIP assays were performed using the MycER cell line. ChIP assays were performed using antibodies against the TFIIIB subunits Brf1 and Bdp1; the TFIIIC subunits TFIIIC 110 and TFIIIC 220; the largest subunit of the polymerase RPC 155 and the pol I TAF, TAF_I48, as a negative control. The TFIIIC subunits TFIIIC 110 and TFIIIC 220 are bound to the $tRNA^{Leu}$ and 5S rRNA genes prior to the activation of the MycER protein. The activation of MycER does not result in further binding of either TFIIIC subunit to the $tRNA^{Leu}$ or 5S rRNA genes (Figure 4.6a). The TFIIIB

Figure 4.5 Analysis of expression of pol III basal transcription factors in response to MycER

Nuclear enriched extracts were prepared from cells containing pBabe (control) or MycER vectors, treated with 4-OHT for the indicated times. Extracts were resolved by SDS-PAGE and blotted using antibodies against Brf1, Bdp1, TFIIIC 110, TFIIIC 220 and RPC 155.


Control Cells
Myc ER Cells
0
4
0
2
4
hrs + 4-OHT
MycER
Brf1
TFIIIC 220
TFIIIC 110
RPC 155

subunits Brf1 and Bdp1 are bound at low levels to $tRNA^{Leu}$ and 5S rRNA genes prior to MycER activation. The activation of MycER results in an enrichment of both Brf1 and Bdp1 levels on the $tRNA^{Leu}$ and 5S rRNA genes 2 hours after MycER activation (Figure 4.6a). The polymerase subunit RPC 155 is bound to the $tRNA^{Leu}$ and 5S rRNA genes at low levels prior to MycER activation, remains at a low level after 2hours of 4-OHT treatment, but its occupancy on $tRNA^{Leu}$ and 5S rRNA is enriched 4 hours after MycER activation (Figure 4.6a). The immunprecipitations of the pol III transcription apparatus in this experiment are specific as no products were detected using specific primers against the promoter of the pol II-transcribed p21 gene (Figure 4.6a).

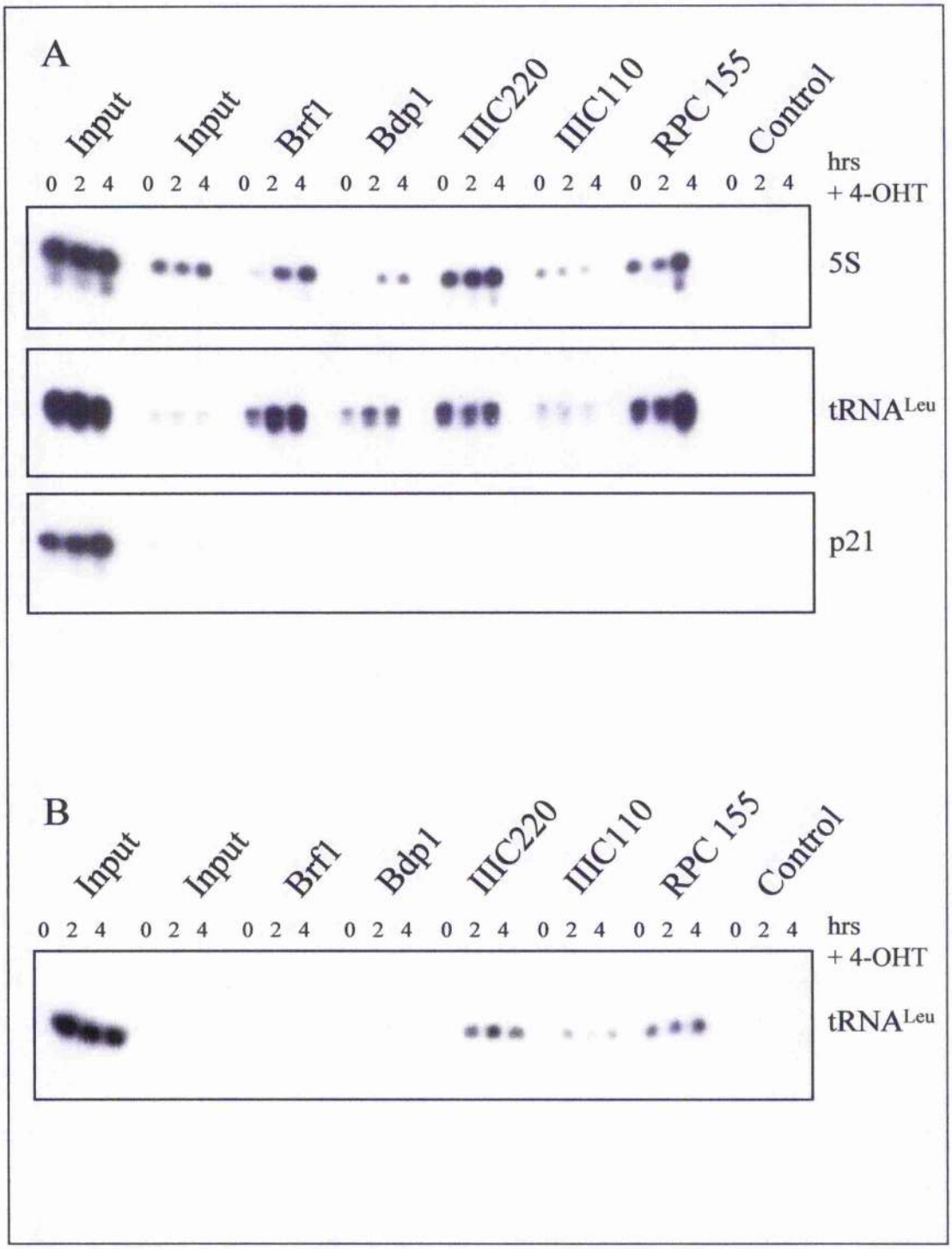
ChIP assays were performed to ensure the differences in promoter occupancies of the pol III specific transcription factors binding to the $tRNA^{Leu}$ and the 5S rRNA gene were due to the activated MycER protein, and not due to the 4-OHT. pBabe cells were treated with 4-OHT and cells harvested at the indicated time points. No differences in the occupancies of the pol III transcription apparatus were observed on the $tRNA^{Leu}$ and the 5S rRNA gene after 4-OHT treatment, as determined by PCR, indicating the effects were specific to the MycER cell line (Figure 4.6b).

4.2.3 TFIIIB and Pol III promoter occupancy is reduced on class III genes in c-myc null fibroblasts

Levels of the pol III-specific transcription factor TFIIIB and the polymerase are enriched on the $tRNA^{Leu}$ and 5S rRNA genes in response to c-Myc activation (Figure 4.6a). To establish the effects of c-Myc deficiency on the promoter occupancy of the

Figure 4.6 MycER activation leads to sequential recruitment of general transcription factors to promoters of pol III-transcribed genes

A. c-myc was induced in MycER fibroblasts by addition of 200nM 4-OHT. ChIP assays were performed at the indicated time points after MycER activation using antibodies against Brf1, Bdp1, TFIIIC 110, TFIIIC 220, RPC 155 and TAF_I48 (control). The samples were normalised by comparing the genomic DNA inputs. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using primers specific to the promoters of 5S, $tRNA^{Leu}$ and p21 genes as indicated. B. ChIPs were performed as above using the pBabe control cell line. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using primers specific to the promoter of the $tRNA^{Leu}$ gene.


A
Input
Input
Brf1
Bdp1
IIIC220
IIIC110
RPC 155
Control
0 2 4 0 2 4 0 2 4 0 2 4 0 2 4 0 2 4 0 2 4 0 2 4
hrs
+ 4-OHT
5S
tRNA^Leu
p21
B
Input
Input
Brf1
Bdp1
IIIC220
IIIC110
RPC 155
Control
0 2 4 0 2 4 0 2 4 0 2 4 0 2 4 0 2 4 0 2 4 0 2 4
hrs
+ 4-OHT
tRNA^Leu

pol III transcription apparatus, c-Myc null fibroblasts were compared to matched c-Myc wildtype cells (Mateyak et al, 1997).

The cells containing wildtype c-Myc display higher expression of pol III-transcribed genes, as compared to the c-Myc deficient cells determined by northern blot (Gomez-Roman et al, 2003). To confirm that the levels of pol III transcription in c-Myc wildtype fibroblasts was higher than the level of that in the c-Myc deficient fibroblasts, RT-PCR analysis of Pol III transcripts was performed using RNA harvested from the cells. The levels of transcription of pre-tRNA^{Leu} and pre-tRNA^{Tyr} were reduced in the c-Myc deficient fibroblasts compared to the wildtype; this effect was specific as no effect was seen on the pol II transcribed ARPPP0 control transcript (Figure 4.7).

To examine what effects c-myc deficiency has on the promoter occupancy of the pol III transcription apparatus, ChIP assays were performed using antibodies against the large Pol III subunit, TFIIIB subunit Brf1, TFIIIC subunit TFIIIC 110 and TFIIB as a negative control. The promoter occupancy of TFIIIC is comparable between the wild type and null cell lines. In contrast the levels of both the polymerase and TFIIIB are enhanced on the tRNA^{Leu} and tRNA^{Tyr} genes in the wildtype cell line compared to that of the null cell line (Figure 4.8).

The abundance of pol III transcription factors was analysed in these matched cell lines. Western blots were performed using antibodies against TBP, Brf1, TFIIC 110, RPC 155 and actin (control). The levels of TBP, TFIIIC and the pol III subunit are comparable between c-Myc null and c-Myc wildtype cell lines (Figure 4.9). The

Figure 4.7 Pol III transcription is compromised in c-Myc deficient fibroblasts

RNA was harvested from c-myc null or wild-type rat fibroblasts. RNA was analysed by RT-PCR for expression of pre-tRNA^{Leu}, pre-tRNA^{Tyr} and ARPP P0 (control) using gene-specific primers.

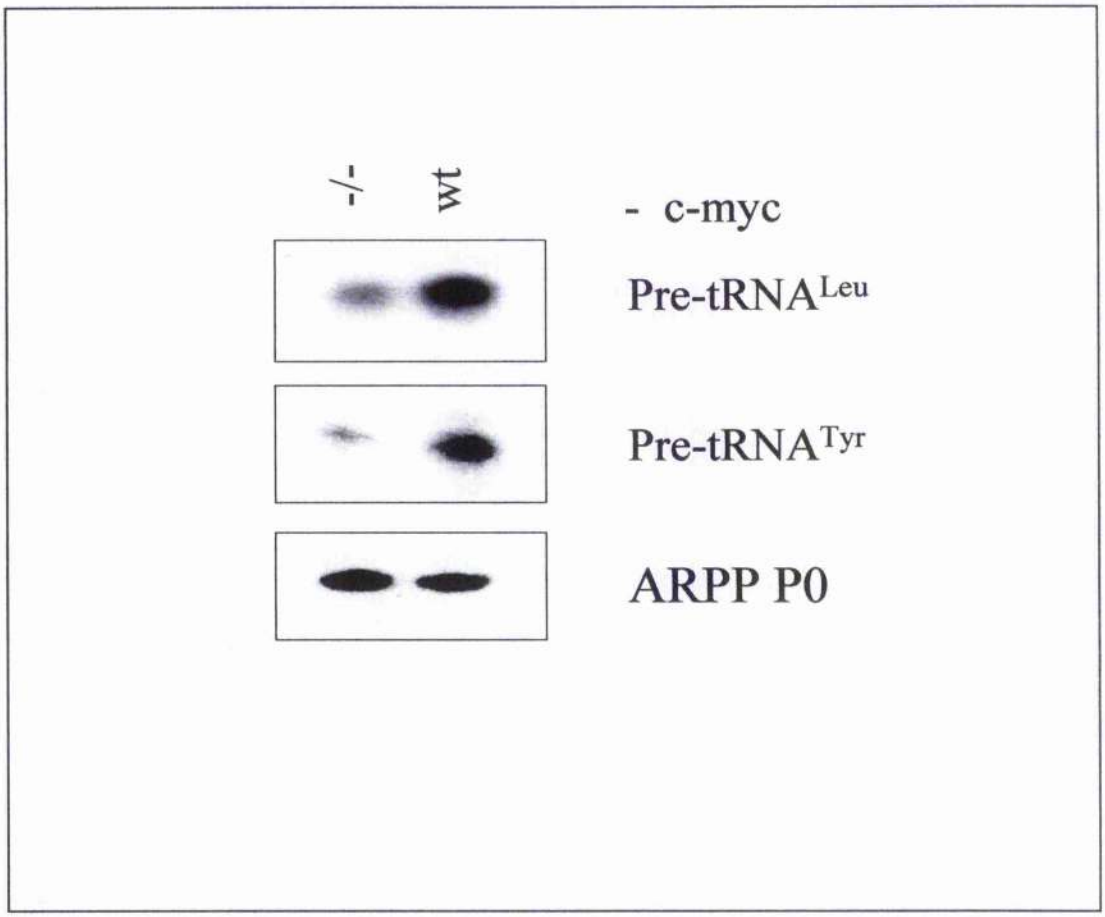

-/-
wt
- c-myc
Pre-tRNA^{Leu}
Pre-tRNA^{Tyr}
ARPP P0

Figure 4.8 Pol III and TFIIIB are enriched on the promoters of Pol III-transcribed genes in vivo in a c-Myc-sensitive manner

The promoter occupancy of TFIIIB and pol III was measured in matched c-myc$^{-/-}$ and c-myc$^{+/+}$ rat fibroblasts. ChIPs were performed using antibodies against Brf1, TFIIIC 110, RPC 155, and TFIIB. The samples were normalised by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using tRNA^{Leu}, tRNA^{Tyr}, and ARPP P0 primers as indicated.

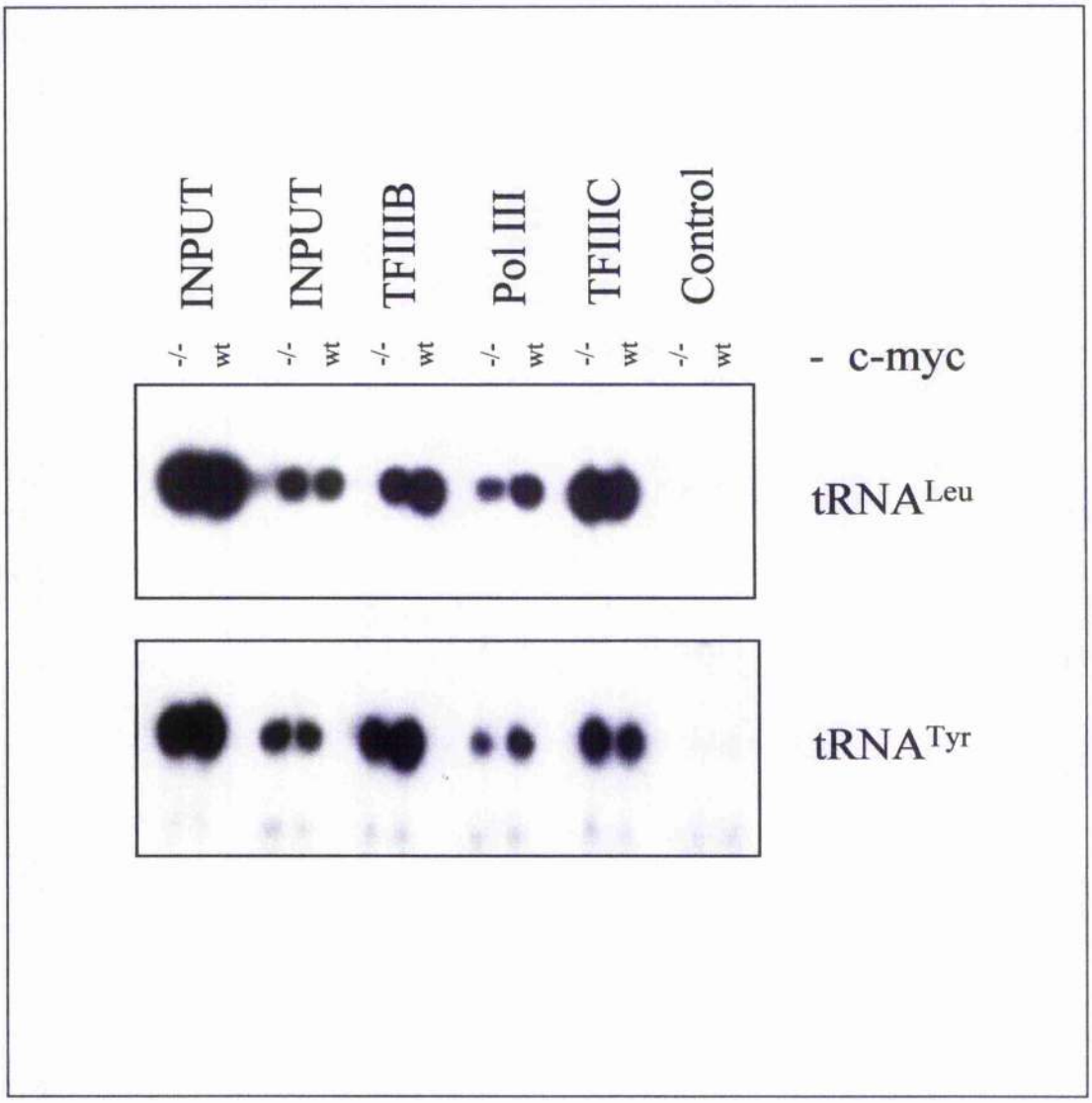
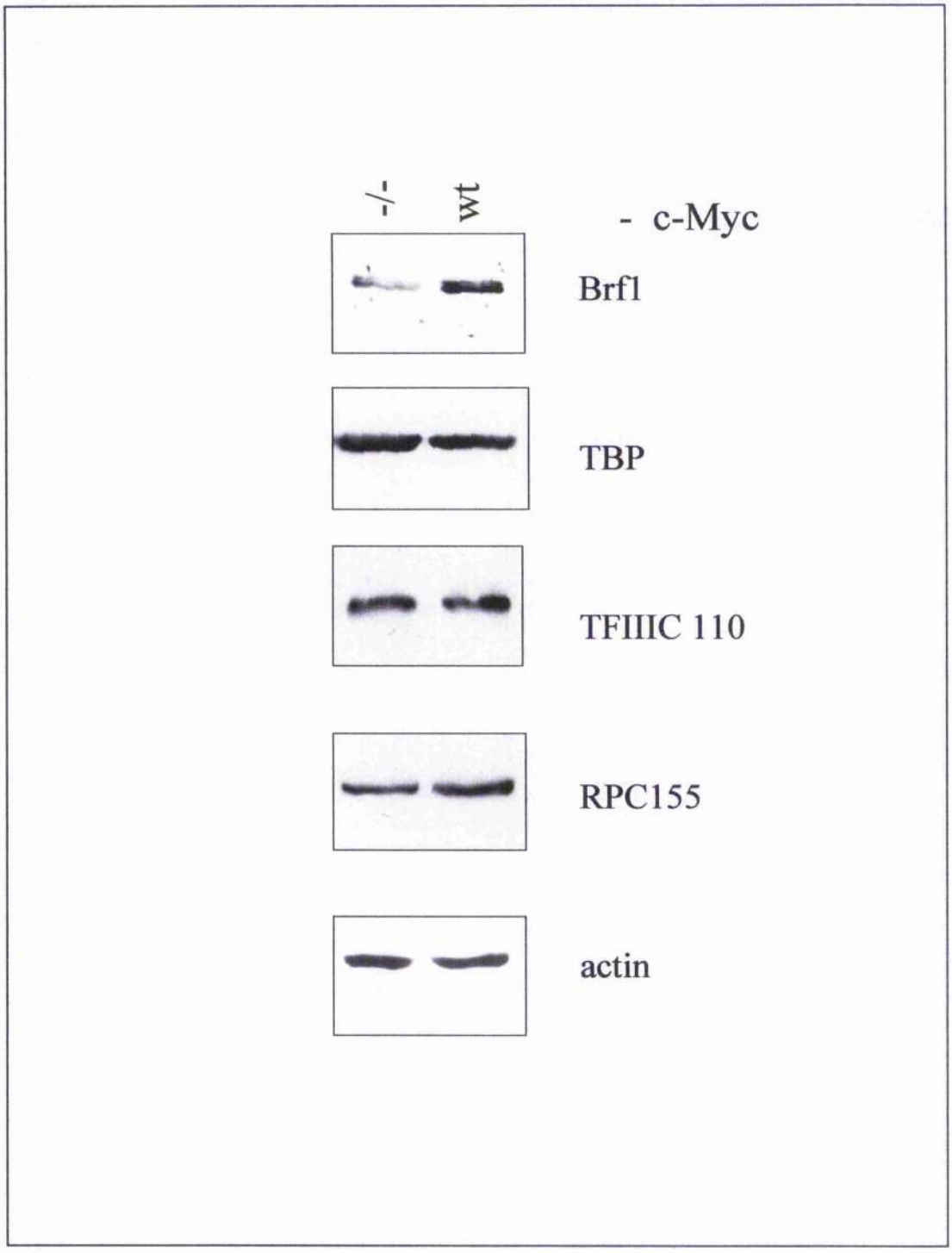

INPUT
INPUT
TFIIIB
Pol III
TFIIIC
Control
-/- wt -/- wt -/- wt -/- wt -/- wt -/- wt
- c-myc
tRNA^Leu
tRNA^Tyr

Figure 4.9 The levels of the Brf1 sub-unit of TFIIIB are lower in c-Myc null fibroblasts

Whole cell extracts were harvested from c-myc null and wild type fibroblasts. Protein extracts were resolved by SDS-PAGE, and western blotting was performed with antibodies against Brf1, TBP, RPC 155, TFIIIC 110 and actin.


