Regulation of pol III transcription by mTOR

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Declaration

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

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Summary

RNA polymerase III (pol III) is dedicated to the transcription of genes involved in protein synthesis (5S rRNA and tRNA genes). Cell division is dependent on the rate of growth, which is dependent on the rate of protein synthesis. Therefore, the rate of pol III transcription plays a fundamental role in cellular growth and proliferation. Regulation is mediated via a number of different mechanisms that can alter the activities of transcription factors, which direct pol III transcription. Work in this project was directed at uncovering potential mechanisms for pol III regulation. A primary target was the mTOR pathway, as it coordinates nutrient availability with cell growth.

Addition of the mTOR inhibitor rapamycin resulted in a decrease in the level of pol III transcripts. Furthermore, inhibition of the mTOR pathway resulted in a decrease in the promoter occupancy of pol III and TFIIIB. This occurred without any changes in the abundance of these two factors. Past studies have shown that mTOR regulates the expression of pol I-transcribed genes through the kinase S6K. Consistent with this, knockdown of S6K reduced the expression of pol I-transcribed genes. However, knockdown of S6K had no effect on the abundance of pol III transcripts. This highlights a difference in the regulation of pol I- and pol III-transcribed genes.

mTOR may regulate gene expression through its direct recruitment to the transcriptional machinery, as raptor, a component of the mTOR complex, was found to co-immunoprecipitate with TFIIIC in this present study. Furthermore, chromatin immunoprecipitation revealed that mTOR is associated with pol III-transcribed genes.

TSC2, Rheb and PKB, upstream components of the mTOR pathway, were all shown to regulate the expression of pol III-transcribed genes. TSC2, a tumour suppressor, was found to have a negative effect on the expression of pol III-transcribed genes. Loss of TSC2 in a knockout cell line resulted in an increase in abundance of the 110kDa subunit of TFIIIC. This is consistent with previous studies where TFIIIC110 was suggested to be the rate-limiting component of pol III transcription. However, work in the present study using a TFIIIC110 inducible cell line was unable to substantiate these claims.

Blocking the mTOR pathway also resulted in a decrease in the acetylation of histone H3 found on pol III-transcribed genes. Furthermore, addition of the drug trichostatin A (TSA), which promotes the acetylation of histones and other cellular proteins, increased the level of pol III transcripts. TSA also increased the promoter occupancy of pol III and TFIIIB, while at the same time increasing the acetylation of histones H3 and H4 on pol III-transcribed genes. In addition to being acetylated, histones are also methylated on pol III-transcribed gene. ChIP analysis has demonstrated the presence histone H3 lysine 4 methylation and histone H3 lysine 9 methylation on pol III-transcribed genes. Further work is needed to characterize the role of these covalent modifications.

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Abbreviations

°C	degrees Celsius
µg	microgram
µM	micromolar
µl	microlitre
A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
ARPP PO	acidic ribosomal phosphoprotein PO
A	adenine
AMP	adenosine monophosphate
ATP	adenosine triphosphate
APS	ammonium persulphate
Arg	arginine
bp	base pairs
Bdp1	B double prime 1
Brf1	TFIIB-related factor 1
Brf2	TFIIB-related factor 2
BSA	bovine serum albumin
C-	carboxy-
ChIP	chromatin immunoprecipitation
cDNA	complementary DNA
Cdk	cyclin-dependent kinase
Da	Dalton

DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTP	2' deoxy (nucleotide) triphosphate
DSE	distal sequence element
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetic acid
g	gram
GAP	GTP-ase activating proteins
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HAT	histone acetyltransferase
HDAC	histone deacetyltransferase
hr	hour
HRP	horseradish peroxidase
ICR	internal control region
Ig	immunoglobulin
K	lysine
kb	kilobases
kDA	kiloDaltons
Leu	leucine
H3 Lys^4	Histone H3 lysine 4
H3 Lys^9	Histone H3 lysine 9
mTOR	mammalian target of rapamycin

M	molar
mg	milligram
min	minutes
ml	millilitre
mM	millimolar
MW	molecular weight
mRNA	messenger RNA
MRP	Mitochondrial RNA processing
N-	amino-
NaOAc	sodium acetate
ng	nanograms
nM	nanomolar
PI3K	phosphoinositide 3-kinase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PKB	protein kinase B
pmol	picomole
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
PP2A	protein phosphatase 2A

PSE	proximal sequence element
PTEN	phosphatase and tensin homologue deleted on chromosome10
Rheb	Ras homology enriched in brain
RNA	ribonucleic acid
RNase	ribonuclease
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcriptase-PCR
s	second
SINE	short interspersed repeat
snRNA	small nuclear RNA
SV40	simian virus 40
SDS	sodium dodecyl sulphate
TAF	TBP-associated factor
TBP	TATA-box binding protein
TBS	Tris buffered saline
TE	tris-EDTA
TEMED	N,N,N',-tetramethylethylenediamine
TFIIIA	transcription factor IIIA
TFIIIC	transcription factor IIIC
tRNA	transfer RNA

TSC1/TSC2	Tuberous sclerosis complex 1/ Tuberous sclerosis 2
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	transfer RNA
TSA	trichostatin A
tyr	tyrosine
U	unit
UBF	upstream binding factor
UCE	upstream control element
UV	ultraviolet
V	volt
v/v	volume per volume
w/v	weight per volume

Chapter 1-Introduction

1.1 Transcription

An important step in understanding the human genome is the identification of transcribed units, and more importantly how they are transcriptionally regulated in relation to cellular function. In eukaryotic cells the task of transcribing nuclear genes has been shared between RNA polymerase I, II and III. This list has been extended recently with the discovery of a fourth nuclear eukaryotic RNA polymerase (Kravchenko et al., 2005). The specificity of the polymerases is often aided by other proteins known as transcription factors, which bind to DNA sequences known as promoters, directing the transcription of specific genes within the genome.