-/-
wt
- c-Myc
Brf1
TBP
TFIIIC 110
RPC155
actin

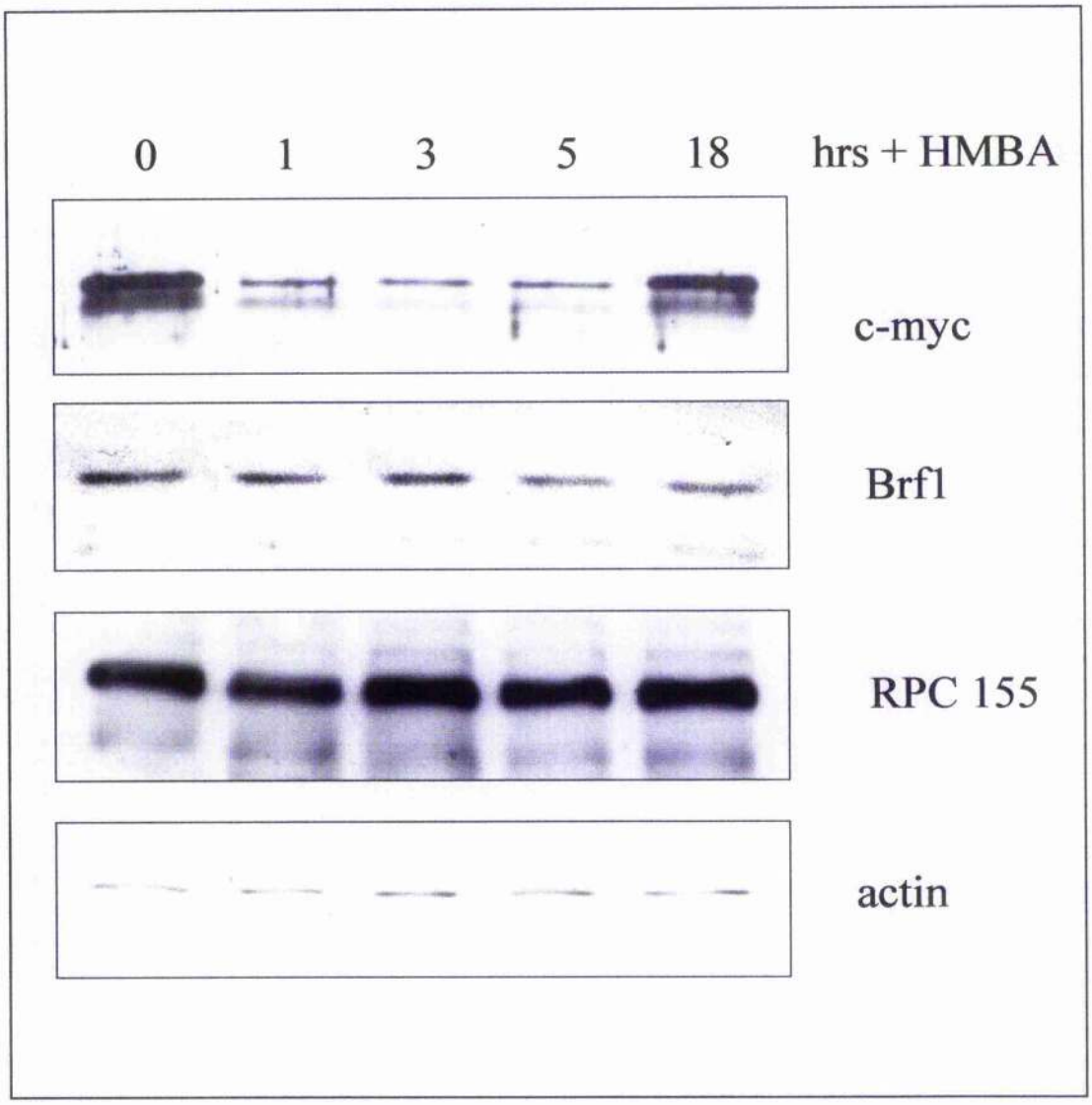
levels of the TFIIIB subunit Brf1 are elevated in c-Myc wildtype cells, compared to c-Myc null cells (Figure 4.9).

4.2.4 Levels of Polymerase on promoters of pol III-transcribed genes is reduced in response to terminal differentiation of MEL cells

To investigate how physiological changes in c-Myc levels correlate with expression of pol III transcribed genes and transcription factor occupancy, experiments were performed using murine erythrleukemia (MEL) cells. . These cells derive from proerythroblasts that were immortalized with the Friend viral complex (Friend, 1978). When MEL cells are treated with 5mM HMBA they are able to re-enter their original differentiation program. The exact nature of how this compound can cause cells to re-enter the differentiation programme is unclear. Within 24 to 48 hours this process leads MEL cells to develop the erythroid phenotype. During this differentiation levels of c-myc protein and mRNA rapidly fall in the early stages of addition of the chemical (1-2 hours), followed by re-expression of the c-Myc gene and re-accumulation of the protein at later time points (Lachman and Skoultchi, 1984). The action of c-Myc in MEL cells is critical to the differentiation program, as disrupting its expression by introducing exogenous c-Myc into the cells inhibits differentiation (Dmitrovsky, 1986; Prochownik and Kukowska, 1986). Western blot analysis revealed that the MEL cells were behaving as previously described, c-Myc levels falling rapidly at the onset of differentiation, and recovering at the later time point (Figure 4.10).

Figure 4.10: c-Myc levels are sensitive to HMBA-induced differentiation of MEL cells

Whole cell extracts were made from MEL cells differentiated by treatment with HMBA after the indicated times. Samples were resolved by SDS-PAGE and western blotting was performed using anti-c-Myc, anti-Brf1, anti-RPC155 and anti-actin antibodies.


0
1
3
5
18
hrs + HMBA
c-myc
Brf1
RPC 155
actin

To address the effects of depleted c-Myc levels in response to HMBA-induced differentiation, we analysed the effects of differentiation and c-Myc down-regulation on levels of pol III transcription in MEL cells 5 hours after treatment with HMBA. RNA was harvested from undifferentiated and HMBA treated cells and levels of pol III transcripts analysed by RT-PCR. This showed that levels of $tRNA^{Leu}$ and $tRNA^{Tyr}$ expression are down regulated during HMBA-induced differentiation of MEL cells (Figure 4.11).

To address if the falling levels of c-myc in differentiating MEL has correlates with a change in the promoter occupancies of pol III transcription factors, ChIP assays were performed using undifferentiated MEL cells and MEL cells treated with HMBA for 5 hours. Initially the level of c-Myc associated with class III genes was looked at. ChIP assays were performed using anti-c-Myc and anti-TFIIB antibodies. The levels of c-Myc associated with the 5S rRNA genes was determined by PCR, and this revealed that the lower levels of the c-Myc protein in the differentiated cells (Figure 4.10) correlates with lower levels of c-Myc associated with a class III gene (Figure 4.12a). ChIPs were then performed using anti-Brf1, anti-Pol III and anti-TFIIB antibodies, and the relative promoter occupancies were compared. The levels of pol III associated with the 5S rRNA and $tRNA^{Leu}$ are lower in the differentiating MEL cells compared to the undifferentiated cells (Figure 4.12b). In contrast, the levels of Brf1 occupancy are comparable in the differentiated and undifferentiated cell lines, indicating that there is no loss of TFIIIB binding in response to lowering of c-Myc levels (Figure 4.12). The levels of expression of Brf1 and the polymerase subunit RPC 155 are unchanged during this stage of differentiation indicating that the

differences in promoter occupancy are due recruitment rather than their levels (Figure 4.10).

Figure 4.11: Pol III transcription is reduced in response to MEL cell differentiation

RNA was harvested from undifferentiated MEL cells and MEL cells treated with 5 mM HMBA for 5 hours. RNA was analysed by RT-PCR for expression of pre-$tRNA^{Leu}$, pre-$tRNA^{Tyr}$ and ARPP P0 (control) using gene-specific primers.

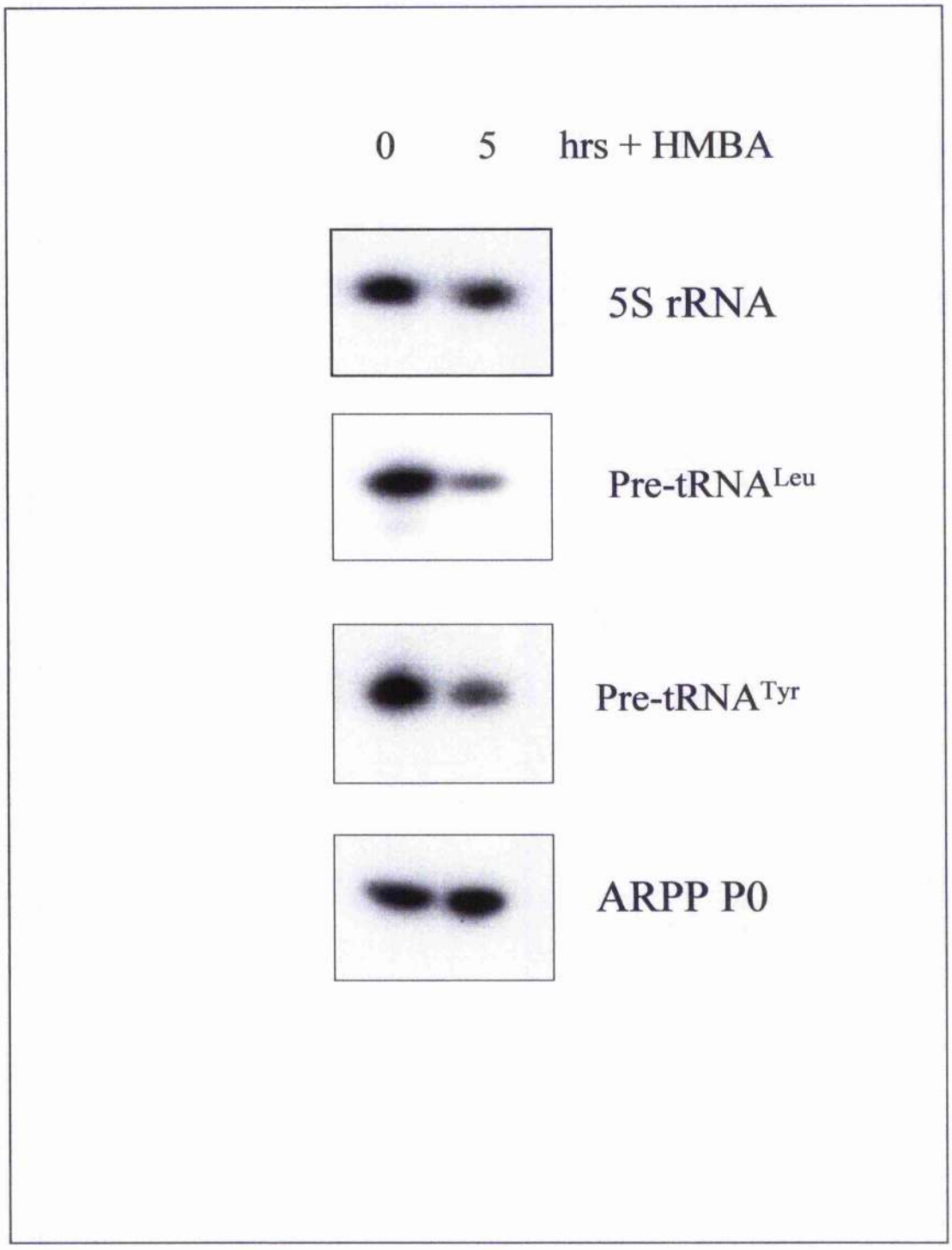
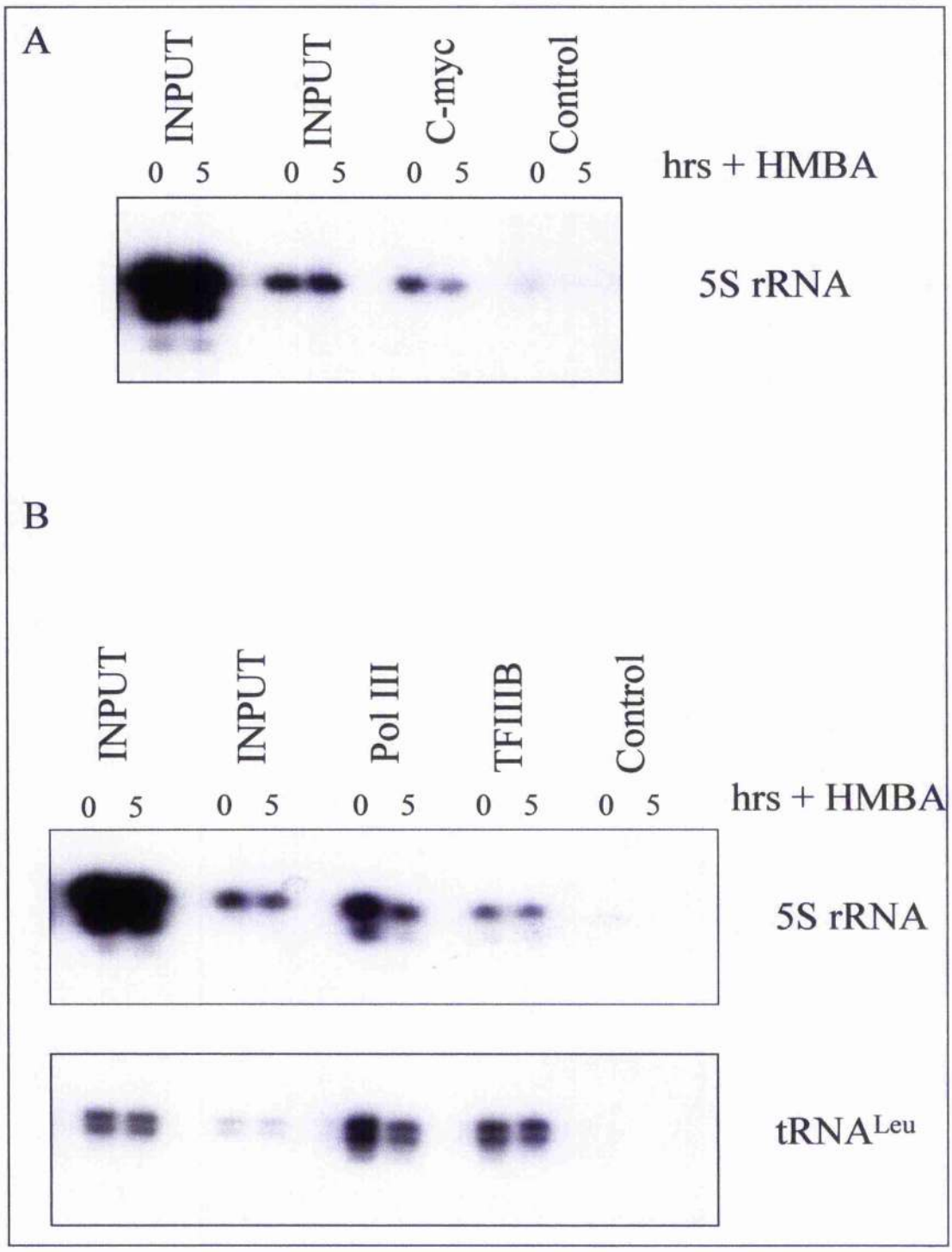

0
5
hrs + HMBA
5S rRNA
Pre-tRNA^{Leu}
Pre-tRNA^{Tyr}
ARPP P0

Figure 4.12: Levels of pol III occupancy are reduced on the promoters of pol III-transcribed genes in differentiated MEL cells

The promoter occupancy of c-Myc, TFIIIB and pol III was measured in undifferentiated compared to differentiated MEL cells. A. ChIPs were performed using antibodies against c-Myc and TFIIB (negative control). The samples were normalised by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using 5S rRNA and $tRNA^{Leu}$ gene-specific primers. B. As in a, but using antibodies against Brf1 (128), RPC 155 (1900), and TFIIB.


A
INPUT
INPUT
C-myc
Control
0 5 0 5 0 5 0 5
hrs + HMBA
5S rRNA
B
INPUT
INPUT
Pol III
TFIIIB
Control
0 5 0 5 0 5 0 5 0 5
hrs + HMBA
5S rRNA
tRNA^Leu

4.3 Discussion

To investigate the effects of c-Myc on pol III transcriptional activation a MycER inducible cell line was used. The MycER protein is rapidly translocated to the nucleus in response to activation of the protein using 4-OHT (Figures 4.1 and 4.2). The nuclear translocation of the c-Myc fusion protein coincides with a recruitment of MycER to the cyclin D2 promoter (Bouchard et al, 2001). The activation of MycER also results in a specific recruitment of the chimeric c-Myc protein to the pol III-transcribed tRNA and 5S rRNA genes (Figure 4.3).

The activation of MycER in rat fibroblasts results in a specific activation of pol III transcription as determined by RT-PCR (Gomez-Roman et al, 2003). I found that activation of c-Myc in A31-derived MycER mouse fibroblasts also results in a specific induction of pol III-transcribed genes 3 hours after MycER activation (Figure 4.4). There is a significant delay between MycER binding to the promoters of pol III-transcribed genes and an activation of transcription. This delay is puzzling and may indicate further unidentified events that occur between c-Myc recruitment to a pol III-transcribed target gene and the subsequent activation of transcription.

In vitro experiments examining pol III-transcribed genes have suggested a step-wise assembly of basal transcription factors and polymerase onto the promoters of target genes to form a pre-initiation complex, followed by transcriptional activation (Geiduschek and Kassavetis, 2001). This is in contrast to mechanisms proposed for transcription complex assembly in vivo. Studies investigating transcription by pol II have suggested that the polymerase can associate stably with components of the basal

transcription machinery in the absence of DNA, and that the assembly of this complex occurs in the nucleoplasm. This is postulated to result in the formation of a holoenzyme, this large complex being recruited to the DNA in a single step (Reviewed in Koleske and Young, 1995). Indeed it has been reported that pol III, TFIIIB and TFIIIC, factors necessary for the transcription of the vast majority of pol III-transcribed genes, form a pol III holoenzyme (Wang et al, 1997).

The data presented in this chapter would suggest that, in the model systems used, c-myc activation of pol III transcription, results in a stepwise recruitment of transcription factors rather than the recruitment of a pre-formed pol III holoenzyme (Figure 4.6a). The promoter occupancy of TFIIIC appears to be independent of levels of the activated, exogenous MycER protein. The activation of the MycER protein and its recruitment to the promoters of the $tRNA^{Leu}$ and 5S rRNA genes coincides with the recruitment of two subunits of the pol III-specific transcription factor TFIIIB after 2 hours, followed by delayed recruitment of pol III at 4 hours after 4-OHT treatment (figure 4.6a). An interaction between TFIIIB and c-Myc has been observed both in vivo and in vitro and the formation of this complex could result in an activation of TFIIIB, leading to enhanced occupation of a c-Myc/TFIIIB complex on the promoters of target genes. The recruitment of the polymerase occurs after c-Myc/TFIIIB binding to the 5S rRNA and $tRNA^{Leu}$ genes, coinciding with the activation of pol III transcription (Figures 4.4 and 4.6a). This lag in polymerase binding suggests that TFIIIB is not associated with pol III prior to TFIIIB binding to the promoters of class III genes, further discounting recruitment of a pol III holoenzyme in this system. The delay in polymerase binding suggests that further events, such as chromatin remodelling, post-translational modification of transcription factors or recruitment of

unidentified co-activator proteins, may be required at the promoters of pol III-transcribed genes after recruitment of c-Myc and TFIIIB. Changes in pol III transcription factor occupancy have been observed in mitosis, ERK inhibition and p53 induction, but this has not previously been reported in the activation of pol III transcription (Fairly et al, 2003; Felton-Edkins et al, 2003a; Crighton et al, 2003). The step-wise recruitment of pol III transcription factors in response to a stimulus has also never previously been documented in vivo.

The promoter occupancies of the pol III transcription factors were measured in c-Myc deficient cells compared to wild type. Similar to the MycER cells, levels of endogenous c-Myc do not appear to alter the promoter occupancy of the TFIIIC complex (Figure 4.8). These results may suggest that TFIIIC is stably associated with class III genes, regardless of whether the gene is being transcribed, suggesting that TFIIIC binding to the promoter is not a step involved in c-Myc activation of a pol III transcribed gene. This is consistent with previous observations of TFIIIC occupancy in mitosis and in response to ERK inhibition (Fairly et al, 2003, Felton-Edkins et al, 2003a).

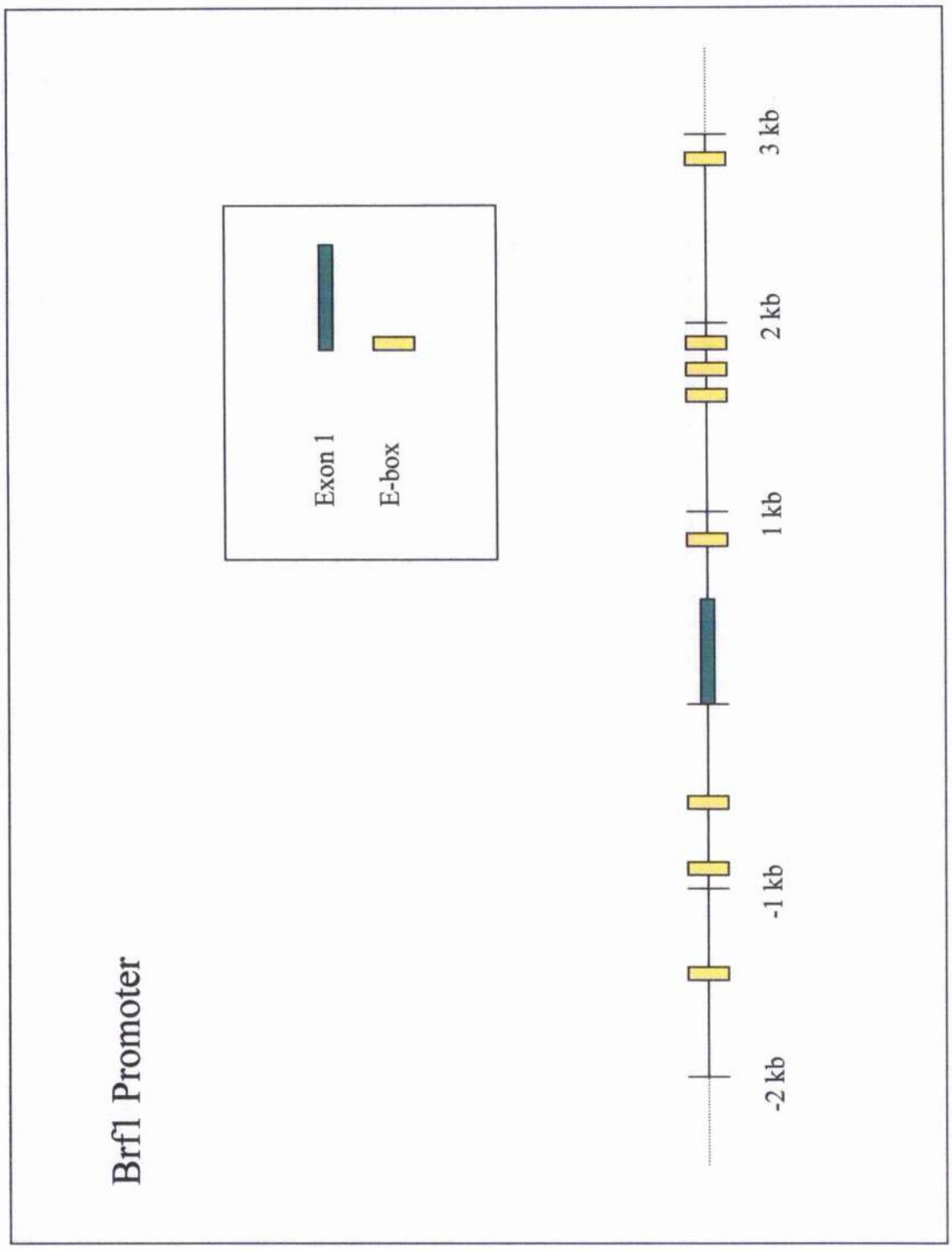
In c-Myc wildtype cells the gene occupancy by TFIIIB and pol III is elevated compared to in the c-Myc deficient cells (Figure 4.8). This agrees with the differences in the levels of pol III transcription, as measured by RT-PCR (Figure 4.7). The increased promoter occupancy of TFIIIB in cells with higher levels of c-myc is similar to the observations using the MycER cells. However the c-Myc deficient cells have lower levels of Brf1 than the c-Myc wildtype cells (Figure 4.9). The lower levels of Brf1 may contribute to the lower levels of pol III transcription in these cells.

A dramatic decrease in pol III transcription is observed when F9 embryonal carcinoma cells are differentiated, and this can be partly attributed to lower level of Brf1 (White et al, 1989; Alzuherri and White, 1998). When activating MycER there was no induction of Brf1 in the short time course looked at, so the differences in expression of Brf1 are likely to be due to long term effects of c-Myc deficiency on the expression of Brf1. It has still to be determined if the gene encoding Brf1 is a direct or indirect target of c-Myc, but sequence analysis of the Brf1 gene promoter reveals several E-box sequences (Figure 4.13). It may be that in tumour formation c-myc could activate pol III transcription not only by directly activating transcription by recruiting the pol III transcriptional apparatus, but also by increasing the levels of pol III transcription factors. Further experiments need to be done to determine c-myc involvement in the expression of Brf1.

To address how down regulation of c-Myc effects pol III transcription in a physiological context, MEL cells were used as a model of differentiation. The level of c-Myc protein falls rapidly in response to chemically-induced differentiation of MEL cells, and this is accompanied by a fall in pol III transcription. Unlike differentiation of the F9 embryonal carcinoma model levels of pol III transcription factors are not sensitive to the differentiation in the time course examined, but levels of c-Myc and pol III associated with the promoters is reduced, consistent with the reduction in protein levels and reduction of transcription, respectively. It is interesting to observe that levels of TFIIIB associated with the promoters of pol III-transcribed genes appear to be unaffected by the loss of c-Myc and polymerase. My experiments in fibroblasts show that c-Myc is involved in recruiting both TFIIIB and pol III to the promoters of target genes, but is only the occupancy of the polymerase

Figure 4.13: The Brf1 promoter contains several high affinity c-Myc/Max binding sites

5 kb of human and rat genomic DNA, spanning the upstream end of the Brf1 gene, was analysed using the transcription factor binding site programme CONSITE (available at www.phlofoot.org). Potential c-Myc/Max binding sites (E-boxes) identified in both species, and their approximate locations, are represented in the diagram.


Brf1 Promoter
Exon 1
E-box
-2 kb
-1 kb
1 kb
2 kb
3 kb

appears to be sensitive to a dramatic fall in c-Myc levels during the early stages of MEL cell differentiation (Figure 4.12b). The difference in might simply reflect the stability of binding of c-Myc, pol III and TFIIIB to the complex. Pol III can dissociate from the transcription complex relatively easily (White, 2002). TFIIIB may be more stably retained due to interactions with TFIIIC and DNA (White, 2002). It may be that it takes more than 5 hours for TFIIIB to dissociate from the class III genes. Further experiments need to be performed looking at the occupancy of TFIIIB after longer time points.

In the model systems used c-Myc is involved in the recruitment of TFIIIB, followed by recruitment of polymerase to the promoters of genes that TFIIIC is stably associated with. The polymerase is sensitive to c-Myc levels, and c-Myc appears to be required for the maintenance of polymerase association with pol III transcribed genes, but high levels of c-Myc can recruit TFIIIB to the promoters of pol III transcribed genes, but do not appear necessary for maintaining promoter occupancy.

Chapter 5 Myc co-activator proteins associated with class III Genes

5.1 Introduction

The data presented in chapter 4 demonstrate that c-Myc is involved in the recruitment of the pol III transcription apparatus to the promoters of target genes, to promote the formation of the pol III transcription initiation complex. Much of the recent work on the mechanisms by which c-Myc can activate target genes has focused on c-Myc recruiting co-activator proteins to their promoters. Many of the co-activator proteins identified have been implicated in altering the localised structure of the chromatin that packages the DNA into the nucleus. Experiments in this chapter will focus on the involvement of c-Myc associated co-activator proteins and their role in the activation of pol III transcription.

5.1.1: Chromatin Organisation

A mammalian cell packages away around 2m of DNA into the chromatin structure that is contained in a nucleus less than 10μm in diameter. In eukaryotic cells, DNA is associated with a family of small basic histone proteins which form a higher order condensed protein:DNA complex termed chromatin. The fundamental base of chromatin is the nucleosome, which consists of 146bp of DNA wrapped twice round an octamer formed from the globular domains of two molecules of each of histones H2A, H2B, H3 and H4 (Luger et al, 1997). The globular domains of the histones

form the core of the nucleosome, and the basic flexible tail regions of the histones protrude from the octamer, and are thought to be associated with the negatively charged DNA wrapped around the outside (Luger and Richmond, 1998). Each nucleosome is connected with 10-80bp of linker DNA associated with a linker histone, such as histone H1 or H5, and the structure is progressively folded into higher order more condensed structures, which remain ill-defined (Felsenfeld and Groudine, 2003).

The structure of higher order packed chromatin is generally repressive for transcription, due to the inaccessibility of the DNA (Felsenfeld and Groudine, 2003). Appropriate gene expression therefore requires interplay with complexes that when recruited by transcriptional activators, or repressors, can adjust the chromatin structure to alter its accessibility (Jenuwein and Allis' 2001; Gamble and Freedman, 2002). There are two main classes of enzyme that perform this task and they are broadly described as chromatin remodelling enzymes. The first class is the ATP-dependent chromatin-remodelling enzymes. These are multi-subunit complexes that utilise energy from ATP to alter the contacts between the histones and the DNA, thus facilitating disruption of the nucleosome structure (Owen-Hughes, 2003; Mohrmann and Verrijzer, 2004). The second class is the enzymes, which can covalently modify the histones. These modifications generally occur on the more flexible and charged histone tails that protrude from the nucleosome, altering the biochemical properties of this region (Luger and Richmond, 1998; Wu and Grunstein, 2000). Within this group there are factors that can acetylate, phosphorylate, methylate, and ubiquitinate the histones (Wu and Grunstein, 2000; Figure 5.1). The many different covalent modifications that can label the histone tails of the nucleosomes are postulated to

create a "histone code" of modifications, that predicts multiple histone modifications acting together or in a sequential manner to specify unique downstream functions (Reviewed in Strahl and Allis, 2000; Turner, 2000).

This chapter will be focussing on the covalent modification of histone proteins by histone acetyl transferases and the ATP-dependent chromatin remodelling enzymes. Enzymes from both categories have been shown to interact with the c-Myc protein to alter transcription of target genes.

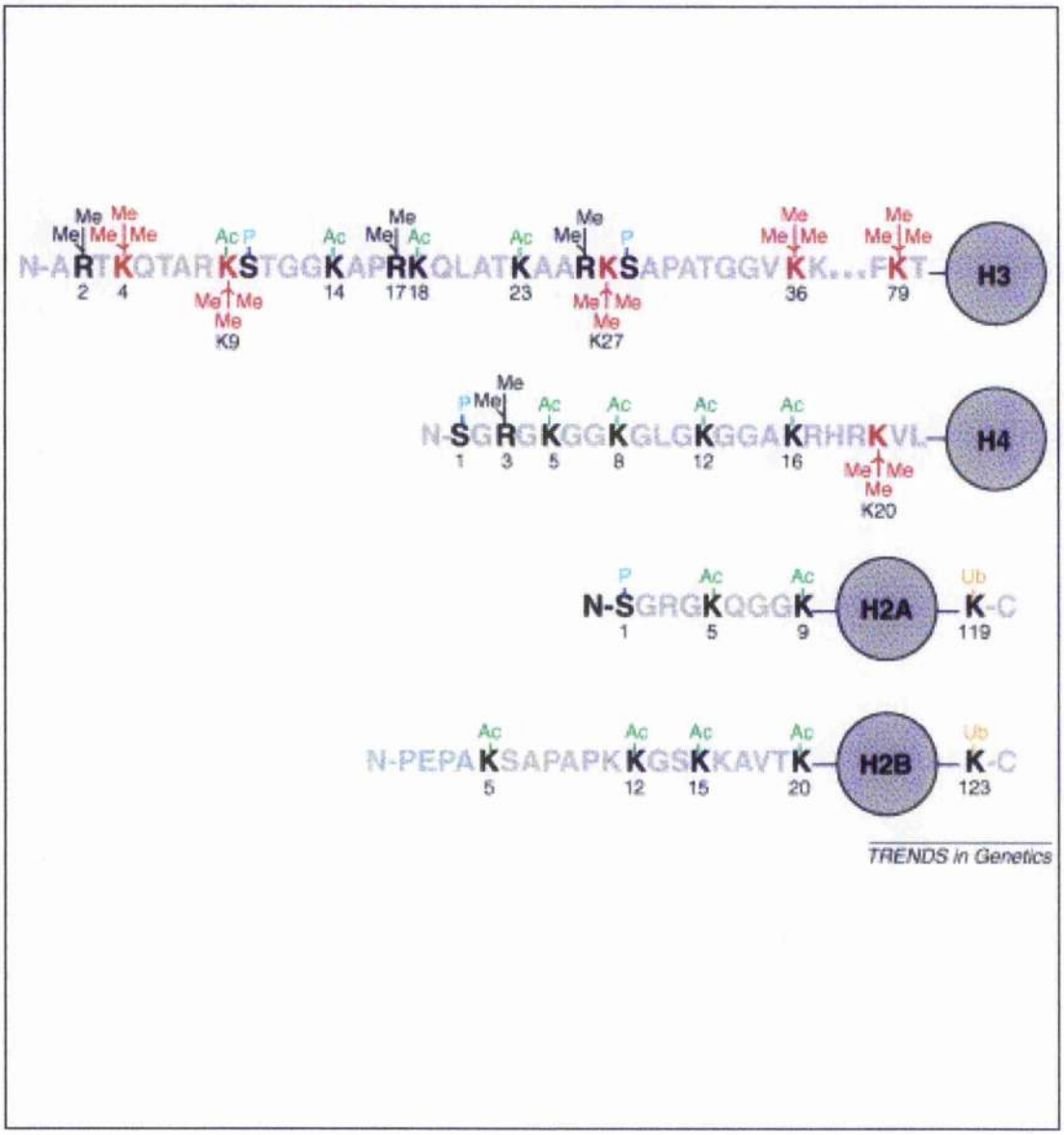
5.1.2 ATP-dependent chromatin remodelling enzymes and c-Myc

The prototype ATP-dependent chromatin remodelling complex, SWI/SNF, was identified in yeast genetic screens investigating mutants in mating type switching (SWI) and sucrose fermentation (sucrose non fermenting, SNF) (Winston and Carlton, 1992; Peterson and Herskowitz, 1992). Mutants identified in these screens not only showed defects in these functions, but altered expression of a wide range of target genes; indeed later studies revealed that mutants in the SWI/SNF complex altered expression of 6% of yeast genes (Winston and Carlton, 1992; Peterson and Herskowitz, 1992; Holstege et al, 1998; Sudarsanam et al, 2000).

The yeast SWI/SNF complex was found to contain 11 subunits, with one of the subunits, Snf2, having motifs that shared homology with DNA and RNA helicases (Gorbalenya et al, 1993; Smith et al, 2003). Consistent with this Snf2, was found to

Figure 5.1: Post translational modifications of the histone tails

The known post-translational covalent modifications of histones (H2A, H2B H3 and H4). Lysine (K) methylation (Me) is represented in red. Acetylation (Ac), phosphorylation (P) and ubiquitination (Ub) are indicated in green, blue and orange, respectively. Arginine (R) methylation is represented in black (figure from Sims et al, 2003).


H3
H4
H2A
H2B
K9
K27
K20
TRENDS in Genetics

be able to hyrolysc ATP in the presence of DNA, but was found to have no helicase activity in strand displacement assays (Côté et al, 1994). Subsequent assays demonstrated that the assembly of histones on the DNA further stimulated this ATPase activity, and the complex was shown to stimulate transcription factor binding to DNA and to alter the positions of the nucleosomes on the template (Reviewed in Owen-Hughes, 2003, Li et al, 2004). The ability of these complexes to mobilise nucleosomes on a DNA template can lead to either a decrease or an increase in the accessibility of the associated DNA sequence (Owen-Hughes, 2003). Consistent with this, ATP-dependent chromatin remodelling enzymes have been shown to both activate and repress transcription of target genes (Holstege et al, 1998; Sudarsanam et al, 2000).

Through homology with the Snf2 protein in yeast, a host of chromatin remodelling complexes have been identified in eukaryotes. In humans the Snf2-related proteins Brg1 and Brm1 have been shown to exist in multiple hSWI/SNF complexes. Other classes of ATP-dependent chromatin remodelling enzymes have been identified, all existing in multi-subunit complexes with a core ATPase necessary for their function (reviewed in Varga-Weisz, 2001).

As described above, the ATP-dependent chromatin remodelling enzymes have been shown to regulate the transcription of certain target genes. It has been demonstrated that SWI/SNF complex can be recruited to promoters through interactions with transcriptional activators (reviewed in Owen-Hughes, 2003, Li et al, 2004). In mammals, SWI/SNF complexes have been shown to play a role in the transcriptional

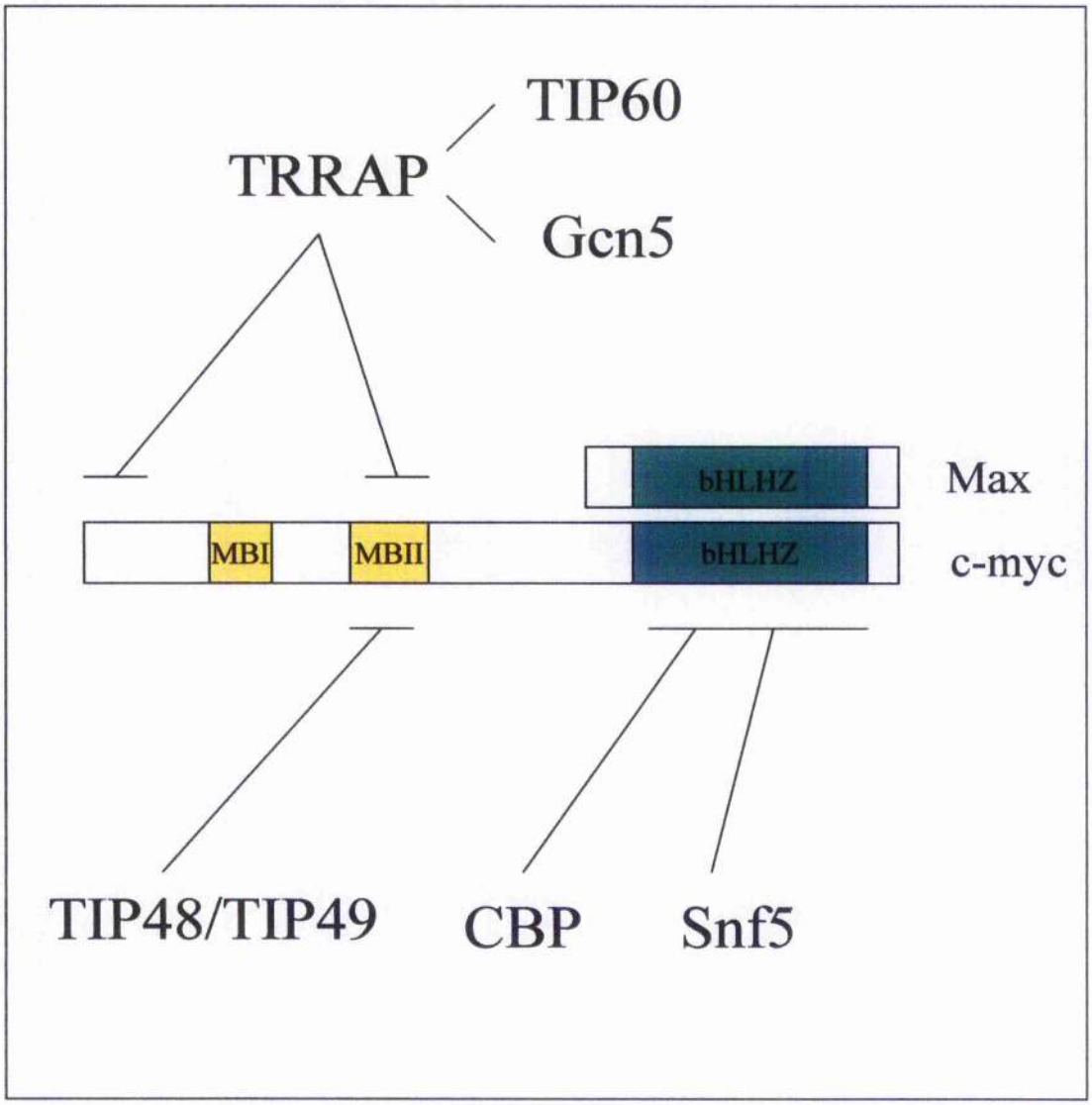
activation of an assortment of genes, including β-interferon, hsp70, β-catenin and retinoblastoma (Owen-Hughes, 2003). One of the best characterised examples of SWI/SNF involved in the activation of a target is a Brg1-containing human SWI/SNF complex mobilising a nucleosome to reveal the TATA box of the β-interferon gene promoter to allow recruitment of the pol II transcription apparatus (Agaloti et al, 2000).

Components of the hSWI/SNF complex have been implicated in cancer development. Snf5, a conserved subunit of hSWI/SNF, and the ATPase subunit, Brg1, are mutated in several different tumours and tumour cell lines (Versteege et al, 1998; Sevenet et al, 1999; Decristofaro et al, 2001; Wong et al, 2000). Homozygous inactivation of Snf5 is embryonically lethal in mice, and a conditional inactivation of Snf5 in mature mice leads to highly penetrant tumours, indicating that Snf5 is a tumour suppressor (Roberts et al, 2003, Roberts and Orkin, 2004). The Brg1 ATPase is a candidate tumour suppressor, as its homozygous deletion also leads to embryonic lethality in mice, and haploid insufficiency has been demonstrated to predispose mice to tumours (Roberts and Orkin, 2004).

The hSWI/SNF complex contains eleven polypeptides including invariant core and also variant subunits. Snf5 is a core sub-unit of the SWI/SNF complex. A search for Snf5-interacting proteins using a two-hybrid screen revealed an interaction between the bHLHz domain of c-Myc and Snf5 (Cheng et al, 1999; Figure 5.2). This interaction was confirmed in vivo, and transient transfection of dominant interfering forms of Snf5 or Brg1 block c-Myc-dependent transactivation as determined by

Figure 5.2: Schematic indicating chromatin remodelling factors binding to c-Myc

Schematic indicating the regions of c-Myc that interact directly with the co-activators TRRAP, Snf5, TIP48, TIP49 and CBP. The conserved Myc Box 1 (MBI) and Myc Box 2 (MBII), as well as the basic helix-loop-helix zipper domain (bHLHz) are indicated on c-Myc as well as its binding partner Max. Also indicated are Gcn5 and TIP60 protein that bind to c-myc indirectly through an interaction with TRRAP.


TIP60
TRRAP
Gcn5
bHLHZ
Max
MBI
MBII
bHLHZ
c-myc
TIP48/TIP49
CBP
Snf5

reporter assays, demonstrating a functional interaction between c-Myc and the SWI/SNF complex (Cheng et al, 1999).

In a study to find novel proteins interacting with the transactivation domain of c-Myc two related ATPase/helicases, TIP48 and TIP49, were identified. Both TIP48 and TIP 49 form complexes with c-Myc *in vivo*, and mutations in TIP49 have been shown to inhibit c-Myc oncogenic activity (Wood et al, 2000). Both TIP48 and TIP49 have been demonstrated to bind to the c-Myc target gene nucleolin in a c-Myc-dependent manner (Figure 5.2; Frank et al, 2003). In yeast, both TIP48 an TIP49 are in complexes with the ISWI-related ATPase Ino80, perhaps representing another functional interaction between c-Myc and a chromatin remodelling machine (Jonsson et al, 2004). They have also been identified as part of a histone acetyl transferase complex, along with another c-Myc interacting protein, TRRAP, which will be addressed later in this chapter (Ikura et al, 2000).

5.1.3: Histone Acetyl Transferases (HATs)

As previously discussed, nucleosomal DNA is generally repressive to transcription. A mechanism for altering the accessibility of the DNA is covalent modification of the histones, especially on the histone tails (Wu and Grunstein, 2000). One of the best-characterised histone modifications is reversible acetylation of lysines residues contained within the tail regions. Histone acetylation and deacetylation are mediated by opposing enzyme families: the HATs and the histone deacetylases (HDACs), respectively. HATs function enzymatically by transferring an acetyl group from acetyl Co-enzyme A (acetyl CoA) to a side chain of a lysine residue of a histone tail (Marmorstein and Roth, 2000; Roth et al, 2001). A separate group of enzymes,

HDACs, can remove the acetyl group from the lysine, to reverse this modification (Kuo and Allis, 1998).

In a large scale screen of the histone modification patterns of a genome of Drosophilia, it was observed that areas of the genome containing active genes (euchromatin) were associated with areas of chromatin hyperacetylated on histone H3 and histone H4 tails, and areas containing inactive genes (heterochromatin) were shown to be associated with deacetylated histones (Schubeler et al, 2004).

The neutralisation of the lysine residues in the histone tails is postulated to weaken the nucleosome:DNA and nucleosome:nucleosome interactions to increase the accessibility of the DNA (Hong et al, 1993; Lee et al, 1993, Luger and Richmond, 1998). The increase in the acetylation state of chromatin affects the higher order structure of the chromatin and leads to a more open and permissive chromatin environment for transcription (Kuo and Allis, 1998; Li et al, 2004).

As well as global increases in histone acetylation being associated with transcriptionally active areas of the genome, more subtle localised changes in nucleosome acetylation surrounding the promoter of genes by recruitment of HAT complexes correlate well with the activation of certain target genes (Kuo et al, 1998, Fernandez et al, 2003). Most of the data showing that increases in localised histone acetylation leads to gene activation is correlative, but in a recent study using a reconstituted system it was demonstrated that HATs could activate transcription by acetylating histones, and that the histone acetylation was necessary for activation of the reporter gene (An et al, 2004).

Recent work has shown that not only are histones HAT substrates, but transcription factors such as p53, c-Myb, E2F1 and c-Myc can be aceteylated to have positive effects on transcription by increasing DNA binding activity and/ or protein stability and half life (Gu and Roeder, 1997; Tomita et al, 1999; Martinez-Balbas et al, 2000; Vervoorts et al, 2003).