Each polymerase is committed to the transcription of a specific set of genes. RNA polymerase I (pol I) is responsible for the transcription of genes encoding the 45S ribosomal (r)RNA, which is subsequently processed into 5.8S, 18S and 28S. Transcription by pol I accounts for 60% of total cellular RNA synthesis (Moss and Stefanovsky, 2002). RNA polymerase II (pol II) transcribes protein-coding genes to produce messenger RNAs (mRNAs) (White, 2001a). mRNAs form targets for the translational machinery where they are subsequently translated into proteins (White, 2001a). Pol II also transcribes many small nuclear (sn)RNAs, which have a role in mRNA processing (White, 2001a). RNA polymerase III (pol III) is dedicated to the transcription of an eclectic mix of genes involved in protein synthesis (5S rRNA and tRNA) and a variety of other essential functions. Pol III accounts for 10% of all nuclear transcription (Moss and Stefanovsky, 2002). RNA polymerase IV (pol IV), a recently discovered nuclear polymerase, is expressed from an alternative transcript of the mitochondrial RNA polymerase gene (Kravchenko et al., 2005). Pol IV is responsible for

the transcription of a number of mRNAs (Kravchenko et al., 2005). Tight control must be exerted over these polymerases to regulate cellular function through coordinated gene expression.

1.2 Transcription and cell growth

The rate of protein synthesis is an important determinant of cellular growth. In animal cells the rate of cellular growth has been shown to be directly proportional to the rate of protein accumulation (Baxter and Stanners, 1978). The process of translation mediates protein synthesis, where ribosomes synthesise proteins from mRNA templates. Therefore, ribosomes play a central role in cellular growth. In fact, ribosomal content is proportional to the rate of growth (Kief and Warner, 1981). This is observed when mitogenic stimulation causes an increase in the synthesis of rRNA and ribosomal proteins, which helps facilitate an increase in protein production and growth (Johnson et al., 1974; Kief and Warner, 1981; Mauck and Green, 1974). During mitogenic stimulation the transcriptional activity of both pol I and pol III increases, this is due to their role in the synthesis of rRNAs (Clarke et al., 1996; Felton-Edkins et al., 2003a; Scott et al., 2001; Stefanovsky et al., 2006; White et al., 1995). Furthermore, the levels of the pol III-transcribed tRNAs have also been shown to play an important role in protein synthesis and growth (Francis and Rajbhandary, 1990). A number of other short untranslated RNAs that play fundamental roles in the biosynthetic capacity of the cell are also synthesised by pol III. Therefore, the regulation of pol III is important in determining the translational aptitude and growth of a cell.

1.3 Class III Genes

Genes transcribed by pol III encode a number of different small RNA molecules, which are not translated. Typically they are less than 400 nucleotides in length and are involved in a number of essential functions in cellular metabolism. Table 1 lists these roles and they are discussed further in the following section.

Pol III Products	Known Functions
tRNA	Protein synthesis as a translational adaptor
5SrRNA	Protein synthesis as a component of ribosomes
U6 RNA	mRNA splicing
H1 RNA	tRNA processing
MRP RNA	rRNA splicing
7SL RNA	Intracellular protein transport (component of SRP)
7SK RNA	Controlling transcriptional elongation by pol II
SINE transcripts	Unknown function
VA RNA	Adenovirus translational control
EBER RNA	Thought to be involved in Epstein-Barr virus translational control

Table 1.1

1.3.1 5S rRNA

The ribosome requires all three RNA polymerases for its synthesis. Pol I makes the 28S, 18S, and 5.8S rRNAs, pol II produces messenger RNAs encoding ribosomal proteins and

pol III synthesises the remaining 5S rRNA. 5S rRNA is 120 bp transcript which is transported to the nucleus where it is processed and integrated into the large ribosomal subunit. Eukaryotic genomes contain multiple copies of 5S rRNA genes, ranging from 140 in the haploid genome of *Saccharomyces cerevisiae*, to more than 20 000 copies in *Xenopus laevis*.(Brown et al., 1971; Elion and Warner, 1984). The majority of *X. laevis* 5S genes are only expressed to sustain rapid growth during development of the oocyte (Wolffe and Brown, 1988). Human cells contain 200-300 5S rRNA genes, with many of these clustering in tandem repeats (Consortium, 2001).

1.3.2 tRNA

tRNAs function as adaptor molecules, which translate genetic information contained within mRNA into a specific amino acid sequence of a protein. Once transcribed, tRNAs are processed into mature tRNAs between 70 to 90 nucleotides in length. Eukaryotic cells contain 50 to 100 distinct tRNA species (Sharp et al., 1984). The human haploid genome contains 497 tRNA genes, though there is considerable redundancy as the average copy number for each amino acid tRNA adaptor is around 10 genes (Consortium, 2001). Each tRNA is covalently linked to specific amino acids. The specificity of tRNAs is determined by a trinucleotide sequence, the anti-codon, which is specific for a particular amino acid. This enables the tRNA to recognise the codon, found in mRNA, via complementary base pairing. This ensures the accurate synthesis of the polypeptide chain encoded by the mRNA nucleotide sequence.

1.3.3 H1 and MRP

Mitochondrial RNA processing (MRP) RNA is found predominately in the nucleolus, where it has an important role in the processing of pre-rRNA (Morrissey and Tollervey, 1995; Schmitt and Clayton, 1993). It is 265 nucleotides in length and forms part of an endoribonuclease called RNase MRP. Another endoribonuclease that shares sequence homology with MRP is H1 (Gold et al., 1989). H1 is a 369 nucleotide RNA that forms part of RNase P, which is involved in processing the 5' termini of pre-tRNA (Lee and Engelke, 1989; Morrissey and Tollervey, 1995).

1.3.4 U6 snRNA

Spliceosomes are multi-subunit complexes consisting of five snRNAs and many proteins that assemble on pre-RNA. Their role is to remove non-coding introns to generate mature mRNAs that are compatible with the translational machinery. Four of these genes are synthesized by pol II, whereas the smallest, U6 at 106 nucleotides is transcribed by pol III (Reddy et al., 1987). Between different organisms U6 is the most highly conserved of the spliceosomal RNAs, highlighting its importance in the splicing process.

1.3.5 7SL

7SL, a pol III-transcribed gene, encodes a 300bp transcript that forms the RNA component of the signal recognition particle. The signal recognition particle plays an essential role in the intracellular localisation of proteins. This is achieved through its involvement in the insertion of nascent polypeptides into the endoplasmic reticulum (Walter and Blobel, 1982), where they can be directed to their final destination.