5.1.4 The Myc/Max/Mad Network and Acetylation

The Mad proteins, which are antagonists of c-Myc function, have been demonstrated to compete with c-Myc to form heterodimers with Max to bind to the same E-Box sequences to repress transcription of target genes (reviewed in Grandori at al, 2000; Chaper 1). All Mad proteins interact with the mammalian Sin3 proteins through an N-terminal Sin3-interaction domain (SID), and the integrity of this domain is required for Mad-mediated transcriptional repression. Sin3 proteins have been demonstrated to interact with HDACs to form co-repressor complexes, and repression of genes by the Mad proteins is sensitive to chemicals that can inhibit HDAC activity (Alland et al, 1997; Hassig et al, 1997; Laherty et al, 1997). Mad-HDAC complexes can bind to the promoters of target genes in an E-box dependent manner to repress transcription, and this repression is attenuated when cells are treated with HCAC inhibitors (Reviewed in Grandori et al, 2000)

With the discovery that Mad proteins could repress transcription through interactions with HDAC complexes, it was postulated that the c-Myc protein may interact with HATs. TRRAP was identified in a screen for novel proteins which interact with the

N-terminal transactivation domain of c-Myc (Figure 5.2; McMahon et al, 1998). TRRAP is a 440kDa polypeptide whose C-terminal domain displays homology to the Ataxia-telangietasia mutated (ATM)/ PI-3 kinase super-family, although mutations in the amino acid sequence suggest that TRRAP cannot function as a protein kinase (McMahon et al, 1998; Vassilev et al, 1998). TRRAP has no apparent DNA binding motif, but can be recruited to the DNA through interactions with transcription factors, for example c-Myc or E2F1 or nuclear receptors, which mediate the targeting of TRRAP to the promoters of target genes (McMahon et al, 1998; Lang et al, 2001; Yanagisawa et al, 2002). The interaction between c-Myc and TRRAP is dependent on the integrity of the essential MBII region and an area in the extreme amino terminal of c-Myc (McMahon et al, 1998, Figure 5.2). By knocking down endogenous TRRAP levels using an antisense approach, McMahon and colleagues showed that the recruitment of TRRAP is essential for transformation of mammalian cells by c-Myc (McMahon et al, 1998). The gene encoding TRRAP is essential, as demonstrated by mouse knock out studies (Herceg et al, 2001). $Trrap^{-/-}$ mice die during embryonic development; cells with the $Trrap^{-/-}$ genotype can be recovered at the blastocyst stage, and when compared to Trrap $^{-/+}$ or Trrap $^{+/+}$ they display severe growth retardation (Herceg el al, 2001). Genome wide analysis of TRRAP knockout cells compared to wildtype, using microarrays, revealed that there can be an increase as well as a decrease in gene expression of target genes, revealing that TRRAP, directly or indirectly, is involved in the activation as well as the repression of transcription (Herceg et al, 2003).

TRRAP and its yeast homologue Tra1p have been demonstrated to be an essential component of several complexes with HAT activity. These include SAGA, PCAF,

TIP60, NuA4, TFTC and STAGA complexes (Grant et al, 1998; Vassilev et al, 1998, Ikura et al, 2000; Allard et al, 1999; Brand et al, 1999; Martinez at al, 2001). The TRRAP/Tra1p-containing HAT complexes can be broadly divided into two categories. The PCAF, SAGA, TFTC and STAGA complexes, that contain the PCAF and Gcn5 HATs, preferentially acetylate histone H3, whereas the NuA4 and Tip60 preferred histone substrate is histone H4 (Sterner and Berger, 2000; Roth et al, 2001).

As discussed earlier in this chapter, TRRAP can interact with the transcription factor c-Myc and, through these interactions, TRRAP has been shown to recruit HAT activity to the promoters of c-Myc target genes (Frank et al, 2001, Bouchard et al, 2001, Fernandez et al, 2003). Further work has demonstrated that c-Myc can bind to both Gcn5 and Tip60 containing HAT complexes, and that these interactions are dependent on TRRAP (McMahon et al, 2000; Frank et al, 2003; Figure 5.2).

It has been shown that both the integrity of TRRAP and the HAT activity of Gcn5 are required for c-Myc mediated transcriptional activation (Liu et al, 2003). A dominant-negative form of Tip60 can delay c-Myc-mediated acetylation of histone H4 (Frank et al, 2003). The evidence suggests that not only can TRRAP act as the adaptor to recruit HAT activity to a gene promoter; it may specify the HAT activity it recruits.

c-Myc can also interact with the HAT CBP, but this interaction is independent of MBII and independent of TRRAP (Vervoorts et al, 2003). CBP can acetylate histones surrounding the promoters of c-Myc target genes (Vervoorts et al, 2003). CBP was also shown to acetylate the c-Myc protein leading to increased c-Myc stability and an increase in its half-life (Vervoorts et al, 2003).

Experiments in this chapter focus on ATP-dependent chromatin-remodelling enzymes and HAT complexes that interact with c-Myc, and are potentially involved in the activation of pol III transcription.

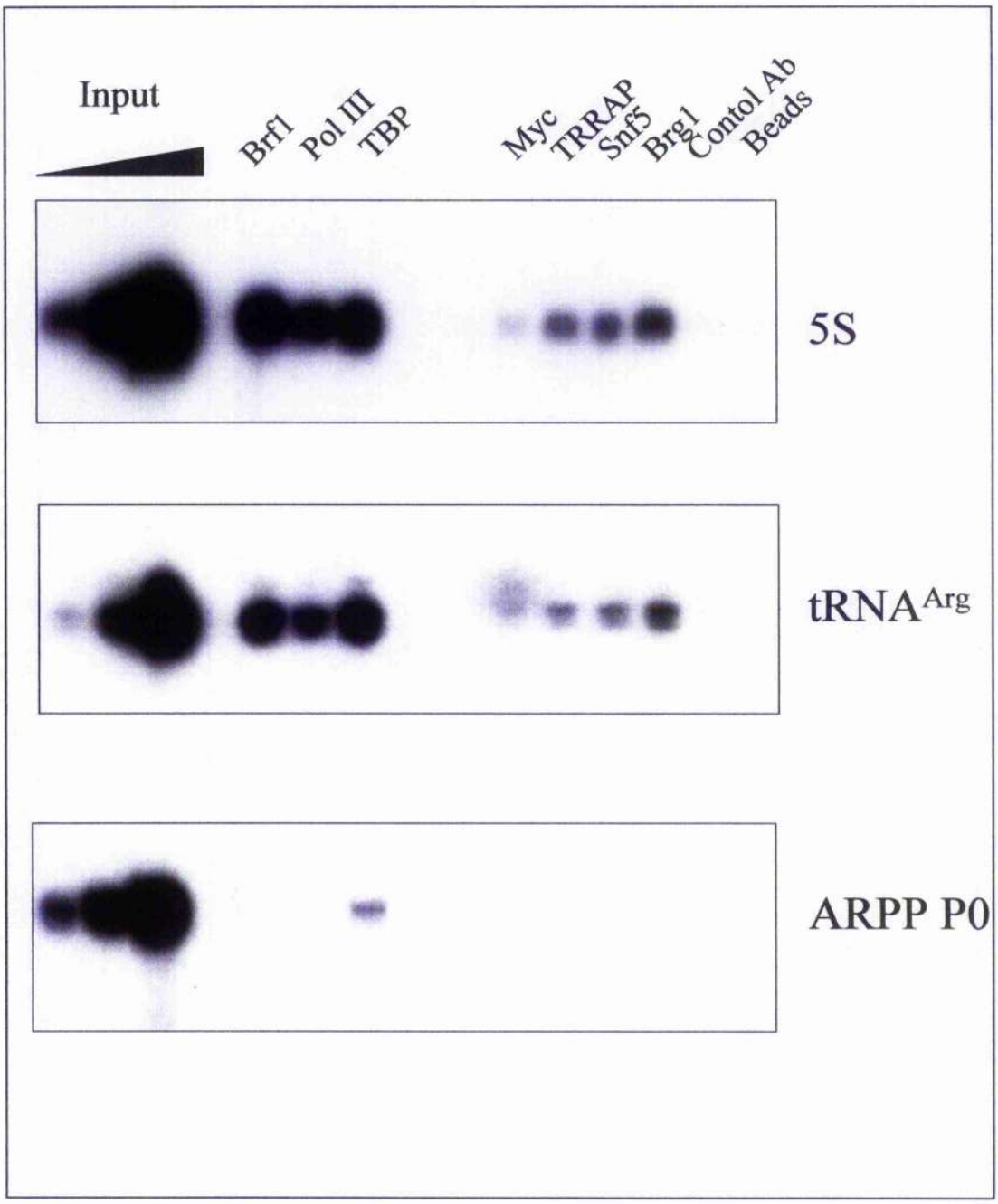
5.2 Results

5.2.1 Swi/Snf and TRRAP are present on pol III-transcribed genes in vivo

As summarised in the introduction to this chapter, there are many co-activator proteins that have been demonstrated to be involved in the transcription of c-Myc target genes. To determine whether any of these factors were involved in pol III transcription, ChIP assays were performed to test for promoter occupancy on pol III-transcribed genes. ChIP assays were performed on asynchronously growing HeLa cells using anti-c-Myc, anti-TRRAP, anti-Snf5 and anti-Brg1 antibodies (Figure 5.3). As positive controls antibodies against the TFIIIB components Brf1 and TBP, along with an antibody against the RPC 155 subunit of the polymerase were used, and a TAF_I48 antibody and beads were used as negative controls. As expected, the TFIIIB subunits Brf1 and TBP and the polymerase subunit RPC 155 are associated with the promoters of pol III-transcribed genes in HeLa cells, shown by their association with the 5S and $tRNA^{Arg}$ genes, but neither the pol III-specific TAF, Brf1, or pol III is associated with the pol II-transcribed ARPP P0 control gene (Figure 5.3). In addition to the association of the Pol III transcription apparatus, TRRAP and two components of the hSWI/SNF chromatin-remodelling complex, Snf5 and Brg1, are associated with pol III-transcribed genes in HeLa cells (figure 5.3). The association of these proteins with pol III-transcribed genes appears to be specific as they are not associated with the pol II-transcribed ARPP P0 gene (Figure 5.3).

Figure 5.3: c-Myc, TRRAP and components of the hSWI/SNF complex bind to class III genes in vivo

ChIP assays were performed on cultured HeLa cells using antibodies against Brf1, TBP, RPC155, c-Myc, TRRAP, Snf5, Brg1, TAF_I48 or with beads alone. As expected, binding of the positive controls, Brf1, RPC155, TBP and c-Myc, was detected at the 5S and $tRNA^{Arg}$ genes, as determined by PCR using gene-specific primers. The c-Myc cofactor TRRAP and two components of the hSWI/SNF complex, Brg1 and Snf5, were found to be specifically associated with the 5S and $tRNA^{Arg}$ genes. The interactions of TRRAP and hSWI/SNF with Pol III promoters is specific as no signal was obtained by PCR using primers specific to the pol II-transcribed ARPP P0 gene.


Input
Brf1
Pol III
TBP
Myc
TRRAP
Snf5
Brg1
Contol Ab
Beads
5S
tRNA^{Arg}
ARPP P0

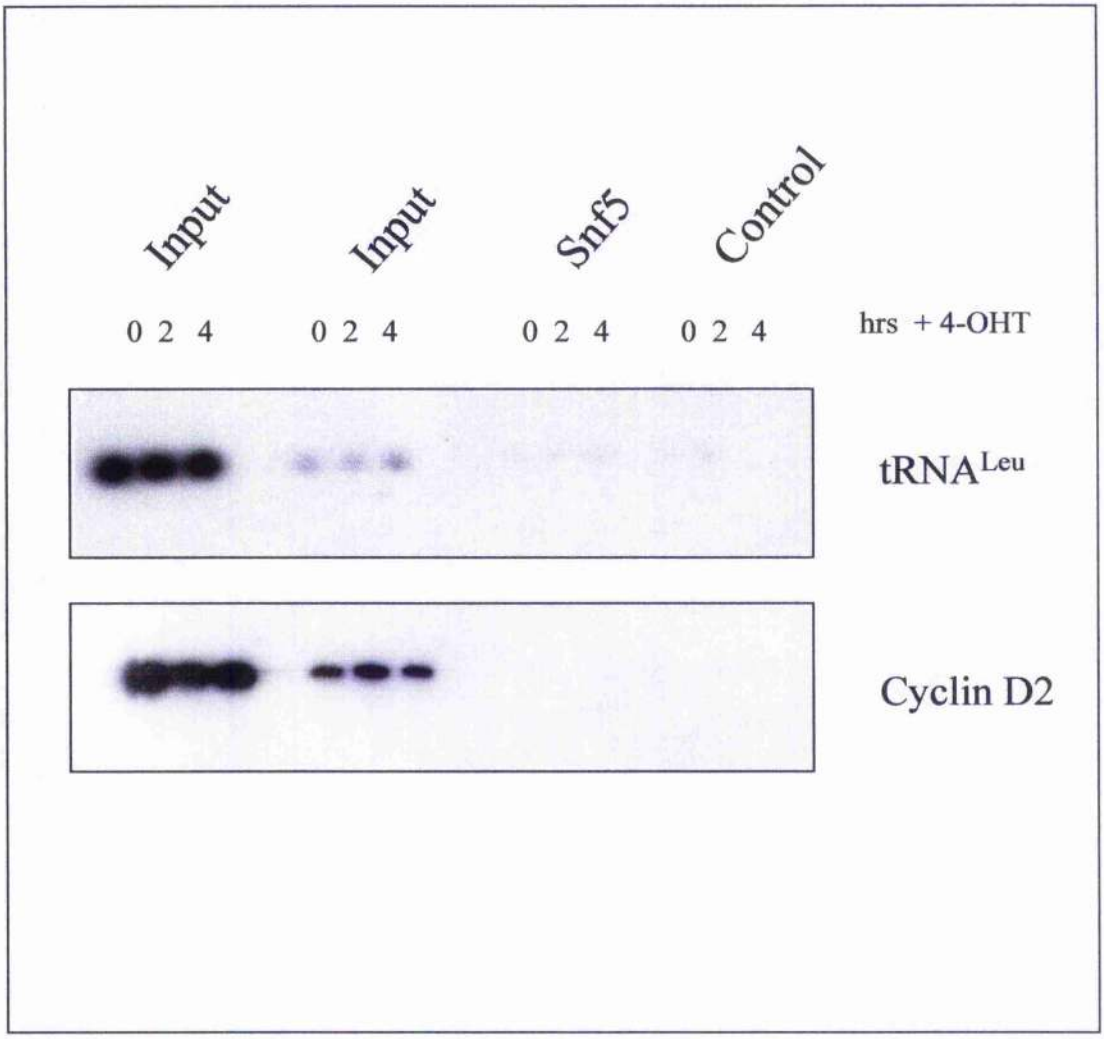
In order to determine whether the components of the hSWI/SNF chromatin-remodelling complex were present in a c-Myc-dependent manner, a c-Myc-inducible cell line was used. The cell line used is the A31 cell line stably transfected with a vector encoding an oestrogen-responsive Myc fusion protein (MycER) that was used and described in chapter 4 (Figure 4.1). The cells were quiesced by serum starvation for 48 hour prior to treatment with 4-OHT, to lower levels of endogenous c-Myc. ChIP assays were performed on these cells after the indicated time points after MycER activation using an antibody against the Snf5 subunit of the hSWI/SNF complex. No binding of the components of Snf5 was observed on the promoter of the $tRNA^{Leu}$ gene (Figure 5.4). This may indicate that Snf5 is not present at the promoters of pol III-transcribed genes in a c-myc dependent manner, or that an insufficient amount of the protein was immunoprecipitated.

To test whether the apparent lack of promoter association of Snf5 is specific to class III gene promoters, the binding of Snf5 to another c-Myc target gene was tested. In contrast to class III genes the cyclin D2 promoter has a consensus E-box sequence required for c-Myc binding (Bouchard et al, 2001). No binding of Snf5 to the promoter of the cyclin D2 promoter was observed, consistent with a failure in the immunoprecipitation (Figure 5.4). Similar results were obtained from ChIP assays in MycER cells looking for promoter occupancy of the Brg1 ATPase subunit of SWI/SNF (data not shown).

To determine whether the interaction of TRRAP with pol III-transcribed genes occurs in other cell lines, ChIP assays were performed using another human derived cell line. ChIP assays were performed using asynchronously growing HEK293 cells, using an

Figure 5.4: Analysis of hSWI/SNF binding to the promoters of class III genes in response to c-myc activation

A. c-Myc was induced in MycER fibroblasts by addition of 200nM 4-OHT. ChIP assays were performed at the indicated time points after MycER activation using antibodies against Snf5 and TAF_I48. The samples were normalised by comparing the genomic DNA inputs. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using primers specific to the promoters of the $tRNA^{Leu}$ and cyclin D2 genes, as indicated.


Input
Input
Snf5
Control
0 2 4
0 2 4
0 2 4
0 2 4
hrs + 4-OHT
tRNA^{Leu}
Cyclin D2

anti-TRRAP antibody. As a positive control an antibody against Brf1 was used, and as negative controls an anti-TFIIB antibody and beads alone. As expected, Brf1 is present on pol III-transcribed genes in these cells, as shown by the association with the 5S, $tRNA^{Leu}$ and $tRNA^{Arg}$ genes, but not the pol II-transcribed ARPP P0 gene (Figure 5.5). In addition to the association of Brf1 with pol III-transcribed genes, TRRAP is also present at the 5S, $tRNA^{Leu}$ and $tRNA^{Arg}$ genes, but not the pol II-transcribed ARPP P0 gene (Figure 5.5).

5.2.2 TRRAP binding to pol III-transcribed genes is enriched in the presence of c-myc

The binding of the TRRAP protein to the promoter of the myc target gene nucleolin is enhanced in c-Myc wildtype compared to myc null rat fibroblasts, as determined by ChIP assays (Frank et al, 2001). To establish whether the binding of TRRAP to pol III-transcribed genes is c-Myc dependent, the binding of TRRAP to class III genes was compared in matched c-Myc wildtype and c-myc null rat fibroblasts (described in Mateyak et al, 1997). It has previously been established that c-Myc-deficient fibroblasts display lower levels of pol III transcription compared with matched c-Myc wildtype (Gomez-Roman et al, 2003; Figure 4.7). ChIPs were performed using anti-pol III RPC155, anti-TRRAP and anti-TFIIB antibodies. In the wildtype cell line the promoter occupancy of the pol III subunit is enriched compared to that of the c-Myc-deficient cell line on the $tRNA^{Leu}$ and $tRNA^{Tyr}$ genes, consistent with the results presented in chapter 4 (Figure 5.6a; Figure 4.7). In the c-Myc wildtype cell line there is also an enrichment of the TRRAP protein on the promoters of the $tRNA^{Leu}$ and

Figure 5.5: TRRAP is present on the promoters of class III genes in HEK 293 cells

ChIP assays using HEK 293 cells were performed to test for association of TRRAP with pol III-transcribed genes. The ChIP assays were performed using anti bodies against Brf1, TRRAP, TFIIB and beads alone. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using 5S, $tRNA^{Leu}$, $tRNA^{Arg}$, and ARPP P0 primers as indicated. The association of both the positive control, Brf1, and TRRAP with pol III-transcribed genes is specific, as no signal is detected by PCR using primers specific to the pol II-transcribed ARPP P0 gene.

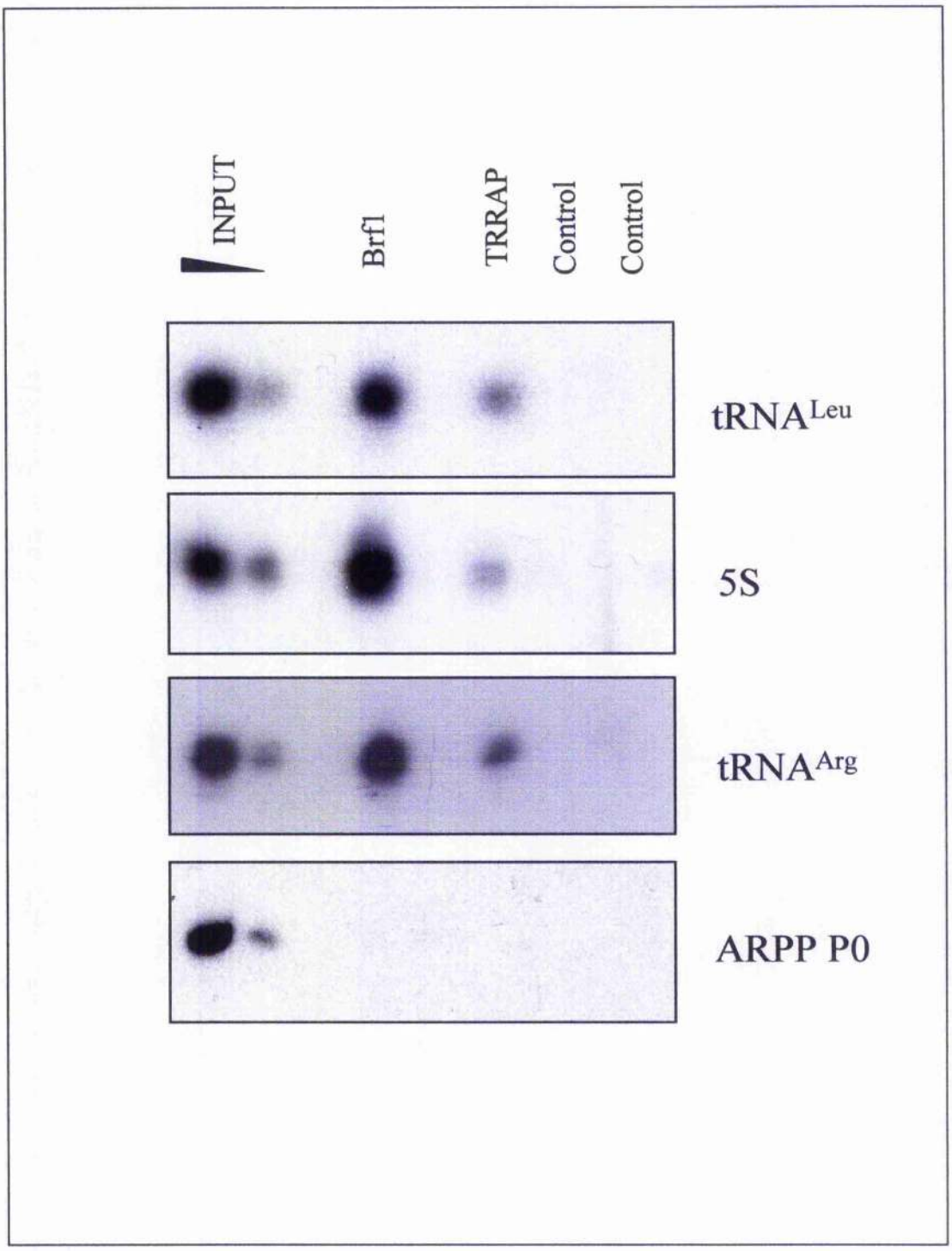
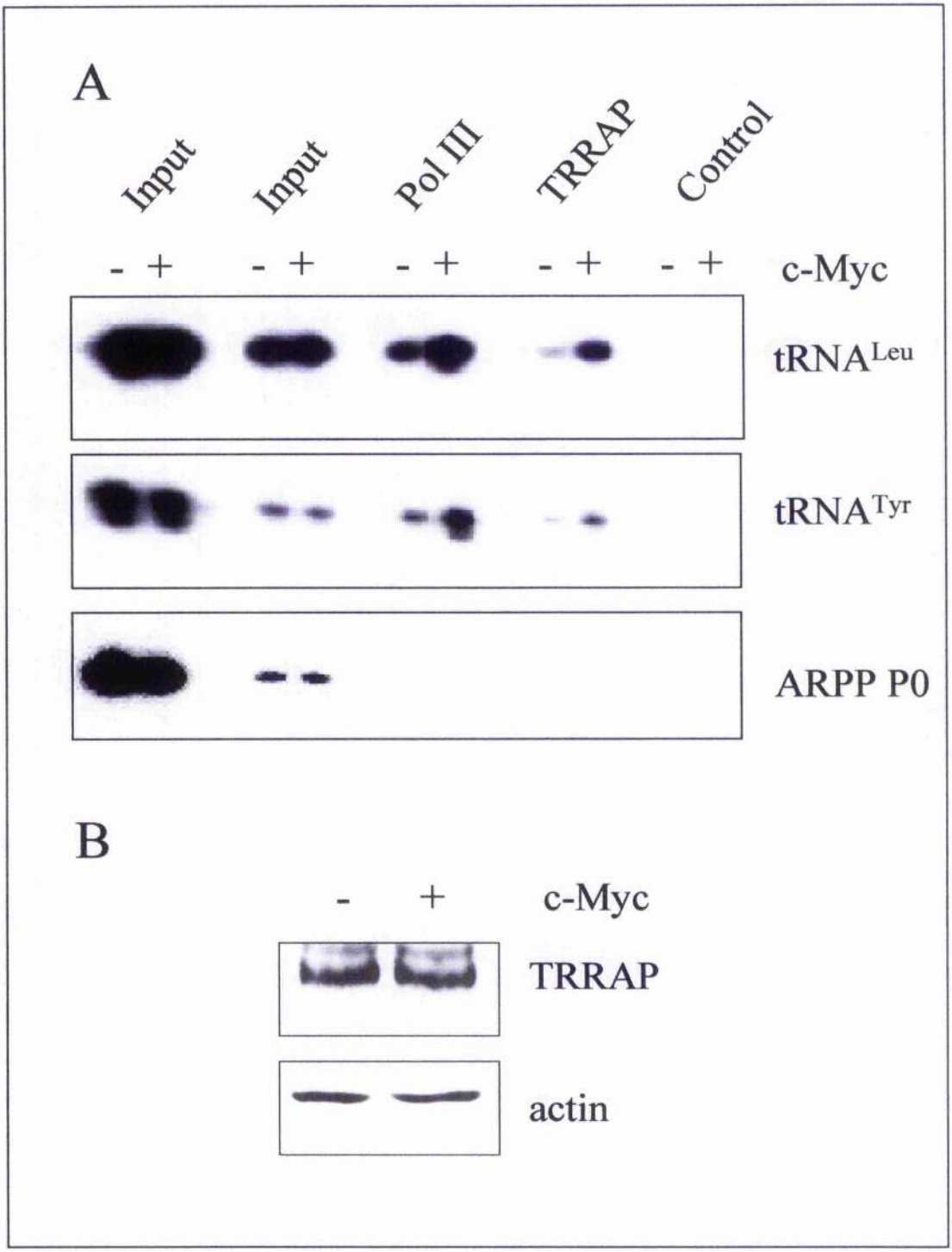

INPUT
Brf1
TRRAP
Control
Control
tRNA^Leu
5S
tRNA^Arg
ARPP P0

Figure 5.6: TRRAP is enriched at the promoters of pol III-transcribed genes in vivo in a c-Myc-sensitive manner A. The promoter occupancy of TRRAP was measured in matched c-Myc$^{-/-}$ and c-Myc$^{+/+}$ rat fibroblasts. ChIPs were performed using antibodies against the large pol III subunit, RPC 155, TRRAP and TFIIB (negative control). The samples were normalised by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using tRNA^{Leu}, tRNA^{Tyr}, and ARPP P0 primers, as indicated. B. Whole cell extracts were made from c-Myc wildtype and c-Myc null cells. The protein extracts were resolved by SDS-PAGE and blotted using antibodies against TRRAP and actin.


A
Input
Input
Pol III
TRRAP
Control
- + - + - + - + - +
c-Myc
tRNA^Leu
tRNA^Tyr
ARPP P0
B
- +
c-Myc
TRRAP
actin

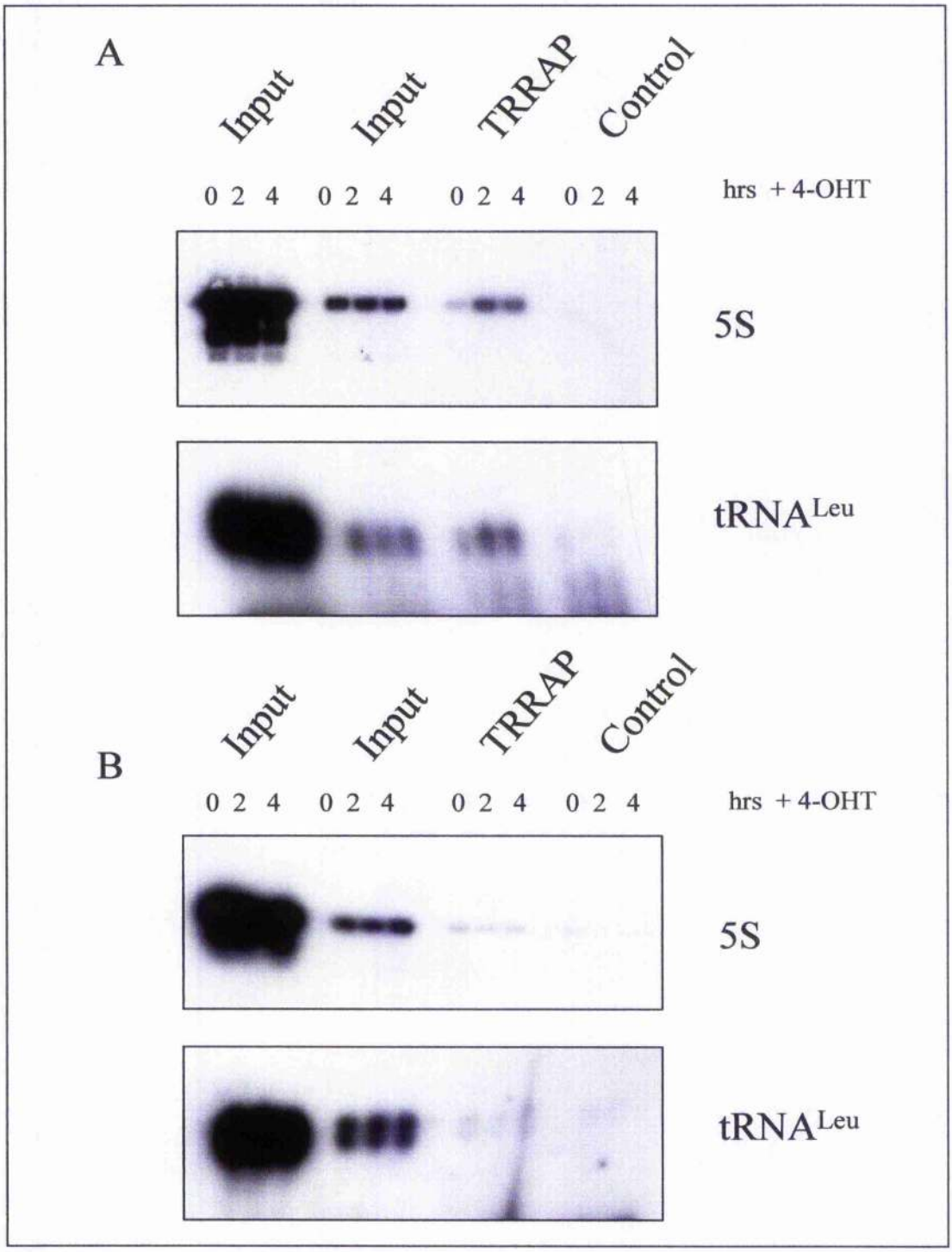
$tRNA^{Tyr}$ genes, compared with the occupancy on the same genes in the c-Myc deficient cell line (Figure 5.6a). This increase is not due to higher levels of TRRAP in the wildtype cell line, as shown by western blotting (Figure 5.6b). This is consistent with c-Myc being involved in the enrichment of TRRAP on pol III-transcribed genes.

5.2.3 Binding of c-myc to pol III-transcribed genes results in recruitment of TRRAP and increased histone acetylation

Bouchard and colleagues examined the levels of TRRAP and localised histone acetylation on the cyclin D2 gene promoter using a cell line with a 4-OHT inducible MycER construct (Bouchard et al, 2001). To address whether TRRAP could be recruited to pol III-transcribed genes in a c-Myc-dependent manner, ChIP assays were performed using the MycER and pBabe (control) cell lines. MycER and pBabe cells were serum starved for 48hrs prior to treatment with 4-OHT, to down regulate the endogenous c-Myc protein. The cells were treated with 4-OHT and harvested at the indicated time points and ChIP assays were performed using anti-TRRAP and anti-TAF_I48 antibodies. Activation of MycER resulted in a recruitment of TRRAP to the promoters of the 5S rRNA and $tRNA^{Leu}$ genes after 2hrs (Figure 5.7a). The effect is not due to 4-OHT, as ChIPs using the pBabe control cell line do not show any recruitment of TRRAP to the 5S rRNA or $tRNA^{Leu}$ genes in response to treatment with the synthetic hormone (Figure 5.7b). The timing of the recruitment of TRRAP is consistent with the protein being recruited with c-Myc, as they are both recruited to pol III-transcribed genes within 2hrs in response to MycER activation (Figure 4.3a and Figure 5.7a).

Figure 5.7: TRRAP is recruited to pol III-transcribed genes in a c-Myc dependent manner in vivo

A. c-Myc was induced in MycER fibroblasts by addition of 200nM 4-OHT. ChIP assays were performed at the indicated time points after MycER activation using antibodies against TRRAP and TFIIB. The samples were normalised by comparing the genomic DNA inputs. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using 5S and $tRNA^{Leu}$ primers as indicated. B. As above, but using the pBabe transfected control cell line.


A
Input
Input
TRRAP
Control
0 2 4 0 2 4 0 2 4 0 2 4
hrs + 4-OHT
5S
tRNA^Leu
B
Input
Input
TRRAP
Control
0 2 4 0 2 4 0 2 4 0 2 4
hrs + 4-OHT
5S
tRNA^Leu

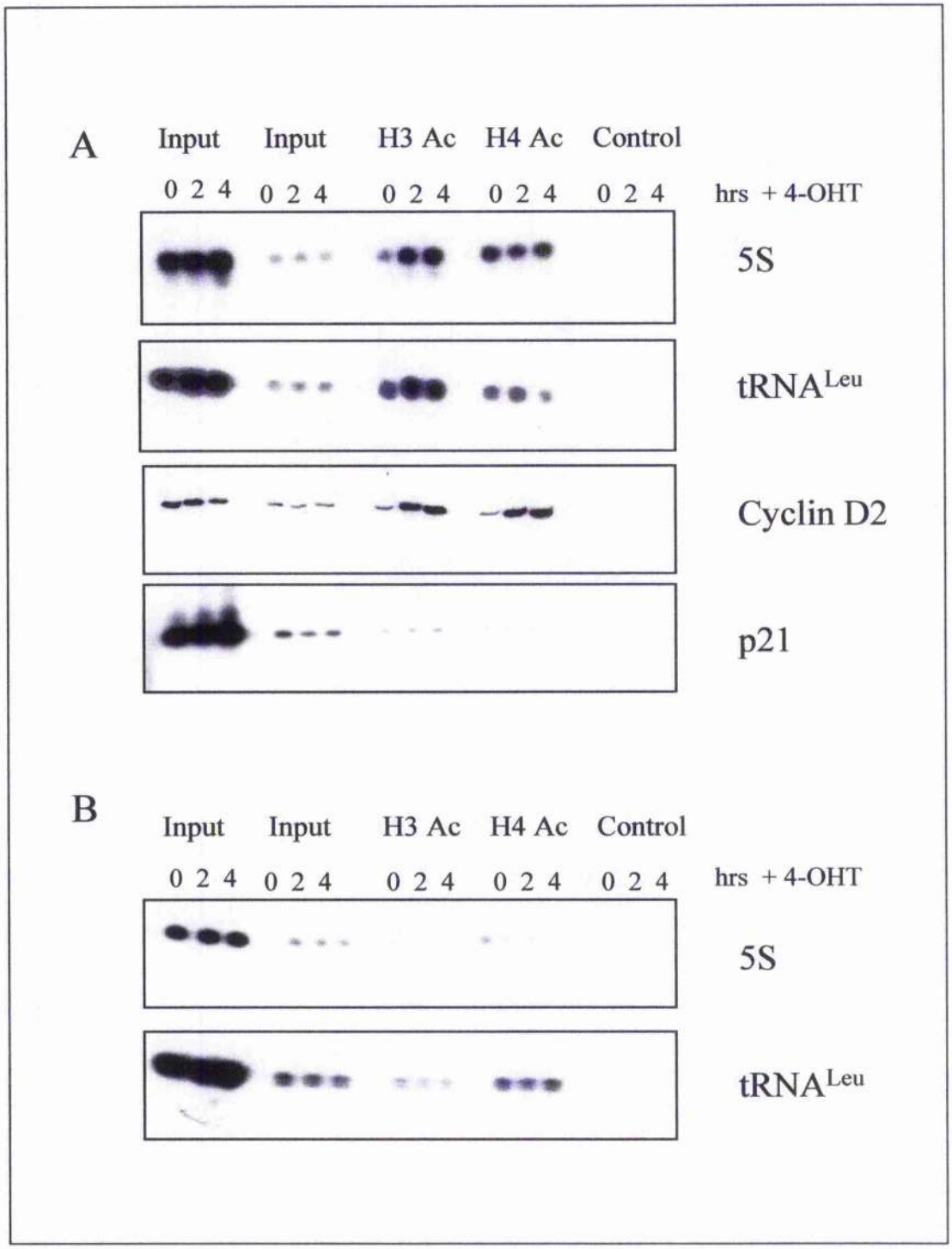
Transcriptional activation of several target genes by c-myc has been shown to alter levels of localised acetylation of the histone tails of nucleosomes surrounding their promoter regions (Bouchard et al, 2001; Frank et al, 2001; Fernandez et al, 2003). To examine whether this was the case in pol III-transcribed genes, ChIP assays were performed using the MycER and pBabe cell lines. The cells were serum starved and myc was induced with 4-OHT, as previously described, and cells were harvested at the indicated time points. Activation of MycER resulted in a localised increase in histone H3 acetylation surrounding the promoters of the 5S rRNA and $tRNA^{Leu}$ genes, but not an increase in the levels of acetylated histone H4 (Figure 5.8a). This is in contrast to the results observed on the promoter of the cyclin D2 gene, where an increase in the levels of localised acetylation of both histones H3 and H4 is observed in response to activation of MycER, suggesting a different method of activation, when comparing the pol II-transcribed cyclin D2 gene and the pol III-transcribed genes (Figure 5.8a; Bouchard et al, 2001). The effect on histone acetylation was not an indirect effect of 4-OHT addition, as no changes in histone acetylation on 5S rRNA and $tRNA^{Leu}$ genes were observed in the pBabe cell line (Figure 5.8b).

5.2.4 Promoter occupancy of c-myc, TRRAP, and acetylated histone H3 are lowered in response to MEL cell differentiation

To further investigate how TRRAP and histone acetylation can contribute to the c-Myc activation of pol III-transcribed genes under physiological conditions I used a mouse erythroleukaemia (MEL) cell line. As described in chapter 4, the MEL cell line can be differentiated using the chemical HMBA; this differentiation results in an

Figure 5.8: Activation of MycER induces localised histone H3 acetylation surrounding the promoters of class III genes

A. c-Myc was induced in MycER fibroblasts by addition of 200nM 4-OHT. ChIP assays were performed at the indicated time points after MycER activation using antibodies against acetylated lysines of histone H3 and histone H4, using an antibody against TAF_I48 as a negative control. The samples were normalised by comparing the genomic DNA inputs. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using 5S, $tRNA^{Leu}$, cyclin D2 and p21 primers as indicated. B. As above, but ChIP was performed using the pBabe-transfected control cell line. Samples were analysed by PCR, alongside input genomic DNA (10% and 1%), using 5S and $tRNA^{Leu}$-specific primers.


A
Input
Input
H3 Ac
H4 Ac
Control
0 2 4 0 2 4 0 2 4 0 2 4 0 2 4
hrs + 4-OHT
5S
tRNA^Leu
Cyclin D2
p21
B
Input
Input
H3 Ac
H4 Ac
Control
0 2 4 0 2 4 0 2 4 0 2 4 0 2 4
hrs + 4-OHT
5S
tRNA^Leu

immediate, dramatic fall in c-Myc levels and can be used as a model system for loss of c-Myc (Figure 4.10).

To investigate any difference in the levels of TRRAP, Max and localised histone H3 acetylation associated with pol III-transcribed genes ChIP assays were performed on undifferentiated and differentiated MEL cells. Levels of c-Myc associated with pol III-transcribed genes are reduced in the differentiated cells compared to the undifferentiated MEL cells (Figure 4.12a). The levels of TRRAP associated with the promoters of the 5S rRNA and $tRNA^{Leu}$ genes were also clearly reduced in response to differentiation (Figure 5.9a). Unlike c-Myc levels, the levels of the TRRAP protein do not change over the time course, so changes in the promoter occupancy were not simply due to TRRAP levels becoming limiting in the cell (Figure 5.9b). The levels of Max associated with pol III-transcribed genes stayed constant, indicating that the presence of Max on a pol III-transcribed gene is not dependent on the presence of c-Myc (Figure 5.9a).

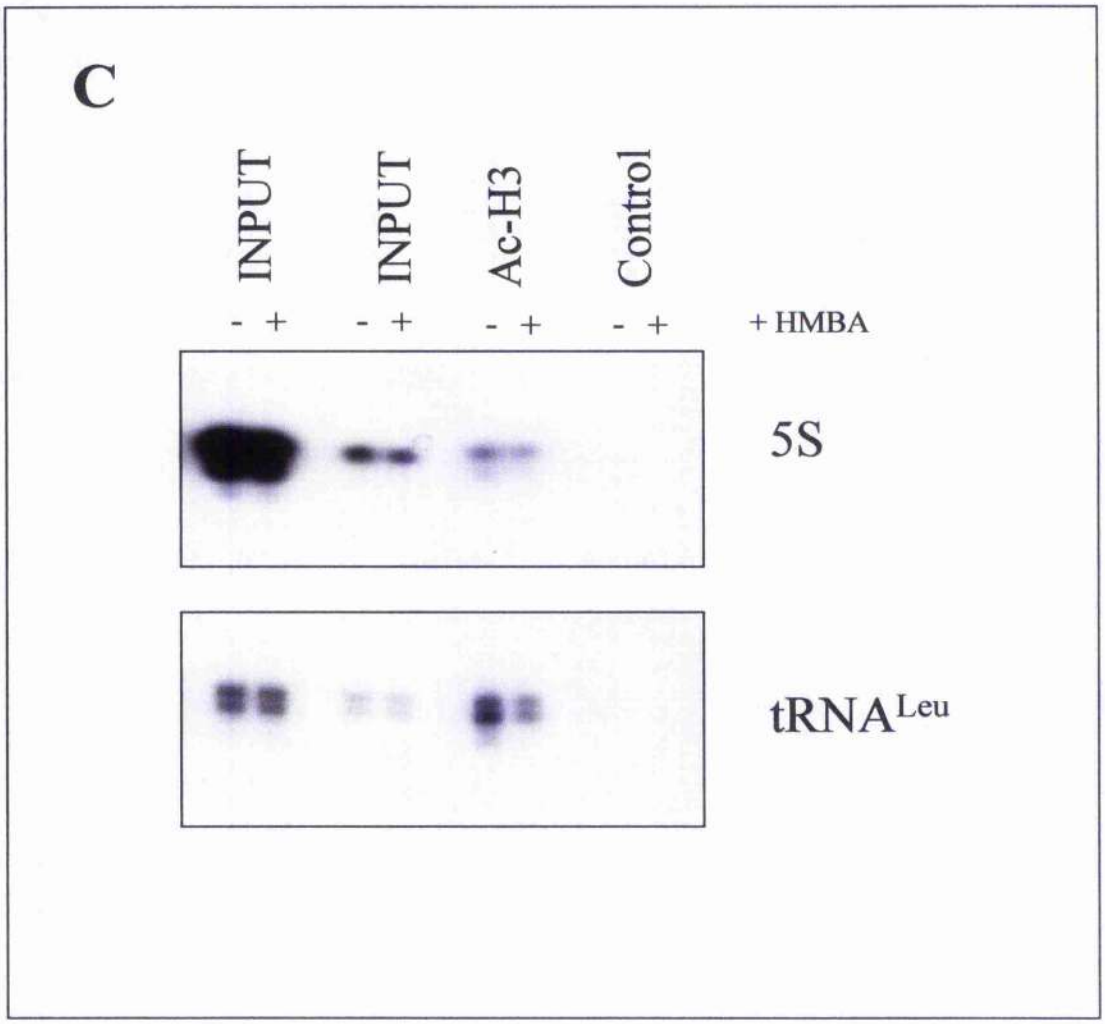
To test if the reduced levels of TRRAP associated with the promoters of pol III transcribed genes correlates with a reduced level of localised histone acetylation ChIP assays were performed on undifferentiated and differentiated MEL cells using an anti-acetyl H3 antibody. In differentiated MEL cells there was a reduced level of acetylated histone H3 associated with $tRNA^{Leu}$ and 5S rRNA genes (Figure 5.9c).

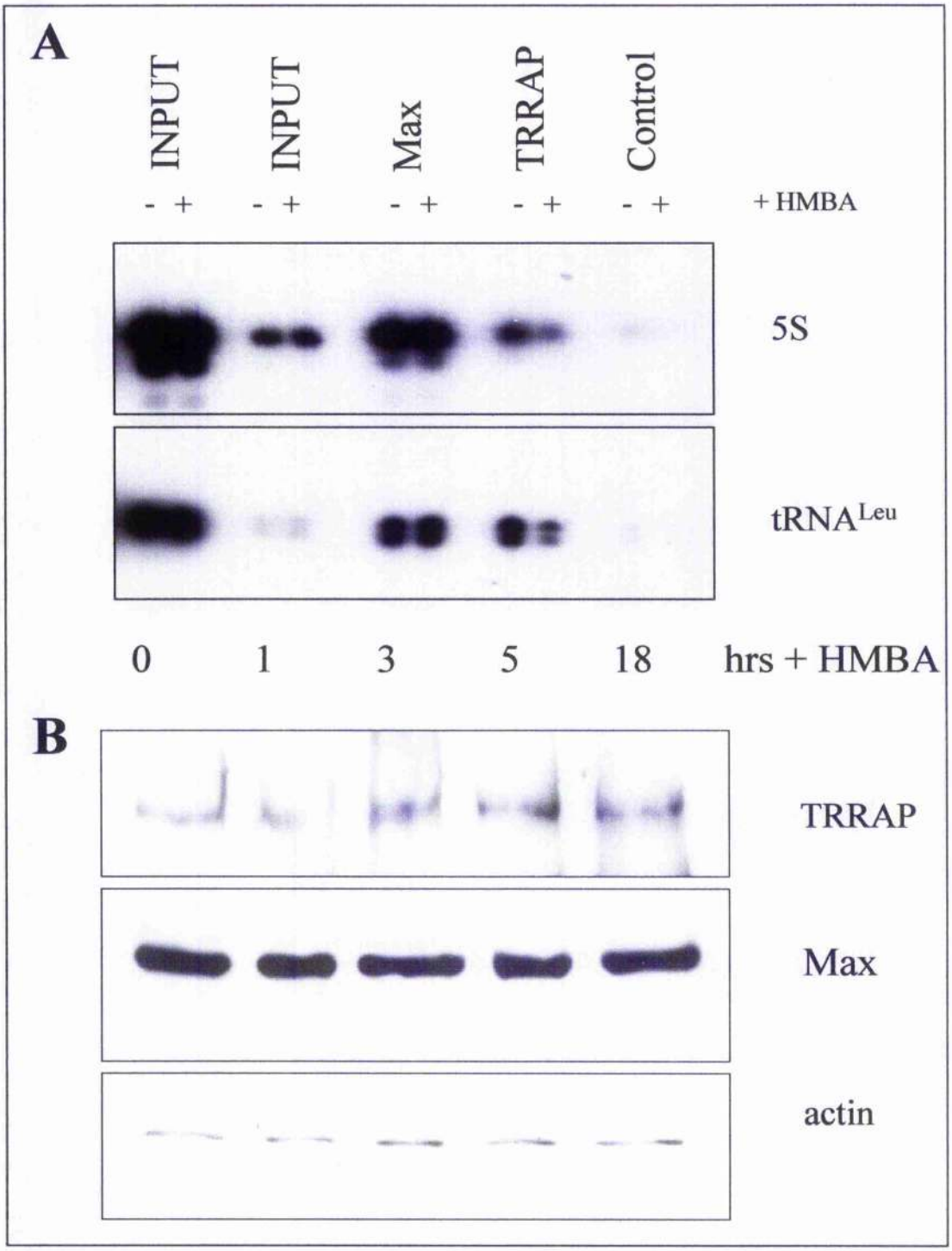
5.2.5 TRRAP interacts with TFIIIB in a c-Myc-dependent manner

The c-Myc protein interacts specifically with the pol III specific transcription factor TFIIIB (Gomez-Roman et al; Chapter 3). To determine if TRRAP associates with

Figure 5.9: Promoter occupancy of TRRAP is reduced in response to chemically induced differentiation of MEL cells

Differentiation of murine erthroleukemia (MEL) cells was induced by treatment with 5mM HMBA for 5hours. ChIP assays were performed on undifferentiated and differentiated MEL cells, using antibodies against Max, TRRAP and TFIIB (negative control). The samples were normalised by comparing the genomic DNA inputs. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using $tRNA^{Leu}$ and 5S rRNA primers as indicated. B. Whole cell extracts were made from MEL cells treated with 5 mM HMBA for the indicated times. The protein extracts were resolved by SDS-PAGE and blotted using antibodies against TRRAP, Max and actin. C. ChIP assays were performed as in A, but using antibodies against acetylated histone H3 and TFIIB (control).