1.3.6 SINEs

Short interspersed nuclear elements (SINEs) constitute the majority of pol III templates in mammals. Examples include the Alu genes, found in primates, and the B1 and B2 elements, which are found within the rodent genome. Alu elements are the most predominant SINE within the human genome. They make up 10% to 11% of the genomic DNA and consists of about 1.2 million copies (Consortium, 2001). Human Alu elements are dimeric, consisting of about 300 bp comprised of two non-identical monomers that are derived from 7SL RNA (Ullu and Tschudi, 1984). In rodents B1, and B2 are the most abundant SINEs, numbering approximately 384,000 and 328,000, respectively. B1 is homologous to Alu and is also thought to have evolved from the 7SL gene, whereas B2 is rodent specific and appears to have evolved from tRNA genes. The dispersion and propagation of these SINEs is believed to occur via retrotransposition, where pol III transcripts are reversed transcribed and converted to DNA. The DNA copy of the pol III-synthesised RNA is then integrated into the genome (Weiner et al., 1986).

Despite the abundance of SINEs, little is known about their function. A proposed role is that they are involved in cellular stress responses, as a number of stress stimuli, such as DNA-damaging agents and heat shock have been shown to induce the transcription of SINEs (Liu et al., 1995; Rudin and Thompson, 2001). The relevance of this has been highlighted in recent studies showing that B2 RNA is involved in the repression of class II gene expression in response to heat shock (Allen et al., 2004; Espinoza et al., 2004). Furthermore, a role for Alu has also been proposed, regulating translation through its

interaction with double stranded RNA-activated kinase PKR (Chu et al., 1998). These studies provide a functional role for SINEs and challenge the notion of them being 'junk DNA' (Makalowski, 2003).

1.3.7 Viral genes transcribed by pol III

A number of viruses that infect cells utilise pol III to transcribe short units within their genome. The best-characterised example is that of the adenovirus, which encodes two pol III-transcribed transcripts, VAI and VAII. Both are approximately 160 nucleotides in length. The role of these transcripts is to stimulate the translation of adenoviral mRNA during the late stages of infection (Soderlund et al., 1976; Thimmappaya et al., 1982). The Epstein-Barr virus (EBV) contains two genes; EBER1 and EBER2, which are also transcribed by pol III. EBER1 and EBER2 are thought to have a similar role to the VA RNAs in subverting the cells translational machinery to allow the synthesis of viral proteins. Furthermore, EBER1 and EBER2 share regions of homology with VA genes and can functionally substitute for VA1 during adenovirus infection (Bhat and Thimmappaya, 1985; Rosa et al., 1981).

The list of genes above illustrates the integral role of pol III in protein synthesis. The assembly of transcription factors at their promoters mediates specificity and recruitment of pol III to these genes. The next section will discus how this specificity and recruitment is achieved.

1.4 Promoter structure

The role of gene promoters is to act as a platform for the recruitment of transcription factors that help facilitate transcription through the recruitment of a specific polymerase. Promoters employed by pol III can be divided into three groups, type 1, 2 and 3 (see figure 1.1 for a schematic representation of promoter structures). Type 1 and 2 promoters are gene internal, which means that they are downstream of the transcriptional start site. Type 3 promoters are different, with their crucial sequence elements found upstream of the transcriptional start site. Each promoter contains a unique set of elements that organise the formation of a stable transcriptional unit able to perform multiple rounds of transcription.

1.4.1 Type1 promoters:

Type 1 promoters consist of three gene internal elements that are unique to the 5S rRNA gene. Its promoter was initially characterised in Xenopus laevis, consisting of an A-block (+50 to +64), an intermediate element (+67 to +72), and a C-block (+80 to +97), collectively known as the internal control region (ICR) (Pieler et al., 1987). These elements are highly conserved between species. Mutations or alterations in the spacing of the elements considerably reduce transcriptional efficiency (Keller et al., 1990). This is in contrast to the flanking regions that display little conservation, showing greater resilience to mutations (White, 2001b).