C
INPUT
INPUT
Ac-H3
Control
- + - + - + - +
+ HMBA
5S
tRNA Leu


A
INPUT
INPUT
Max
TRRAP
Control
- + - + - + - + - +
+ HMBA
5S
tRNA^Leu
0 1 3 5 18 hrs + HMBA
B
TRRAP
Max
actin

TFIIIB, co-immunoprecipitation assays were performed. HeLa nuclear extracts were mixed with reticulocyte lysate containing ^{35}S-labelled in vitro transcribed and translated Brf1, these extracts were immunoprepitated with anti-TRRAP antibody or anti TFIIA antibody as a negative control. The recovered protein complexes were resolved by SDS-PAGE and subject to auto-radiography to test for co-immunoprecipitation of the Brf1 component of TFIIIB. The binding of TFIIIB to TRRAP was confirmed by the co-immunoprecipitation of Brf1 and TRRAP, this interaction is specific, as a TFIIA antibody does not immunoprecipitated Brf1 (Figure 5.10a).

To determine if the interaction between TFIIIB and TRRAP is c-Myc dependent, co-immunoprecipitation experiments were performed comparing the interaction between TRRAP and ^{35}S-labelled Brf1 in extracts made from c-Myc null and c-Myc wildtype fibroblasts. In the extracts from c-Myc wildtype fibroblasts the specific interaction between TFIIIB and TRRAP is observed, but this interaction is not observed when the same experiment was performed in extracts from c-Myc null fibroblasts, indicating that this interaction is sensitive to the presence of c-Myc (Figure 5.10b).

5.2.6 Knocking down TRRAP levels using RNAi results in lowering levels of Pol III transcription

RNA interference was used to test whether or not transcription of tRNA genes was sensitive to the level of endogenous TRRAP. A pool of vectors encoding short-hairpin RNAs against the TRRAP transcript were transiently transfected into HeLa cells (Brummelkamp et al, 2002; Berns et al, 2004). To confirm knockdown of the

Figure 5.10 : TRRAP interacts with TFIIIB in a c-Myc-dependent manner

A. Reticulocyte lysate (5µl) containing in vitro-translated Brf1 was added to 300µg HeLa cell nuclear extract and then immunoprecipitated using anti-TRRAP or anti-TFIIA antibodies. Proteins retained after wash steps were resolved by SDS-PAGE and then visualised by autoradiography. 5% of the extract was run as an input B. Reticulocyte lysate (5µl) containing in vitro-translated Brf1 was added to 250µg of whole cell extracts prepared from either c-myc$^{-/-}$ or c-myc$^{+/+}$, then immunoprecipitated using anti-TRRAP or anti-TFIIA antibodies. Proteins retained after wash steps were resolved by SDS-PAGE and then visualised by autoradiography. 5% of each of the original extracts were run as inputs.

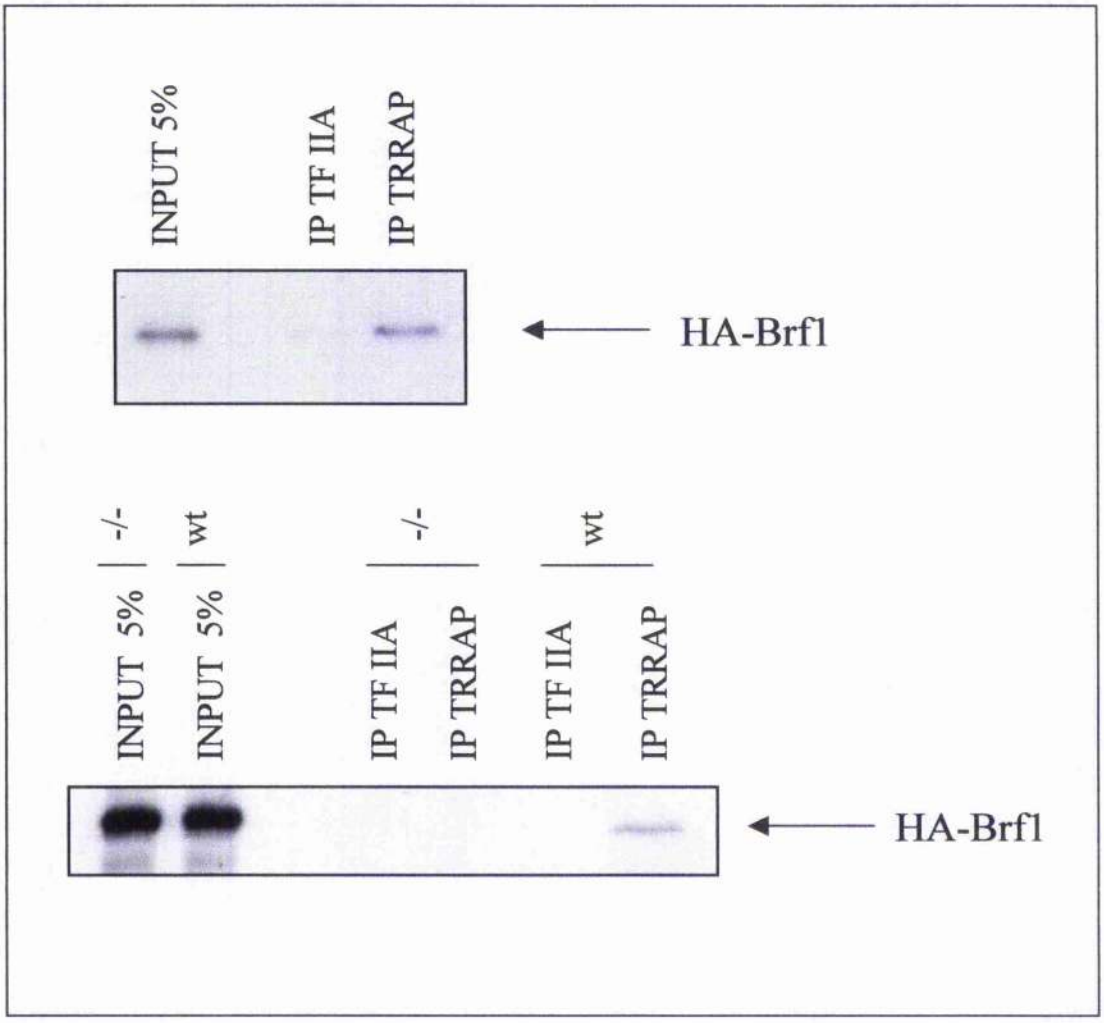
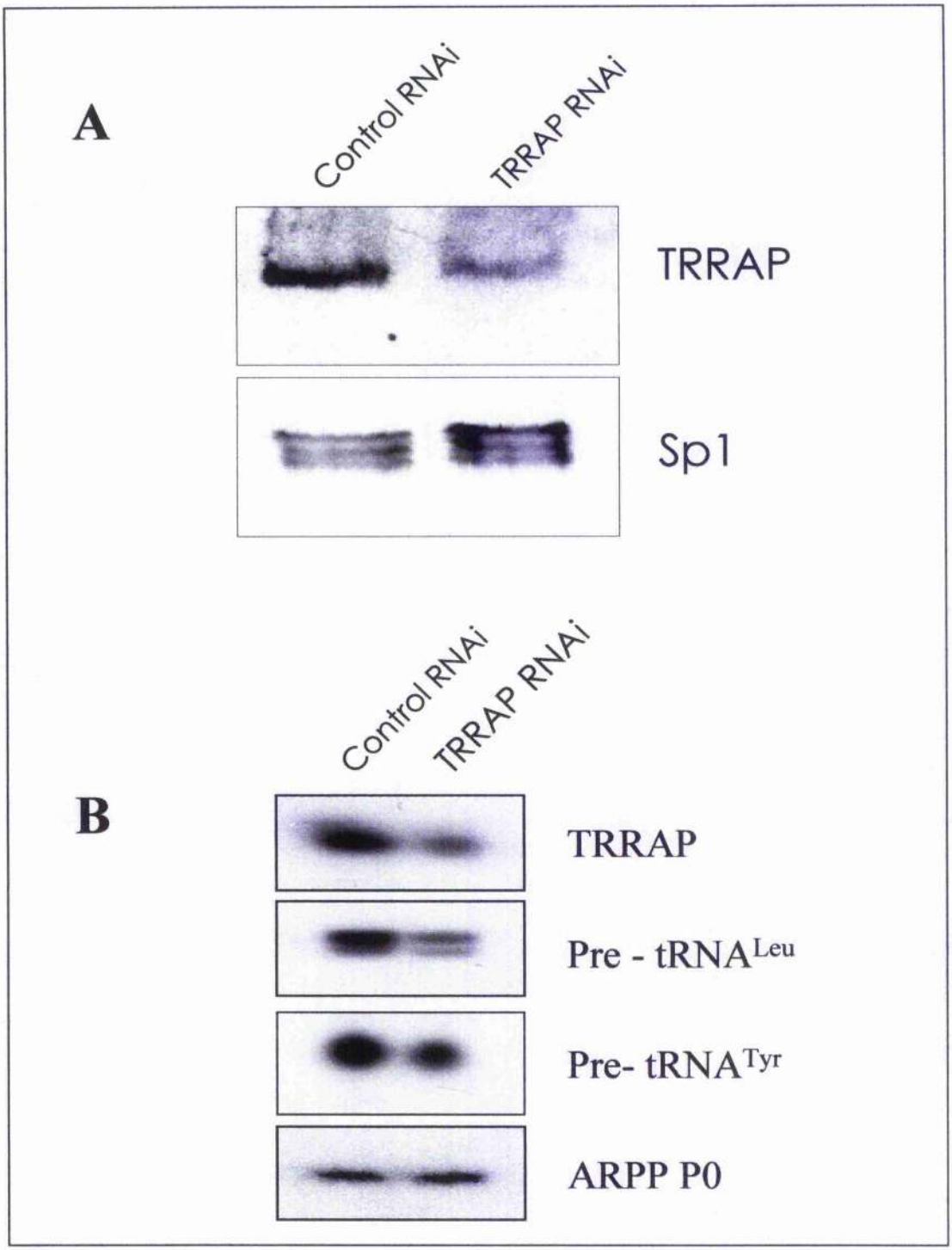

INPUT 5%
IP TF IIA
IP TRRAP
HA-Brf1
-/-
wt
INPUT 5%
INPUT 5%
IP TF IIA
IP TRRAP
IP TF IIA
IP TRRAP
HA-Brf1

Figure 5.11: Depletion of endogenous TRRAP levels in HeLa cells by RNA interference selectively reduces class III gene expression in HeLa cells

Growing HeLa cells were transfected with pSuper vectors encoding short hairpin RNAs (shRNAs) against TRRAP or MyoD (control). A. Whole cell extracts were harvested from transfected cells 48 hours after transfection and analysed by western blot, using antibodies against TRRAP or Sp1 (control). B. RNA was harvested from transfected cells 48 hours after transfection and analysed by RT-PCR for the expression of TRRAP, $tRNA^{Leu}$, $tRNA^{Tyr}$ and ARPP P0 (control).genes are lower in cells with less TRRAP as shown by RT-PCR (Figure 5.11b). This effect of the TRRAP shRNA was specific, as confirmed by the levels of the control transcript ARPP P0 remaining constant (Figure 5.11b).


A
Control RNAi
TRRAP RNAi
TRRAP
Sp1
B
Control RNAi
TRRAP RNAi
TRRAP
Pre - tRNA^{Leu}
Pre- tRNA^{Tyr}
ARPP P0

TRRAP protein levels, western blots were performed using extracts from TRRAP shRNA-transfected cells, and cells transfected with shRNA vectors against MyoD, a muscle specific transcription factor, as a control. The levels of the TRRAP protein are partially, but clearly knocked down in TRRAP shRNA-transfected cells compared to the control (Figure 5.11a). The levels of transcription of the 5S, $tRNA^{Leu}$ and $tRNA^{Tyr}$ genes are reduced in the cells with lower levels of the TRRAP protein (5.11b). This effect is specific as levels of the pol II-transcribed ARPP P0 transcript do not change in these cells (5.11b).

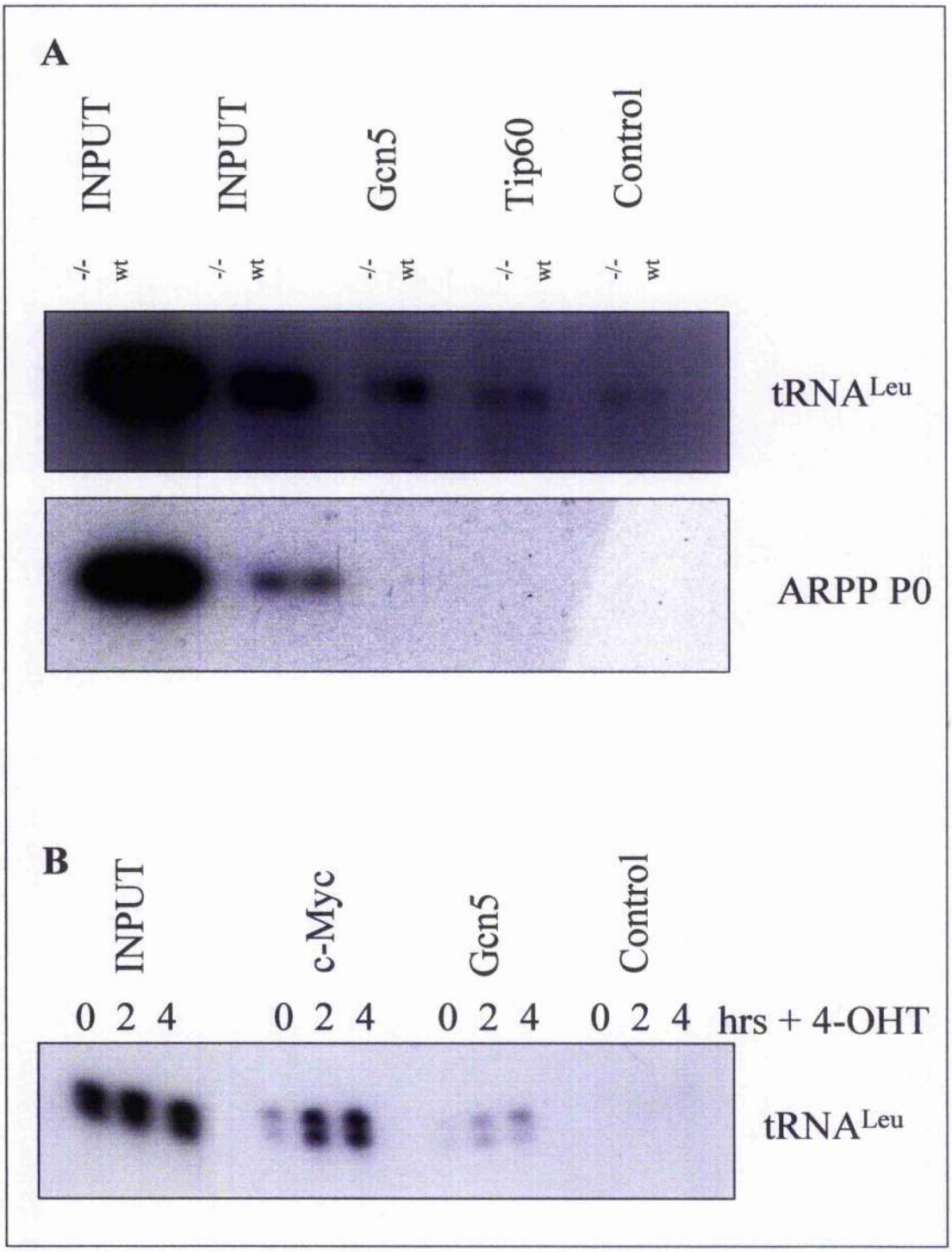
5.2.7 The HAT Gcn5 is recruited to pol III-transcribed genes in a c-Myc dependent manner

TRRAP is a common component of several different HAT complexes associated with c-Myc that have been implicated in the transactivation of c-Myc target genes. Myc has been shown to be in complex with Gcn5 and Tip60, through an interaction with the TRRAP protein (McMahon et al, 2000; Frank et al, 2003). To test if these HATs are present at the promoters of pol III-transcribed genes, ChIPs were performed using matched c-Myc wildtype and c-Myc null fibroblasts. ChIP assays were performed using anti-Gcn5, anti-Tip60 and anti-TFIIB antibodies. The level of Gcn5 associated with the $tRNA^{Leu}$ gene is enriched in the c-Myc wildtype fibroblasts compared to the c-Myc deficient fibroblasts (Figure 5.12a). The levels of Tip60 are marginally above the control IP; thus it may be associated with class III genes, but its binding to pol III-transcribed gene promoters is not enriched in the presence of c-Myc (Figure 5.11a).

The association of Gcn5 with the pol III-transcribed gene is specific, as no binding was observed on ARPP P0 gene (Figure 5.12a).

Figure 5.12: Gcn5 is associated with the promoters of class III genes in a c-Myc-sensitive manner

A. The promoter occupancy of Gcn5 was measured in matched c-Myc$^{-/-}$ and c-Myc$^{+/+}$ rat fibroblasts. ChIPs were performed using antibodies against Gcn5, TIP60 and TFIIB (negative control). The samples were normalised by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using tRNA^{Leu} and ARPP P0 (control) primers as indicated. B. c-Myc was induced in MycER fibroblasts by addition of 200nM 4-OHT. ChIP assays were performed at the indicated time points after MycER activation using antibodies against c-Myc, Gcn5 and TAF$_I$48. The samples were normalised by comparing the genomic DNA inputs. The samples were then analysed by PCR, alongside input genomic DNA (10% and 1%), using tRNA^{Leu} primers as indicated.


A
INPUT
INPUT
Gcn5
Tip60
Control
-/- wt -/- wt -/- wt -/- wt -/- wt
tRNA^Leu
ARPP P0
B
INPUT
c-Myc
Gcn5
Control
0 2 4 0 2 4 0 2 4 0 2 4 hrs + 4-OHT
tRNA^Leu

To confirm the presence of Gcn5 on a class III gene in a different system, ChIP assays were performed on MycER cells to test for c-Myc-dependent recruitment. ChIP assays were performed using anti-c-Myc, anti-gcn5, and anti-TAF$_I$48 antibodies. c-Myc is recruited to pol III-transcribed genes in response to 4-OHT induction, consistent with the results outlined in chapter 4. (Figure 4.3a). The recruitment of the HAT Gcn5 coincided with the recruitment of c-myc and TRRAP to a $tRNA^{Leu}$ gene (figure 5.12b)

5.3 Discussion

The data presented in this chapter confirm that, in the model systems used, factors that modify chromatin structure are recruited to the promoters of class III genes and implicated in the c-Myc-dependent activation of pol III transcription.

c-Myc directly interacts with the Snf5 component of the SWI/SNF chromatin remodelling complex, via an interaction with its C-terminal basic helix-loop-helix zipper domain, and expression of an inactive catalytic subunit, Brg1, resulted in c-myc transactivation being inhibited in a reporter assay (Cheng et al, 1999). Both Snf5 and the catalytic subunit Brg1 are present at the promoters of pol III-transcribed genes in vivo, as confirmed by ChIP assays, indicating that chromatin remodelling enzymes may play a role in organising the localised chromatin structure surrounding pol III-transcribed genes to regulate transcription. In an attempt to explore the possibility that Snf5 and Brg1 were recruited to class III genes in a c-Myc-dependent manner, ChIP assays were performed using a c-Myc inducible cell line. However, as discussed previously, these assays were unsuccessful, perhaps due to an inability to immunoprecipitate these proteins in a rodent cell line. The SWI/SNF complex was not detected on the well-characterised c-Myc target gene cyclin D2 in these assays, further suggesting a problem with the immunoprecipitation, or that SWI/SNF is not recruited to this promoter.

The potential involvement of SWI/SNF in the c-Myc-dependent activation of pol III transcription is puzzling, due to the fact that components of the complex have been demonstrated to be tumour suppressors, for example the c-Myc-interacting protein

Snf5 (Roberts et al, 2003). To address this further, experiments are required to assay whether SWI/SNF is involved in the regulation of pol III-transcribed genes including experiments to assess pol III activity in cells deficient in SWI/SNF subunits, or to target sub-units with RNA interference to selectively knock down their expression. These techniques could be used to uncover any involvement of SWI/SNF on expression of class III genes.

The c-myc associated protein, TRRAP, has been shown to be critical for myc transforming activity by being necessary for the activation of a subset of c-Myc target genes (McMahon et al, 1998; Herceg et al, 2001; Herceg et al, 2003). TRRAP is a component of several HAT complexes that are recruited to the promoters of c-myc target genes (Bouchard et al, 2001; Frank et al, 2001). The data presented have not only demonstrated TRRAP association with pol III-transcribed genes, but also that enhanced TRRAP binding to the promoter coincides with c-Myc binding, and precedes pol III transcriptional activation, as confirmed by ChIP analysis in the MycER cell line (Figure 5.6a; Figure 4.3a). Confirming the c-Myc dependent TRRAP protein association with Class III genes, TRRAP is enriched on the promoters of pol III-transcribed genes in c-Myc wildtype, compared to c-Myc deficient cell lines (Figure 5.5a).