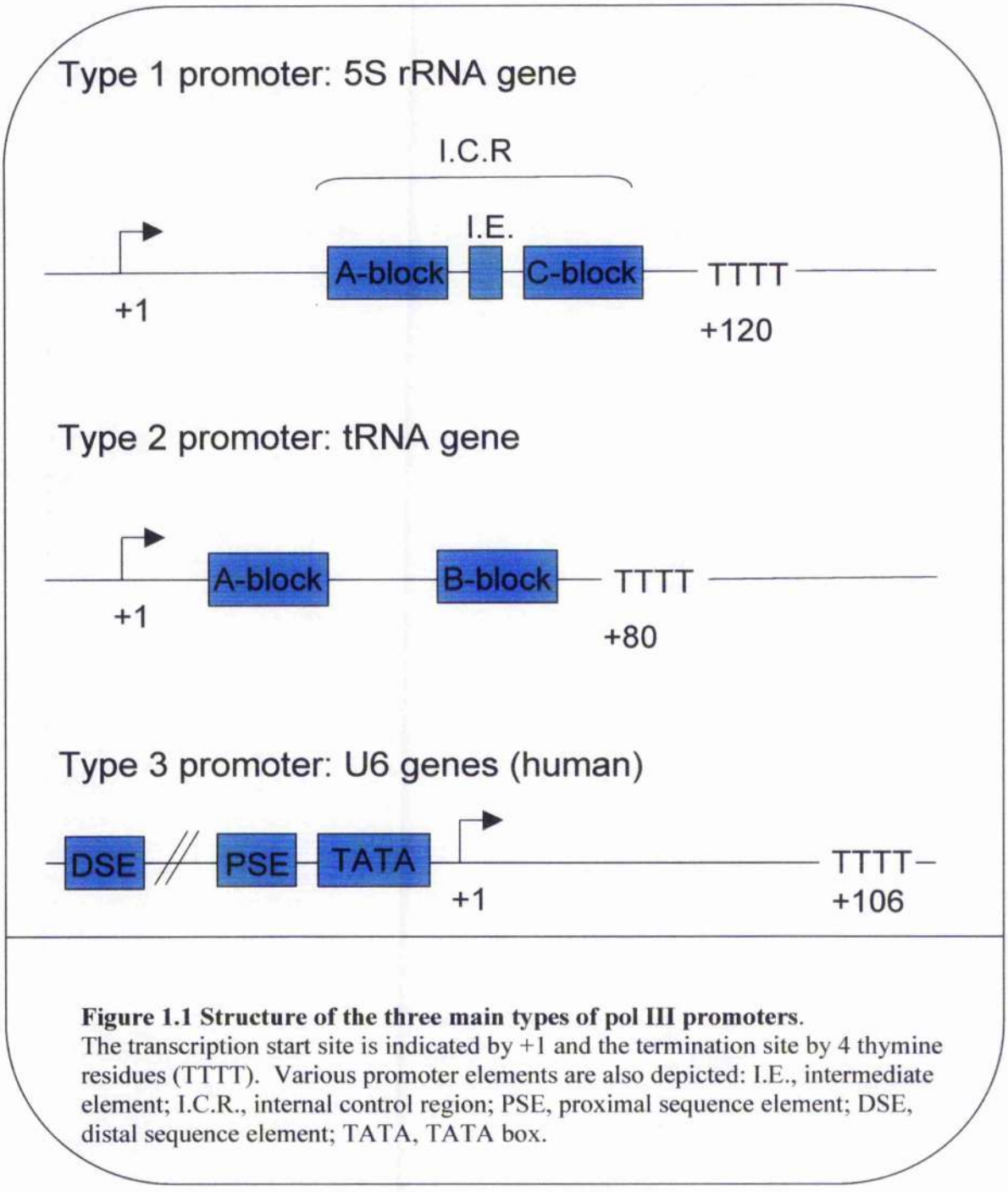


Figure 1.1 Structure of the three main types of pol III promoters.
The transcription start site is indicated by +1 and the termination site by 4 thymine residues (TTTT). Various promoter elements are also depicted: I.E., intermediate element; I.C.R., internal control region; PSE, proximal sequence element; DSE, distal sequence element; TATA, TATA box.

1.4.2 Type 2 promoters:

Type 2 promoters consist of two highly conserved sequence elements of about 10bp each; an A- and a B- block (Galli et al., 1981). It is this arrangement that is the most commonly observed in pol III-transcribed genes, including the tRNA genes, the adenovirus VA genes and a number of middle repetitive genes such as Alu, B1 and B2 (White, 2001b). The A-block is homologous to a type 1 promoter A-block, and in some species it is functionally interchangeable (Ciliberto et al., 1983). The B-block is positioned downstream of the A-block, though its positioning is highly variable (Baker et al., 1987). The A- and the B- blocks are typically separated by 30-60bp; however, a distance of up to 365bp can still support transcription (Baker et al., 1987; Fabrizio et al., 1987).

1.4.3 Type 3 promoters:

Type 3 promoters consist of three elements: a TATA box, a proximal sequence element (PSE) and a distal sequence element (DSE). As previously stated these elements are found upstream of the transcriptional start site. Type 3 promoter structures encompass 7SK, MRP and U6 genes. The human U6 promoter is the best-characterised type 3 promoter. Its TATA box is found approximately –27 upstream of the transcriptional start site, this is followed by the PSE at –56.

1.5 Transcription of class III genes

For transcription to take place, a number of processes must occur. Initial steps require the recruitment of transcription factors to a gene's promoter. This pre-initiation complex provides a platform for the polymerase to be brought in, where first initiation, then elongation occurs which is then followed by transcriptional termination.

1.5.1 Complex assembly on type 2 promoters (see figure 1.2)

Transcription factor IIIC (TFIIIC) is a multi-subunit complex that binds directly to the intragenic promoter sequence of type II promoters (Lassar et al., 1983). Human TFIIIC was originally resolved into two components, TFIIIC1 and TFIIIC2 (Yoshinaga et al., 1987). *In vitro*, both are required for the transcription of 5S and tRNA genes (type 1 and 2 promoters), whereas only TFIIIC1 is required for the transcription of 7SK and U6 genes (type 3 promoters) (Lagna et al., 1994; Oettel et al., 1997; Yoon et al., 1995). TFIIIC2 is composed of five polypeptides, known as TFIIIC220, 110, 102, 90 and 63 according to their molecular mass (Kovelman and Roeder, 1992). TFIIIC220 and TFIIIC110 form a sub-complex that is capable of DNA-binding via the B-block. The TFIIIC-DNA interaction is thought to be further aided by TFIIIC63 interaction with the A-block (Hsieh et al., 1999b). TFIIIC90 interacts with TFIIIC220, TFIIIC110 and TFIIIC63, providing a bridge between the two DNA binding components of TFIIIC (Hsieh et al., 1999a). Although both the A- and the B- blocks are contacted by TFIIIC, it is the B- block that is the predominant determinant of binding affinity (Baker et al., 1986). In contrast to TFIIIC2, TFIIIC1 is relatively ill-defined in terms of its precise function and composition.

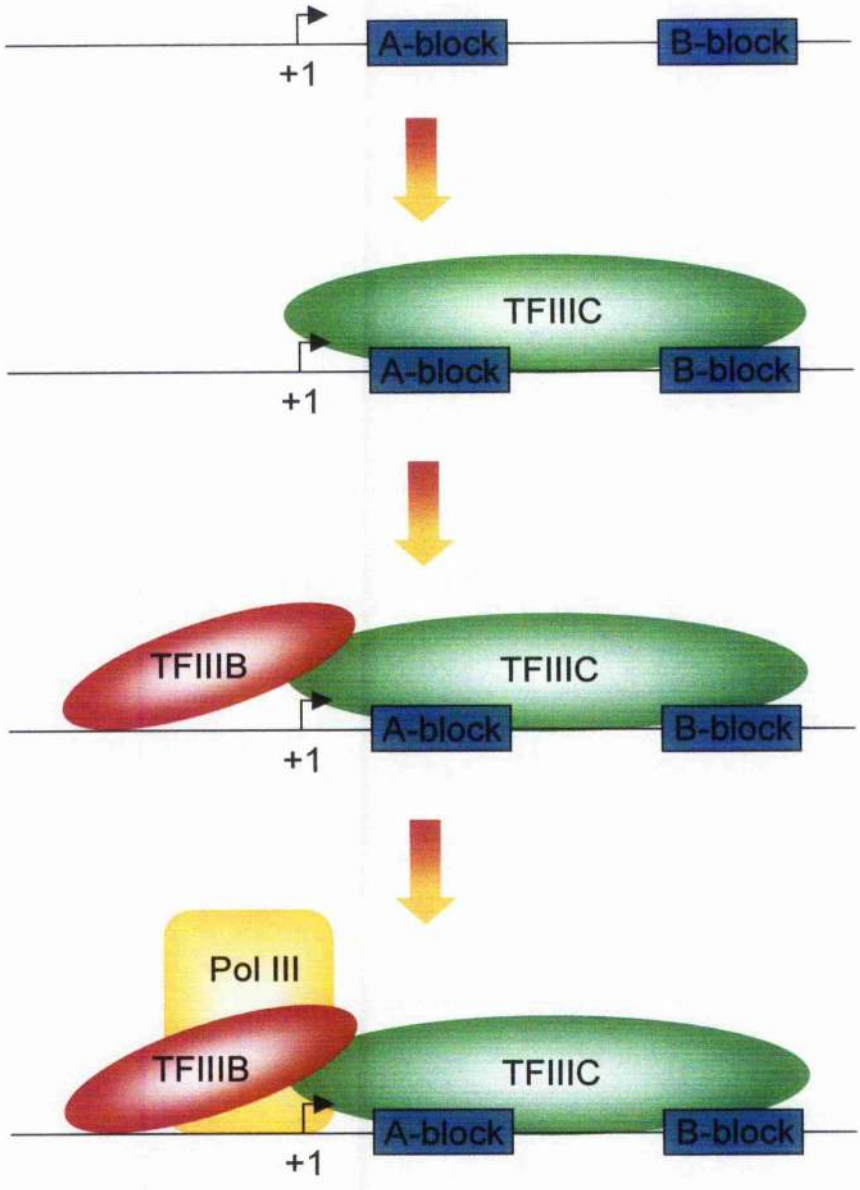


Figure 1.2 Transcriptional complex on a type 2 promoter. TFIIIC binds to the A- and B-block directly. This acts as a platform to recruit TFIIIB, via protein-protein interactions. Following recuitment, pol III can be brought in, and transcription is initiated at the transcriptional start site (+1).

Once TFIIIC is at the promoter, it serves to recruit TFIIIB, positioning it just upstream of the transcriptional start site. TFIIIB consists of three components: TATA-binding protein (TBP), TFIIB-related factor (Brf1) and B double prime (Bdp1), with molecular masses of 34, 90 and 160kDa, respectively. TBP is a general factor, used by pol I, II and III (Cormack and Struhl, 1992; Kim and Roeder, 1994), whereas Brf1 and Bdp1 are specifically required for pol III transcription (Schramm and Hernandez, 2002). TBP and Brf1 form a tight association with each other (Wang and Roeder, 1995). This is in contrast to Bdp1, which has a weak interaction with this complex (Kassavetis et al., 1995).

Work in both yeast and human cells have demonstrated the high degree of homology in the recruitment and interaction of TFIIIC, TFIIIB and pol III. DNA-bound TFIIIC was initially shown to contact Brf1, a subunit of TFIIIB. The TFIIIC subunit responsible for this interaction was identified in *S. cerevisiae* as the human equivalent of TFIIIC102 (Schramm and Hernandez, 2002). Subsequently, a number of other interactions were also identified between TFIIIC and TFIIIB. In human cells, TFIIIC63 and TFIIIC90 interact with Brf1. TBP also directly interacts with TFIIIC, with both TFIIIC102 and TFIIIC63 binding to this subunit of TFIIIB (Hsieh et al., 1999a; Hsieh et al., 1999b).

Pol III recruitment can only take place once TFIIIB is bound to TFIIIC. All three subunits of TFIIIB are required for polymerase recruitment. However, only Brf1 and TBP have been shown to make direct contact. Brf1 directly interacts with three pol III subunits, RPC32, RPC39 and RPC63, with TBP also binding to RPC39 (Wang and

Roeder, 1995). In addition to these interactions, TFIIIC has been shown to interact with the pol III subunit RPC62 via TFIIIC63 (Hsieh et al., 1999b).

1.5.2 Complex assembly on Type 1 (5S rRNA) promoters (see figure 1.3)

Type 1 promoters differ from type 2 promoters, as they require the presence of an additional factor, TFIIIA. This is due to the fact that type 1 promoters lack a functional B-block, which is the major determinant of TFIIIC DNA binding affinity. As a result, TFIIIA serves as an adaptor factor that facilitates TFIIIC recruitment (Schramm and Hernandez, 2002; White, 2002). TFIIIA is a single polypeptide of approximately 40kDa that contains 9 zinc finger domains. Separate clusters of these zinc fingers bind to the A-block, the intermediate element and the C-block (Clemens et al., 1992; Nolte et al., 1998). However, it is its interaction of three zinc fingers with the C-block that contributes the majority of its DNA binding affinity (Clemens et al., 1992; Nolte et al., 1998). Once TFIIIA is bound, TFIIIC can be recruited, though the way in which this occurs remains unclear. TFIIIC recruitment allows TFIIIB to bind just upstream of the transcriptional start site, which is then followed by the recruitment of pol III.