The c-Myc protein is associated with the pol III-specific transcription factor TFIIIB, and it is this interaction which is thought to be responsible for its association with pol III-transcribed genes (Gomez-Roman et al, 2003, Felton-Edkins et al, 2003b) Co-immunoprecipitation assays were performed that demonstrated that TRRAP can interact with the Brf1 component of TFIIIB (Figure 5.9a). This interaction is

dependent on c-Myc, as the interaction is not observed in c-Myc deficient fibroblasts (Figure 5.9b). Although the TRRAP/ TFIIIB interaction is dependent on the presence of c-Myc, there is evidence that low levels of TRRAP are associated with the promoters of pol III transcribed genes in a c-Myc independent manner (Figure 5.5). This may be due to a non-specific background signal obtained from the antibody, or a secondary uncharacterised interaction between TRRAP and the pol III transcription apparatus.

As discussed previously in this chapter the TRRAP protein is a common subunit of several histone acetyltransferase complexes, and recruitment of TRRAP coincides with an increase in histone H3 acetylation (figure 5.7a). This may represent a requirement for a localised increase in histone acetylation surrounding the promoters of pol III-transcribed genes to lead to an increase in pol III transcription. In studies performed *in vitro* on a 5S rRNA gene, increased histone acetylation and disruption of higher order chromatin structure leads to a 15-fold increase in the level of transcription of this template (Tse et al, 1998). Preliminary data demonstrate that treatment of mammalian cells with the HDAC inhibitor TSA leads to an increase in localised histone acetylation surrounding the promoters of class III genes and an activation of their transcription (Ramsbottom and White, unpublished observations).

The pattern of the histone acetylation is surprising; the majority of work on this area has focused on a subset of well-characterised pol II-transcribed genes, which mainly exhibit localised changes in histone H4 acetylation and some changes in histone H3 (Frank et al, 2001; Fernandez et al, 2003). c-Myc has also been shown to enhance localised histone acetylation surrounding the ribosomal DNA and stimulate

transcription by Pol I (Grandori et al, 2005; Arabi et al, 2005). The activation by c-myc in this case has been followed by a recruitment of TRRAP to the pol I-transcribed large ribosomal RNA genes. (Arabi et al, 2005). Again, the studies on c-Myc activation of Pol I transcription reveal an increase in levels of predominantly histone H4 acetylation surrounding the promoters of the pol I-transcribed genes (Grandori et al, 2005; Arabi et al, 2005). The significance of the preferred acetylation of histone H3 is unclear, but it would suggest that the activation of pol III-transcribed genes by c-Myc involves different TRRAP/HAT complexes at the promoters of pol III target genes than it when activating transcription of genes transcribed by pol I and pol II.

The specific acetylation of histone H3 indicated that it was likely that the HAT recruited by c-Myc and TRRAP to the promoters of pol III-transcribed genes would have substrate specificity for histone H3. The HAT Gcn5 was demonstrated to be associated with pol III-transcribed genes in a c-Myc-sensitive manner. The Gcn5 HAT and TRRAP are recruited to pol III-transcribed genes at the same time as c-Myc in the MycER cell line. Gcn5 complexes have been demonstrated to prefer the Histone H3 tail as their preferred substrate and this result fits with the observation that c-Myc induces localised acetylation of histone H3 associated with certain class III genes (Sterner and Berger, 2000; Roth et al, 2001). The human form of Gcn5 has been demonstrated to exist in two distinct HAT complexes with TRRAP, TFTC and STAGA; but the nature of the complex associated with class III genes has yet to be determined.

The HAT Gcn5 has been shown to be present on pol III-transcribed genes in a c-myc sensitive manner. In a cell line where the Gcn5 HAT has been deleted using a gene targeting technique, the Gcn5 deficient cells have shown to have a reduced growth rate and deficiencies in progressing through the cell cycle (Kikuchi et al, 2005). As the rate of protein synthesis correlates with the growth rate of a cell (Zetterberg and Killander, 1965), the deficiency in the growth rate in Gcn5 null cells may be due to a reduced supply of pol III-transcribed factors involved in protein synthesis.

The target of the TRRAP/HAT complex may not be solely a histone substrate, as there have been many studies published showing that HATs can act on transcription factors to increase transcription of target genes (Kouzerides, 2000). c-Myc itself can be acetylated by HAT complexes (Vervoorts et al, 2003: Patel et al, 2004). It has been demonstrated that acetylation of c-Myc can lead to c-Myc stabilisation and an increase in its half-life (Patel et al, 2004). c-Myc's affinity for target genes may also be increased in response to acetylation, in a manner analogous to that observed with p53 (Gu and Roeder, 1997). The results here show that an increase in c-Myc/TRRAP and associated HAT, leads to an increase in histone H3 acetylation at the promoters of class III genes, but does not answer the question whether non-histone substrates are also targets.

Class III genes do not have any E-box sequences in their promoters, which are required for c-Myc/Max binding to the DNA, instead c-Myc is thought to be associated with promoters via an interaction with TFIIIB (Gomez-Roman et al, 2003). Immuno precipitation experiments have shown that TRRAP is also associated with the TFIIIB complex (Figure 5.9). This interaction with TFIIIB is dependent on the

presence of c-Myc, as shown by further immunoprecipitations using extracts from c-Myc null and c-Myc wild type cell lines (Figure 5.9). These results suggest that the recruitment of a TRRAP/Gcn5 HAT complex is through an indirect interaction with TFIIIB, through a bridging interaction with c-Myc, or perhaps the TRRAP can bind to TFIIIB weakly and c-Myc can act to stabilise it. The timing of the recruitment of TFIIIB, c-Myc, TRRAP and Gcn5 and the c-Myc dependent recruitment of these factors, may suggest they are recruited to the promoter as a complex, with c-Myc being the limiting factor.

Although do not know if the histones are the only substrates of the c-Myc recruited TRRAP/Gcn5 HAT complex, TRRAP is involved in the activation of class III genes. Partially knocking-down TRRAP protein levels in a human cell line, by using vectors encoding shRNAs against the TRRAP transcript, results in lower levels of pol III transcription of the $tRNA^{Leu}$ and $tRNA^{Tyr}$ genes, showing a requirement for the TRRAP protein for activation of class III genes (Figure 5.10). TRRAP null cells display reduced proliferation and growth rates and have been demonstrated to differentially regulate a whole host of genes compared to matched wildtype cells (Herceg et al, 2003). The data above strongly suggest that pol III-transcribed genes are regulated, in part, by the TRRAP protein, and some of the growth defects in TRRAP null cells could be attributed to repression of pol III transcription.

The promoter occupancy of the TRRAP protein and localised acetylation of Histone H3 is reduced in response to chemical differentiation of MEL cells (Figure 5.8c). This reduction of TRRAP levels associated with the promoter coincides with reduced levels of c-Myc and pol III. It is interesting that the levels of promoter-bound TFIIIB

do not change in response to chemically-induced differentiation. This would indicate that if TFIIIB, c-Myc, TRRAP and Gcn5 are recruited as a complex, they do not dissociate from the promoter as one. The rapid reduction of the levels of histone H3 acetylation not only agrees with the reduction in c-Myc and TRRAP levels, but may also indicate the recruitment of an HDAC complex to class III genes in the absence of c-Myc.

The promoter-bound levels of the c-Myc binding partner Max also remain constant after differentiation (Figure 5.8). c-Myc recruitment to pol III-transcribed genes is thought to be through an interaction between TFIIIB and the transactivation domain of c-Myc (Gomez-Roman et al, 2003). Differentiating MEL cells have lower levels of the c-Myc protein than non-differentiated cells and this is reflected in the promoter occupancy of c-Myc on class III genes (Figure 4.10 and 4.12a). The observation that Max levels do not change on the promoters of class III genes indicates that Max can bind independently of c-Myc (Figure 5.8). As Max does not contain a homologous transactivation domain, this indicates it binds to class III genes through a novel interaction, and may suggest that other members of the Myc/Max/Mad family of transcription factors may influence transcription by pol III. The levels of the c-Myc antagonist Mad proteins increase in response to MEL cell differentiation, and perhaps Mad/Max complexes may replace c-Myc/Max complexes to recruit HDAC complexes to class III genes (Kime and Wright, 2003). This switch from Myc/Max to Mad/Max complexes in response to differentiation has been observed on c-Myc target genes transcribed by pol II (Bouchard et al, 2001; Xu et al, 2001). Indeed, preliminary data suggests that Mad proteins are associated with class III genes in vivo (Robertson and White, unpublished observations).

The activities of ATP-dependent chromatin remodelling enzymes and HATs are not exclusive and independent. Synergy between these factors has been demonstrated in the activation of a variety of genes. The ATPase subunits of several SWI/SNF ATP-dependent chromatin remodelling complexes, including the Brg1 ATPase that is associated with class III genes in vivo, have been shown to contain bromodomains (Sif, 2004). Bromodomains recognise acetylated lysines, and through this motif proteins can be recruited to hyper-acetylated histones, representing a mechanism by which SWI/SNF chromatin remodellers can be recruited to active genes (Bottomley, 2004; Horn and Peterson, 2001). The observation that both ATP-dependent chromatin remodelling complexes and HAT complexes are present on class III genes raises the possibility that they may work in concert to enable the recruitment of pol III-specific transcription factors to the promoters of these target genes.

Chapter 6 Final Discussion

Cancer is a disease that will affect 1 in 3 of the population of the United Kingdom and will eventually lead to the death of 1 in 4 (statistic from the Cancer Research UK web site, www.cancerresearchuk.org). Studying the mechanisms through which normal cells can become cancerous is critical to understanding tumour formation and the development of therapeutic agents. One of the key features of cancer cells is their ability to grow and proliferate in an uncontrolled manner (Hanahan and Weinberg, 2000). Analysis of the genetic alterations in cancer cells has revealed a large number of proto-oncogenes and tumour suppressor genes that encode proteins that are deregulated in tumour cells and can lead to aberrant growth and proliferation. The vast majority of work on the products of these tumour suppressor and oncogenes has focussed on their ability to control the cell cycle, and comparatively little work has been done on their ability to regulate cell growth.

Cell growth requires an accumulation of protein, which is dependent on the increase in the translational capacity of the cell. Translational capacity is determined by the availability of a number of factors including ribosomal proteins, rRNAs, tRNAs and limiting translation factors (outlined in Chapter 1). Products of the genes transcribed by pol III are critically involved in the biosynthetic capacity of the cell, and the understanding of the mechanisms behind their regulation is essential to understanding cell growth (White, 2002). Pol III transcription is elevated in many transformed and tumour cell types and its activity regulated by a number of tumour suppressors and oncogenes (White, 2004).

The c-Myc proto-oncogene is mutated or deregulated in a high proportion of tumours and can lead to the formation of tumours in experimental model systems (Dang, 1999; Nesbit et al, 1999). In fact, deregulated expression of Myc is found in 1 in 7 of all US cancer deaths (Dang, 1999; Nesbit et al, 1999). Initial characterisation of Myc target genes revealed many of its functions could be attributed to regulating the cell cycle, leading to the view that regulation of cell division is the principle mechanism for c-Myc dependent transformation of cells (Grandori and Eisenman, 1997). It is now becoming clear that c-Myc not only regulates genes involved in the cell cycle, but also has a profound influence on cell growth though activation of genes critical in this process (Gomez-Roman et al, 2005). c-Myc can potently and directly activate transcription by pol III and this accumulation of essential components, critical for protein synthesis, may be key to c-Myc-induced growth (Gomez-Roman et al, 2003).

The work in this thesis examined the mechanisms behind the activation of pol III transcription by c-Myc, and identified co-activator proteins involved in this process. An understanding of how oncogenes can activate cell growth is critical to our understanding of cancer and with c-Myc being proposed as an attractive therapeutic target in cancer (Hermeking, 2004; Prochownik, 2004), it is important to fully understand its action.

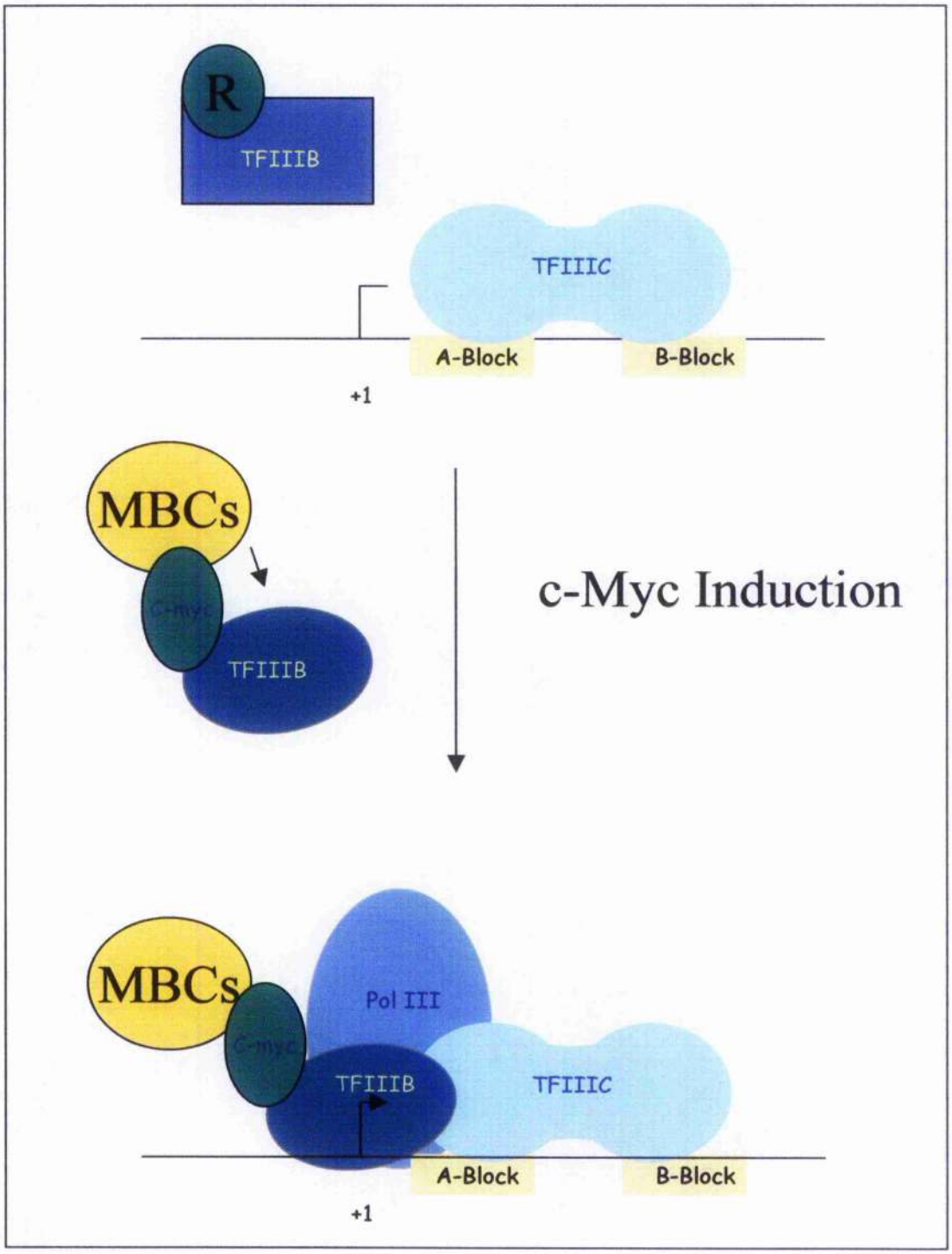
6.1 Mechanisms behind the c-Myc-dependent activation of pol III transcription

6.1.1 c-Myc can bind to TFIIIB and contributes to its recruitment to pol III promoters

The transcription of a class III gene is dependent on the recruitment of the general transcription factors followed by recruitment of the polymerase (Schramm and Hernandez, 2002). For the majority of genes transcribed by pol III, this is determined by the binding of TFIIIC and TFIIIB to the promoter regions and subsequent binding of the polymerase (Schramm and Hernandez, 2002). I have demonstrated that c-Myc can facilitate the formation of this complex by promoting the recruitment of TFIIIB and pol III to the promoters of 5S rRNA and tRNA genes in vivo, but does not alter the promoter occupancy of TFIIIC (Figures 4.6 and 4.8). The mechanism by which c-Myc can do this is unclear, as neither 5S rRNA nor tRNA genes contain E-box sequences to which c-Myc could bind and recruit the transcription machinery (Gomez-Roman et al, 2003). The observation that c-Myc and TFIIIB can bind in the absence of DNA through co-immunoprecipitation experiments (Figure 3.8; Gomez-Roman et al, 2003), may suggest that their initial interaction is in the nucleoplasm and precedes DNA binding; this binding could lead to the activation of the TFIIIB complex that increases its affinity for TFIIIC. I have further characterised the interaction between c-Myc and TFIIIB, demonstrating that c-Myc can bind to TBP and Brf1 in vitro (Figure 3.2). The activation of TFIIIB by c-Myc may be through a host of mechanisms, from precluding a TFIIIB/repressor complex interaction, altering the conformation of TFIIIB, through binding or post-translational modification of TFIIIB by Myc interaction complexes (Figure 6.1).

Figure 6.1 Activation of TFIIIB by c-Myc

Induction of c-Myc may activate TFIIIB and promote the formation of the TFIIIB-TFIIIC complex in several ways; competing for binding sites with repressor (R) proteins, inducing a conformational change in TFIIIB and/or post-translational modification by one of the many c-Myc-binding complexes (MBCs).


R
TFIIIB
TFIIIC
A-Block
B-Block
+1
MBCs
c-myc
TFIIIB
c-Myc Induction
MBCs
c-myc
Pol III
TFIIIB
TFIIIC
A-Block
B-Block
+1

6.1.2 c-Myc activation of pol III transcription by a sequential recruitment of general transcription factors

The formation of the transcription complex on class III genes is well studied in vitro and the order of binding relatively well defined (Figures 1.2, 1.3, 1.4; Schramm and Hernandez, 2002). However, this step-wise ordered recruitment of transcription factors onto a pol III-transcribed gene may not happen in vivo. In the formation of the transcription machinery of pol II, there is considerable evidence that factors may be assembled off of the DNA and may therefore be recruited to the promoters as a pre-formed complex. Various groups have identified these holoenzyme complexes and have proposed that pol II transcription complex formation may bypass some of the stages of the sequential recruitment of factors to a gene (Thomson et al, 1993; Ossipow et al, 1995). TFIIIB, TFIIIC and pol III have also been found to be associated in the absence of DNA in Drosophilia and human cell extracts (Burke and Soll, 1985; Wingender et al, 1986). The formation of a pol III holoenzyme complex containing TFIIIB, TFIIIC and pol III has been proposed (Wang et al, 1997).

During the activation of 5S rRNA and tRNA transcription by c-Myc there is no evidence of the recruitment of a preformed holoenzyme complex. ChIP analysis reveals that the promoter occupancy of TFIIIC remains constant, regardless of the presence of c-Myc (Figure 4.4 and 4.6). The activation of c-Myc results in a recruitment of TFIIIB to 5S rRNA and tRNA genes, that precedes the activation of transcription (Figures 4.4 and 4.6). The recruitment of pol III is delayed and occurs after TFIIIB recruitment (Figure 4.6). In the experimental conditions, c-Myc does not alter the levels of the pol III general transcription factors examined; therefore the

changes in occupancy are dependent on c-Myc dependent recruitment (Figures 4.5 and 4.6). This evidence would suggest that, at least in response to activation by c-Myc, the recruitment of a pol III transcription complex to the promoters of target genes involves a step-wise formation of the pre-initiation complex.

6.1.3 c-Myc recruitment leads to increases in localised histone acetylation surrounding pol III transcribed genes

The correlation between histone acetylation and increased transcription has been known for many years (Allfrey et al, 1964). In recent years it has become apparent that changes in the localised histone acetylation state surrounding the promoters of genes can have effects on their transcription (Lee et al, 1993; Mizzen and Allis, 1998). High levels of acetylation are generally associated with active genes, with areas of the genome that are hypo-acetylated associated with a repressed state of transcription (Jenuwein and Allis, 2001; Eberharter and Becker, 2002). c-Myc can directly interact with several HATs, including CBP and TRRAP-containing HAT complexes, and can recruit these complexes to the promoters of target genes (Bouchard et al, 2001; Frank et al, 2001; Vervoorts et al, 2003). The recruitment of c-Myc to 5S rRNA and tRNA genes leads to a significant increase in levels of acetylated histone H3, but no change in the levels of histone H4 acetylation (Figure 5.8). This observation is surprising, as the majority of the work performed on c-Myc dependent acetylation of the promoters of target genes implicated both histone H3 and H4 as being acetylated, with histone H4 being the primary target (Frank et al, 2001; Bouchard et al, 2001; Nikiforov et al, 2002; Fernandez et al, 2003; Grandori et al, 2005; Arabi et al, 2005).

The acetylation of histones surrounding the promoters of genes is postulated to activate transcription of genes in two ways. Acetylation of the histone tails can weaken the histone-DNA and histone-histone interactions to lead to the opening of the chromatin to facilitate the association of transcription complexes with the DNA (Strahl and Allis, 2000; Turner, 2000; Fischle et al, 2003). The other mechanism is that the acetylated histone tails form interaction domains for the binding of other effector molecules that contain bromodomains, which can specifically bind to acetylated lysine residues (Ornaghi et al, 1999; Dhalluin et al, 1999; Bottomley, 2004). As TFIIIC is associated with almost the entire length of a tRNA or 5S gene, and the occupancy of this factor does not change throughout the experiment, it is difficult to imagine the need for alleviating chromatin-mediated repression of pol III transcribed genes, albeit it may be necessary at the start site of transcription and upstream of the gene to allow TFIIIB and pol III binding (Schramm and Hernandez, 2000; White, 2002). The localised histone acetylation may in fact form binding domains on the chromatin for unidentified bromodomain-containing complexes to act as co-activators for pol III transcription (Roth et al, 2001; Bottomley, 2004). This additional unidentified step may explain the delay in binding of the polymerase. It is interesting to note that the Brg1 ATPase, the catalytic subunit of a SWI/SNF complex found associated with pol III-transcribed genes, contains a bromodomain (Sif, 2004), and the histone acetylation may recruit ATP-dependent chromatin remodelling enzymes.