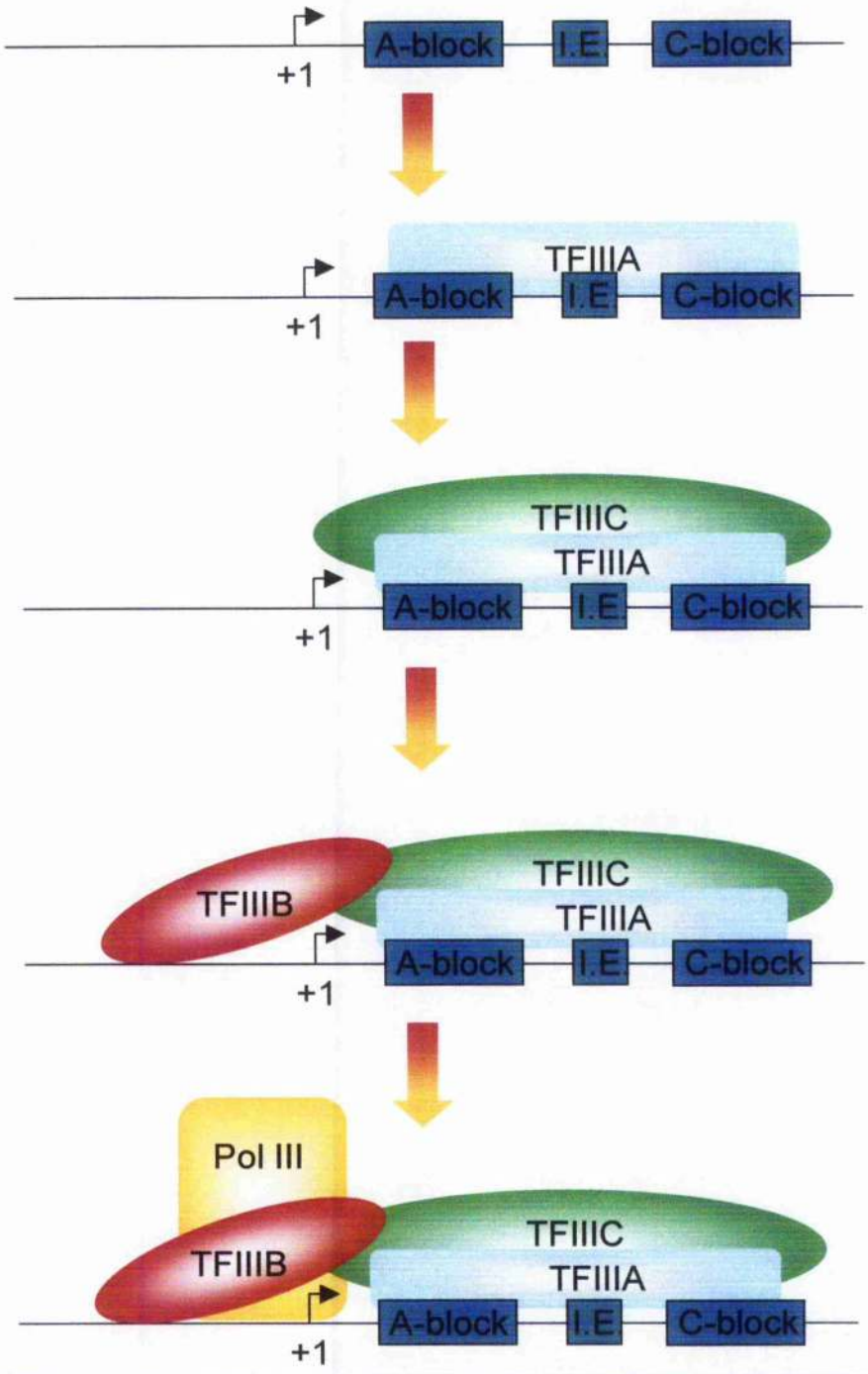


Figure 1.3 Transcriptional complex on a type 1 promoter. TFIIIA binds to the internal control region of the gene. TFIIIC can then be recruited, followed sequentially by TFIIIB and pol III. Transcription can then begin at the transcriptional start site (+1).

1.5.3 Complex assembly on type 3 promoters (see figure 1.4)

The recruitment of TFIIIB to type 3 promoters occurs independently of TFIIIC, which distinguishes it from most pol III templates. Furthermore, TFIIIB is different from the one present at type 1 and 2 promoters as Brf1 is replaced with a related factor known as Brf2 (Schramm et al., 2000). Type 3 promoters contain two sequences upstream of the transcriptional start site; TATA box and PSE. The TATA box and PSE elements are recognised by TBP (a component of TFIIIB) and $SNAP_c$ (a five-unit factor), respectively. The independent DNA binding affinity of these factors is weak. However, the protein-protein interactions between TFIIIB and $SNAP_c$ greatly enhance their recruitment to the promoter (Mittal and Hernandez, 1997). TFIIIB and $SNAP_c$ recruitment is further enhanced by an additional transcription factor known as Oct-1. Oct-1 binds to the DSE, a sequence found upstream of the PSE and TATA box. It enhances the recruitment of the TFIIIB/$SNAP_c$ complex via its direct interaction with $SNAP_c$ (Mittal et al., 1996). However, its presence is not essential for basal transcription from type 3 promoters (Hu et al., 2003). Once the TFIIIB/$SNAP_c$ complex is in place, pol III can be recruited and transcription can occur.

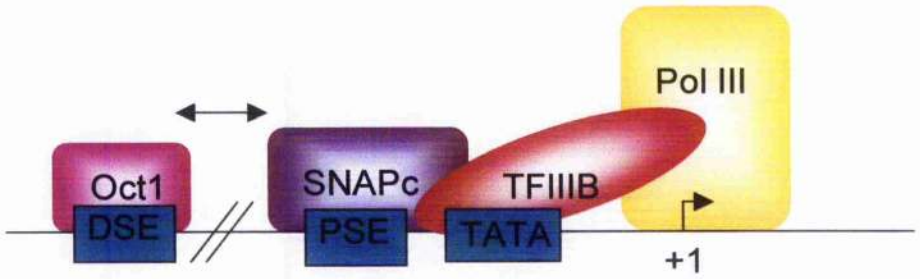


Figure 1.4 Transcriptional complex on a type 3 promoter. TFIIIB and SNAPc cooperatively bind to the TATA box and PSE of type 3 promoters, respectively. Binding of Oct1 to the DSE enhances SNAPc/ TFIIIB recruitment. Following SNAPc/TFIIIB recruitment, pol III binds and transcription comences from the transcriptional start site (+1).

1.6 Pol III

Pol III is the largest and most complex nuclear RNA polymerase. It consists of 17 subunits in yeast and humans, amassing a size of 600-700Kda (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002; White, 2002). Of the 17 subunits identified in yeast, 16 have been demonstrated to be essential for function and yeast viability (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002; White, 2002). Pol III shares a number of common subunits with pol I and pol II. This is of no surprise, as each performs a similar role in the faithful transcription of DNA to produce a complementary RNA strand. Five of the 17 subunits are shared between all three RNA polymerases. A further two are common to both pol I and pol III, and the ten remaining subunits are unique to pol III (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002; White, 2002).

1.7 Transcription, initiation to termination

Once the transcription complex is in place, pol III melts the double stranded DNA around the initiation site (Kassavetis et al., 1992; Kassavetis et al., 1990). TFIIIB is also thought to be of importance in strand separation, because some mutations in Bdp1 and Brf1 prevent strand separation at the promoter complex even though normal polymerase recruitment has taken place (Geiduschek and Kassavetis, 2001; Kassavetis et al., 1998).