6.1.4 TRRAP and Gcn5 are recruited to pol III-transcribed genes by c-Myc and are involved in activation of transcription

TRRAP was originally identified as a c-Myc co-activator protein that was necessary for c-Myc-mediated transformation, and forms the scaffold of a variety of HAT complexes with different catalytic subunits (McMahon et al, 1998; Ogryzko et al, 1998; Ikura et al, 2000). Work in this thesis has demonstrated that TRRAP is present on the promoters of pol III-transcribed genes in five different cell lines, and its occupancy correlates with the presence of c-Myc (Figures 5.3, 5.5, 5.6, 5.7 and 5.9). When using a c-Myc-inducible cell line, TRRAP is recruited to 5S rRNA and tRNA genes in a c-Myc-dependent manner and this correlates well with the hyperacetylation of histone H3 and recruitment of Gcn5, a H3-specific HAT (Figures 5.7, 5.8 and 5.12). When the endogenous TRRAP protein is knocked down by RNAi, this results in a specific down-regulation of pol III transcription, indicating that TRRAP is indeed involved in the activation of class III genes (Figure 5.11). TRRAP can bind to TFIIIB through an interaction that is dependent on c-Myc, and this may represent a mechanism through which TRRAP is bound to the promoters of class III genes (Figure 5.9). Another line of evidence suggesting that TRRAP is involved in the activation of pol III transcription is the lack of any transcriptional activation from a mutant Myc protein that fails to interact with TRRAP. The mutant missing the MBII region can efficiently bind to TFIIIB, but cannot activate the transcription of a pol III reporter gene in cells (Figures 3.8 and data not shown). MBII is critical for the binding of c-Myc to TRRAP-containing complexes, and the inability of this mutant to interact with the HAT complex may explain the lack of transcriptional activation (McMahon et al, 1998).

The target of the acetylation may not only be histones; there is a variety of evidence now that many cellular proteins including transcription factors can be acetylated and this acetylation can alter their activities (Kouzarides, 1998). For example, the pol II basal transcription factors TFIIB, TFIIEβ and TFIIF can be acetylated to regulate their activities (Chol et al, 2003; Imhof et al, 1997). The c-Myc protein may form a complex with TFIIIB, prior to binding to the DNA, and recruit HAT's to acetylate subunits of this complex to alter TFIIIB activity. This needs to be investigated, but would offer a mechanism for the activation of TFIIIB by c-Myc. Also a possibility is that c-Myc/TRRAP/Gcn5 recruitment may lead to acetylation of other factors of the pol III transcription machinery to activate transcription.

The deletion of the MBI region of c-Myc can also prevent c-Myc-dependent activation of pol III transcription, but not binding to TFIIIB (Figures 3.8 and data not shown). Why deletion of this region prevents activation pol III transcription is unclear, but may indicate another protein complex that can bind to c-Myc in a MBI-dependent manner and act as a co-activator for pol III transcription.

Although its presence is enhanced on pol III-transcribed genes, there is evidence that TRRAP can bind to pol III-transcribed genes in the absence of c-Myc (Figure 5.6). TRRAP is homologous to the yeast Tra1 protein, which is an essential protein in yeast and, like its mammalian equivalent, is also a scaffold for essential HAT complexes (Saleh et al, 1998; Grant et al, 1998; Allard et al, 1999; Brown et al, 2001). As there is no c-Myc homologue in yeast, it would be interesting to test if Tra1 disruption would have any effect on pol III transcription in these cells. In mammalian cells,

TRRAP not only binds to c-Myc but has also been demonstrated to interact with the E2F-1 transcription factor and to nuclear receptors (McMahon et al, 1998; Yanagisawa et al, 2002; Fan et al, 2004), so there may be an unidentified interaction with other pol III transcription factors that can explain its presence at the promoters of 5S and tRNA genes in the absence of c-Myc. Further work needs to be done to determine if TRRAP can and act on transcription in the absence of c-Myc.

6.1.5 Is TRRAP a regulator of cell growth?

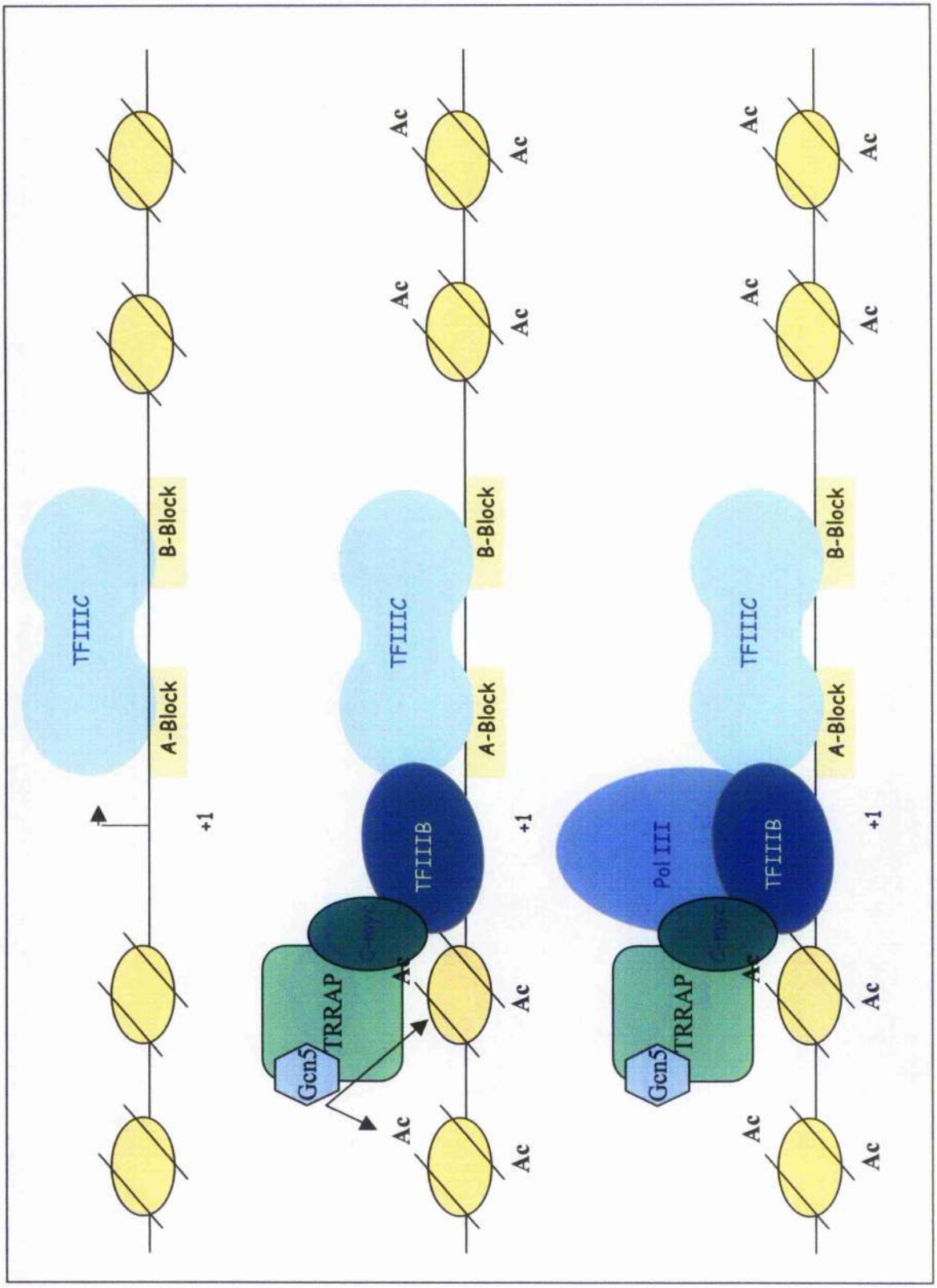
Work in this thesis has demonstrated that the TRRAP protein is involved in the activation of pol III transcription. Recent work on c-Myc activation of pol I transcription has demonstrated that TRRAP is also recruited to the rDNA repeats in a c-Myc dependent manner (Arabi et al, 2005). TRRAP knockout mice die at less than 7.5 embryonic days (<7.5E) and TRRAP $^{-/-}$ blastocysts recovered at 3.5E displayed severe growth retardation and the absence of an inner cell mass (Herceg et al, 2001). The regulation of pol I and pol III transcription may contribute to this growth retardation, as their products are essential for protein biosynthesis. Primary cells with TRRAP deleted are not viable, but conditional TRRAP knockout embryonic stem cells were developed and global gene expression was analysed using a cDNA microarray (Herceg et al, 2003). A number of genes down-regulated by TRRAP were involved in protein biosynthesis, including a polymerase subunit shared by pol I, II and III (Herceg et al, 2003). Although pol I and pol III transcripts were not looked at in this study, it would be interesting to discover if the rates of transcription of their respective products are affected by this deletion.

6.1.6 Proposed mechanism of c-Myc activation of pol III-transcribed genes

The data summarised above have led to the following model of the c-Myc dependent activation of pol III transcription. Mitogenic stimulation of cells can lead to the induction of the c-Myc protein. c-Myc can then interact with the TFIIIB complex to promote the formation of the TFIIIB-TFIIIC interaction. The c-Myc protein can recruit TRRAP and Gcn5 to TFIIIB, and they are present on 5S rRNA and tRNA genes in vivo. This recruitment of the TRRAP/Gcn5 complex correlates with a localised increase in the levels of histone H3 acetylation, followed by the recruitment of pol III and the subsequent activation of pol III transcription (Figure 6.2)

Figure 6.2 Activation of a tRNA gene by c-Myc

A. Inactive tRNA gene with TFIIIC bound. B. c-Myc induction leads to recruitment of the of c-Myc, TFIIIB and the TRRAP/ Gcn5 HAT complex. This leads to localised acetylation of histone H3. C. The recruitment of the HAT complex is followed by recruitment of the polymerase and activation of transcription.


TFIIIC
B-Block
A-Block
+1
TFIIIB
TRRAP
Gcn5
c-myc
Ac
Pol III

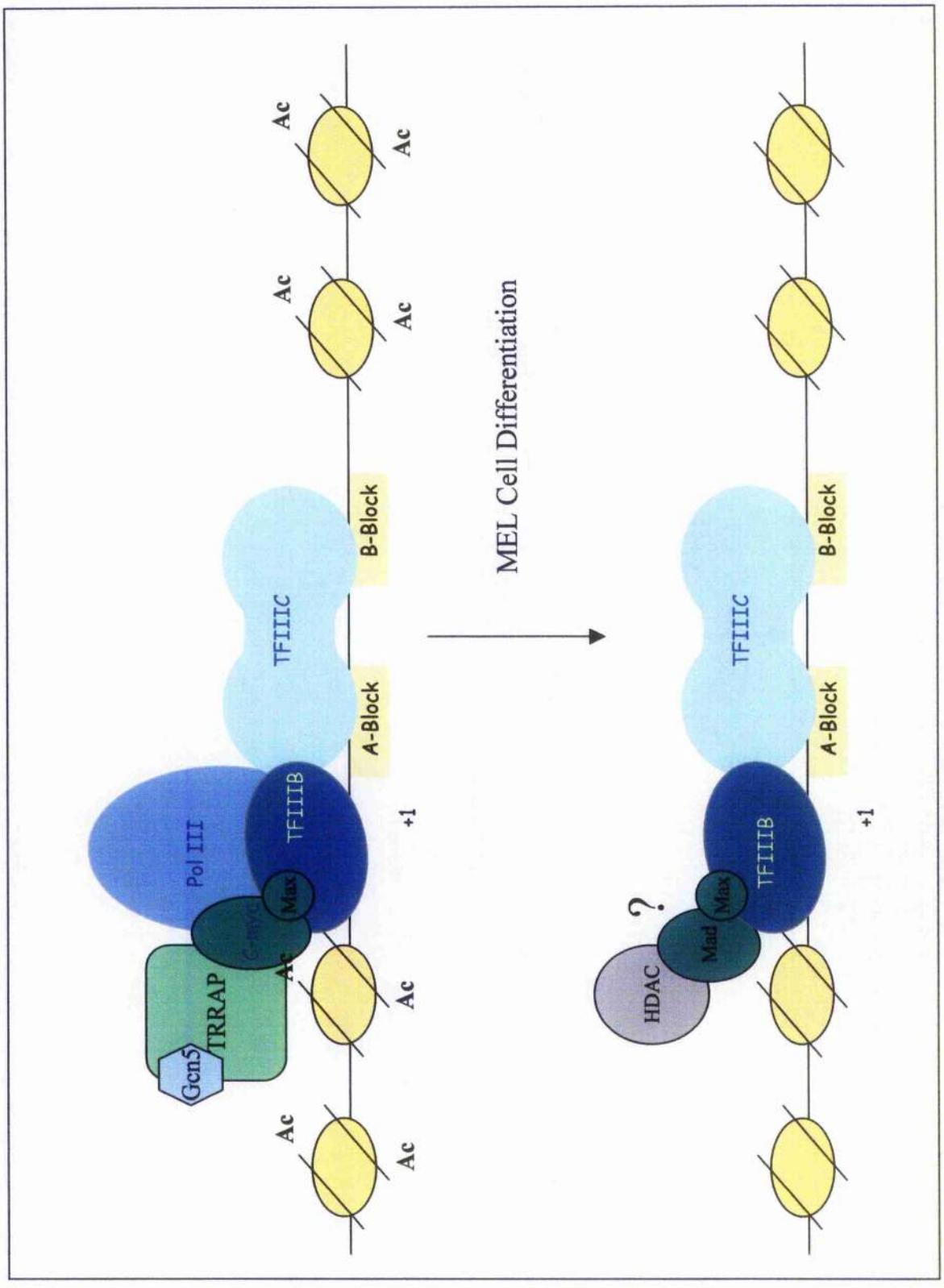
6.2 c-Myc may activate pol III transcription directly and indirectly

It has been unequivocally demonstrated that c-Myc can bind to pol III-transcribed genes and directly activate their transcription (Gomez-Roman et al, 2003). In the context of a cell, c-Myc may also indirectly contribute to the activation of pol III transcription. It has been demonstrated in this thesis that c-Myc$^{-/-}$ fibroblasts have lower levels of the Brf1 protein than the matched wildtype; this may be a direct effect as the Brf1 promoter contains good matches for E-box sequences (Figures 4.9 and 4.13). This is an interesting observation, as levels of the Brf1 protein can be limiting for pol III transcription in certain cell types (Fairley et al, 2003, Felton-Edkins et al, 2003a). It has been previously demonstrated that c-Myc can directly activate the transcription of the pol III subunit BN51 (Figure 6.3; Greasley et al, 2000; Fernandez et al, 2003). The BN51 subunit of pol III has been demonstrated to be critical for cell cycle progression in both yeast and mammals, with temperature-sensitive mutations of BN51 in a hamster cell line and the BN51 homologue in *S. cerevisiae* causing G1 arrest at the non-permissive temperature (Ittmann et al, 1987; Ittmann et al, 1993; Jackson et al, 1995; Mann et al, 1992). It may be that c-Myc can alter the levels of limiting pol III-specific transcription factors to stimulate pol III transcription, and further work may reveal that Brf1 and BN51 are not isolated examples of this. Also it is well established that c-Myc can stimulate cell cycle progression through activation of CDKs and repression of CDK inhibitors (Figure 6.3; Dang et al, 1999; Eisenman, 2001; Adhikary and Eilers, 2005). This stimulation of cell cycle progression will result in the hyper-phosphorylation and inactivation of RB and the related pocket proteins, leading to a derepression of pol III transcription (Figure 6.3; Scott et al, 2001). RB and c-Myc may also compete for binding sites on the TFIIIB complex

(discussed in Chapter 3). Although the interaction domains of c-Myc and RB on Brf1 are clearly distinct, they are both in the same region of the protein, and perhaps due to steric interactions RB and c-Myc may not be able to bind to TFIIIB at the same time (Figures 3.4 and 3.5). It would be interesting to determine if they could complete for binding sites on TFIIIB, thus changing its activation state (Figure 6.1). Work still has to be done to elucidate any indirect effects of c-Myc and their contribution to the activation of pol III transcription.

Figure 6.3 Indirect and direct activation of pol III transcription

c-Myc may work in several ways to activate pol III transcription. A. Activation of c-Myc is directly involved in the induction of cyclins and CDKs, as well as inhibiting the expression of CDK inhibitors. These events will lead to phosphorylation of RB and the related pocket proteins, and would relieve Rb- and p130-dependent repression of pol III transcription. B. c-Myc can bind to pol III-transcribed genes and directly activate transcription. C. c-Myc may promote the expression of limiting factors in pol III transcription: it has previously been demonstrated that the pol III subunit BN51 is a direct target of c-Myc and work in this thesis has suggested that Brf1 may also be induced by c-Myc.


Ac
Ac
Ac
Ac
Ac
Ac
TFIIIC
B-Block
A-Block
+1
Pol III
TFIIIB
Max
C-myc
Ac
Ac
Ac
TRRAP
Gcn5
MEL Cell Differentiation
Ac
Ac
TFIIIC
B-Block
A-Block
+1
TFIIIB
Max
Mad
?
HDAC

6.3 Are other members of the Myc/Max/Mad family involved in the regulation of pol III transcription during differentiation of MEL cells?

During the terminal differentiation of MEL cells there is a marked reduction in the levels of c-Myc and this is accompanied by a reduction in the levels of pol III transcription (Figures 4.10 and 4.11). This is reflected on the promoters of these genes with the reduced occupancy of c-Myc and pol III, as confirmed by ChIP analysis (Figure 4.12). In the same experiment, the promoter occupancy of Max was also tested, and surprisingly the levels associated with the 5S rRNA and tRNA genes stayed constant throughout the experiment (Figure 5.9). This would suggest that Max can bind to pol III-transcribed genes in the absence of c-Myc, and perhaps it is present there in complex with other binding partners.

Also witnessed during the differentiation of the MEL cells is a reduction in the levels of histone H3 acetylation surrounding the 5S rRNA and tRNA genes (Figure 5.9). This may simply be due to the dissociation of the c-Myc/TRRAP complex, but the short time course of this experiment may suggest that the deacetylation is an active process and that HDAC complexes are recruited (Figure 5.9). During the differentiation of these cells the levels of the Mad proteins have been shown to increase as the level of c-Myc decreases (Cultraro et al, 1997; Kime and Wright, 2003). Mad proteins can interact with HDAC complexes via an interaction with mSin3, through the SID in Mad (Ayer et al, 1995; Hassig et al, 1997; Sommer et al, 1997), and these complexes can be recruited to the E-boxes of target genes during differentiation of HL-60 cells (Bouchard et al, 2001; Xu et al, 2001). It may therefore be that HDACs can be recruited to pol III-transcribed genes during MEL cell

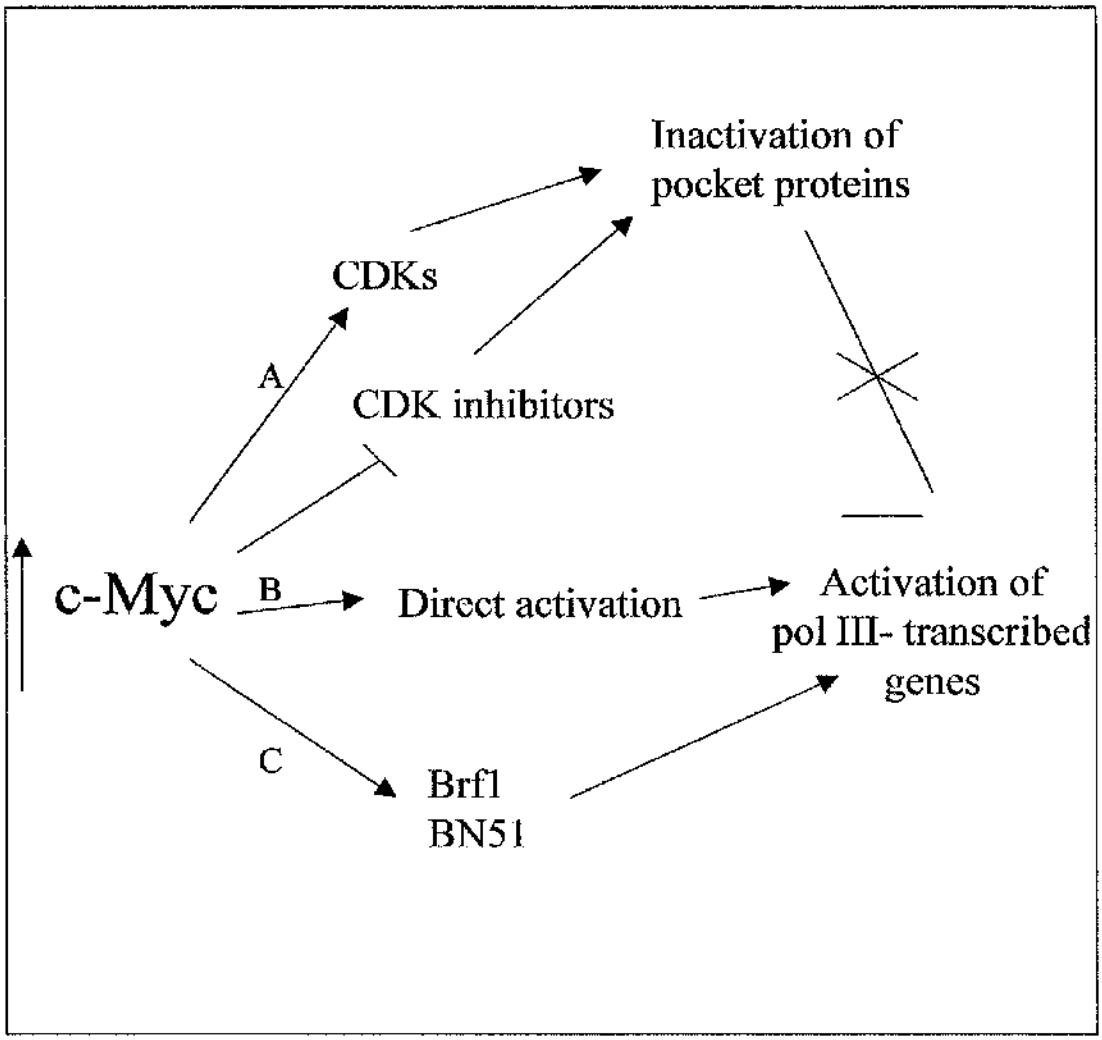
differentiation (Figure 6.4). This switching from c-Myc and associated HATs bound to an active hyperacetylated gene to Mad1 and HDAC binding to repressed hypoacetylated genes has been demonstrated on genes transcribed by pol II (Bouchard et al, 2001; Xu et al, 2001). This may happen on the promoters of pol III-transcribed genes, but still needs to be investigated.

This model leaves the intriguing question as to how Max alone or a Mad/Max complex can associate with pol III-transcribed genes. The TFIIIB complex is still present on the promoters in these genes and it could be that Mad family proteins can bind to TFIIIB proteins through an unestablished interaction domain with a TFIIIB subunit.

In summary the work in this thesis describes novel insights into how c-Myc can activate pol III transcription. I have demonstrated that c-Myc can recruit HAT complexes to pol III-transcribed genes and alter the levels of localised histone acetylation, which correlates with an activation of pol III transcription. This work further improves our understanding of the regulation of pol III transcription by the c-Myc protein in vivo.

Figure 6.4 Model for differentiation of the MEL cell line

During differentiation of the MEL cell line the levels of the c-Myc protein fall sharply and this correlates with a reduction in pol III transcription. At the promoter level, occupancy of c-Myc and pol III are reduced during differentiation. Histone H3 acetylation is also lower in the differentiated cells. The levels of the c-Myc binding protein Max and TFIIIB stay constant during this process. As Max levels do not reduce, it may suggest that the reduction in histone acetylation is perhaps due to the recruitment of Mad and an associated HDAC complex.


Inactivation of
pocket proteins
CDKs
A
CDK inhibitors
c-Myc
B
Direct activation
Activation of
pol III- transcribed
genes
C
Brf1
BN51

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