Once transcription begins, pol III can dissociate from the promoter-bound TFIIIB and progress along the DNA. The polymerase continues until it encounters the termination signal, which for pol III genes consists of a cluster of four or more T residues. This is in

contrast to pol I and pol II, where accessory factors are required to terminate transcription (Geiduschek and Kassavetis, 2001). At the point of termination, pol III can be recycled, so that multiple rounds of transcription of the same gene can take place. This is much quicker than the original round of replication, because pol III does not have to dissociate from the template, avoiding the slow step of polymerase recruitment (Dieci and Sentenac, 1996). Recycling is aided by TFIIIB and, under certain circumstances, TFIIIC is also required (Ferrari et al., 2004). It is thought that pol III transcription factors facilitate a bend in the DNA that brings the transcriptional start site close to the end of the gene. This allows multiple rounds of transcription once the transcriptional complexes are assembled.

1.8 Regulation of pol III activity

1.8.1 Regulation of pol III activity in proliferating cells:

Cell growth (increased cell mass and size) is a prerequisite for proliferation (increased cell number). The rate of transcription by pol III plays a fundamental role in cellular growth and proliferation. Pol III produces molecules such as tRNA and 5S rRNA, both required for protein synthesis in growing cells. Protein production varies at different points in the cell cycle, therefore the relationship between the cell cycle regulators and the transcriptional activity of pol III is of no surprise.

The retinoblastoma protein (RB) is a negative regulator of cellular proliferation. Its regulation is tightly linked to the cell cycle. Loss or inactivation of RB is a major mechanism by which tumour formation occurs. RB binds and regulates a variety of

transcription factors that are required for a cell's entry into S phase. A prominent example is the E2F family of transcription factors that regulate the transcription of a number of pol II-transcribed genes (Dyson, 1998). In addition to regulating genes transcribed by pol II, RB can also repress transcription by pol I and pol III (Cavanaugh et al., 1995; Sutcliffe et al., 2000). RB regulates pol III activity by its ability to bind and sequester TFIIIB (Scott et al., 2001; Sutcliffe et al., 2000). RB-TFIIIB interaction is controled by the phosphorylation of RB (Scott et al., 2001). During G_0 and early G_1, RB is in a hypophosphorylated state and is bound to TFIIIB. This blocks TFIIIB interaction with TFIIIC and disrupts its' interaction with pol III (Sutcliffe et al., 2000), thus preventing the expression of genes transcribed by pol III. Repression is relieved during the G1-S phase transition of the cell cycle, when RB becomes phosphorylated by cyclin D- and E-dependent kinases. TFIIIB is released, leading to an increase in the expression of genes transcribed by pol III prior to S phase entry (Scott et al., 2001).

Another protein that regulates pol III activity and one that has an extensive role in cancer is the oncogene c-Myc. Its involvement in cellular transformation is achieved through its diverse roles in cell cycle regulation, apoptosis, metabolism, differentiation and cell adhesion (Eisenman, 2001). c-Myc is recruited to the pol III transcription machinery via TFIIIB, where it activates the expression of pol III transcribed genes (Gomez-Roman et al., 2003).

A number of different kinases have been identified as having a direct role in the regulation of pol III activity. These respond to mitogenic stimuli and are part of a

signalling cascade that communicates with the pol III transcriptional machinery through phosphorylation. One such example is Erk (extracellular signal-regulated kinase). Erk responds to mitogens through a signalling cascade consisting of Ras, Raf and MEK (Downward, 2002). Erk mediates its effect through the phosphorylation of Brf1 a component of TFIIIB. This results in an increase in the expression of genes transcribed by pol III (Felton-Edkins et al., 2003a). TFIIIB appears to be an important target of another kinase, CK2, also phosphorylates Brf1 (Johnston et al., 2002). CK2 is a highly conserved enzyme that forms part of the Wnt signalling pathway (Song et al., 2000). It is associated with cellular growth and proliferation. Overexpression of CK2 is associated with cellular transformation and tumourigenesis (Faust et al., 1996b; Munstermann et al., 1990). It therefore follows that the phosphorylation of Brf1 by CK2 leads to an increase in the transcription of genes transcribed by pol III (Johnston et al., 2002). The effect of kinases on pol III transcriptional activity is not only limited to a positive effect, as phosphorylation can decrease pol III transcriptional activity in mitotic cells. Cyclin dependent kinases (cdks) from mitotic frog extracts inhibit the expression of pol III-transcribed genes through their kinase activity (Gottesfeld et al., 1994; Hartl et al., 1993; Leresche et al., 1996). In comparison, repression is not observed in cdks derived from interphase extracts (Gottesfeld et al., 1994; Hartl et al., 1993). Work in human cells has shown that repression during mitosis is the result of the phosphorylation of Brf1 (Fairley et al., 2003). This results in the structure of TFIIIB being compromised, with Bdp1 being selectively released from the TFIIIB complex (Fairley et al., 2003). Although the kinase responsible is not defined, it is clear that kinases help modulate the expression of pol III-transcribed genes at specific points of the cell's cycle.

1.8.2 Regulation of pol III-transcribed genes in response to cellular stress

Stress responses can be defined as a change in cellular activity in response to a condition that deviates from the norm. This may include nutrient deprivation, hypoxia or genotoxic stress. Nutrient deprivation or stress lead to a rapid decrease in the transcription of pol III transcribed genes (Ghavidel and Schultz, 2001; Roberts et al., 2006).

Mammalian target of rapamycin (mTOR) acts as a key nodal point at which a number of different nutrient and mitogenic signalling pathways converge. mTOR responds to these inputs by regulating growth and proliferation (Inoki et al., 2005; Wullschleger et al., 2006). Key downstream targets of mTOR are proteins involved in protein synthesis, such as S6K and 4E-BP1 (Inoki et al., 2005; Wullschleger et al., 2006). However, work in mammals indicates it has a direct role in regulating ribosomal gene transcription, and that synthesis of rRNA is rapamycin sensitive (Majahan, 1994). Recent studies have demonstrated that mTOR regulates rRNA gene expression by the phosphorylation of two components of the pol I transcriptional machinery (Hannan et al., 2003; James and Zomerdijk, 2004; Mayer et al., 2004; Zang et al., 2005). Yeast TOR regulates pol III transcription (Zaragoza et al., 1998). Both rapamycin and TOR mutants cause the transcriptional repression of genes transcribed by pol III (Zaragoza et al., 1998). However, the mechanism that is responsible for pol III activation by TOR has yet to be determined.

Maf1, a protein identified in *S. cerevisiae*, is another example of a factor that regulates pol III in response to stress (Upadhya et al., 2002b). Cells that lack Maf1 are unable to repress the transcription of pol III-transcribed genes when exposed to nutrient deprivation, DNA damage or oxidative stress (Desai et al., 2005; Upadhya et al., 2002b). Recent studies have demonstrated that under favourable conditions Maf1 is phosphorylated, whereas diverse unfavourable conditions lead to the rapid dephosphorylation of Maf1 (Oficjalska-Pham et al., 2006; Roberts et al., 2006). Dephosphorylation is mediated by the protein phosphatase type 2A (PP2A) (Oficjalska-Pham et al., 2006). In its dephosphorylated state, Maf1 accumulates in the nucleus and represses pol III via a direct interaction (Oficjalska-Pham et al., 2006). Although homologues of Maf1 have been discovered in higher eukaryotes (Pluta et al., 2001), a role for its regulation thus far has only been seen in yeast.

p53 can induce cell cycle arrest or cell death in response to a number of different stresses, including hypoxia, radiation and oncogenic stimuli (Vousden and Lu, 2002). More than half of all cancers either have lost p53 or have a mutated form of p53 (Hollstein et al., 1991), highlighting its importance as a tumour suppressor. As with RB, p53 plays an important role in G1 arrest (El-Deiry et al., 1993) and has the ability to alter the transcriptional activity of a number of genes (Vousden and Lu, 2002). Pol III transcriptional activity is repressed by p53 (Crighton et al., 2003). p53 acts in a similar manner to RB, binding and sequestering TFIIIB away from the promoter (Crighton et al., 2003). This interaction is mediated through TBP (Crighton et al., 2003).

For a summary of pol III regulators, see figure 1.5

1.8.3 Pol III and cancer:

Many of the proteins involved in pol III regulation are either oncogenes or tumour suppressors. The link between pol III and cancer has been observed *in vivo*, with pol III transcription showing a level of deregulation in tumours. This would make sense, as pol III regulation is tightly linked to cellular growth and proliferation (Larminie et al., 1998), both important factors in tumour progression.

A partial explanation for this deregulation is found in the tumour suppressors RB and p53. It has been well-established that RB function is compromised in many human malignancies (Mulligan and Jacks, 1998). Further to this, mutations found within RB in carcinomas prevent its function of inhibiting transcription by pol III (White et al., 1996). Transforming factors, such as the adenovirus oncoprotein E1A and the large T antigen of the DNA virus SV40, disrupt RB function, releasing TFIIIB from repression (Larminie et al., 1999; Mulligan and Jacks, 1998; White et al., 1996). Therefore, infection by adenovirus or SV40 decreases the level of RB-mediated pol III repression. As with RB, mutations found within p53 can diminish its ability to inhibit pol III transcription. One such mutation, R175H, a common mutation in cancer, converts p53 from a pol III repressor to an activator (Stein et al., 2002). Oncoproteins also play a role, with papillomavirus E6 and cellular hdm2 being able to reduce the inhibition of pol III, via the neutralisation of p53 (Stein et al., 2002). Recent work has also shown that infection by

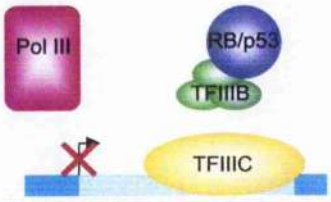
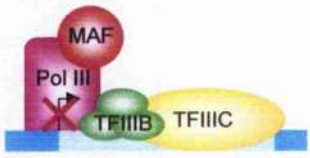
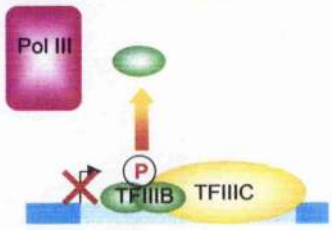
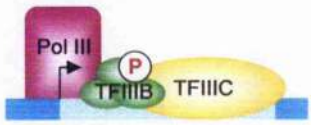
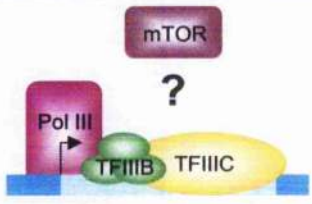
Cartoon of promoter	Effect on transcription	Description
	**Negative**	**P53/RB**: The binding of p53 or RB to TFIIIB prevents its recruitment to the promoter. This inhibits TFIIIB's role as a bridging factor between TFIIIC and pol III.
	**Negative**	**MAF**: MAF in an hypophosphorylated state binds to pol III and inhibits its transcriptional activity.
	**Negative**	**Mitotic kinase**: Phosphorylation of Brf1 (a component of TFIIIB) causes the dissocation of Bdp1 from TFIIIB, inhibiting its function.
	**Positive**	**CK2/ERK**: Phosphorylation by CK2 or ERK promotes the recruitment of TFIIIB to the promoter.
	**Positive**	**c-Myc**: c-Myc binding to TFIIIB via Brf1 promotes the transcription of pol III transcribed genes.
	**Positive**	**mTOR**: mTOR activates the transcription of pol III transcribed genes by an as yet undefined mechanism.

Figure 1.5 Summary of the different ways in which the transcription of pol III transcribed-genes are regulated.

high-risk types of human papillomavirus (HPV), such as HPV16, cause an increase in the expression of genes transcribed by pol III (Daly et al., 2005). HPV16 expresses the oncoproteins E6 and E7, which can inactivate p53 and RB, respectively (Dyson et al., 1989; Munger et al., 1989; Werness et al., 1990). HPV16 is associated with cervical cancers and their progression to an invasive state (zur Hausen, 2000; zur Hausen, 2002). Another example of an oncogene with a prominent role in regulating pol III-transcribed genes is c-Myc (Gomez-Roman et al., 2003). c-Myc is different from the oncoproteins mentioned above, in that it directly binds to the pol III transcriptional machinery. As mentioned previously, c-Myc exerts a positive effect on pol III transcriptional activity through its interaction with TFIIIB (Gomez-Roman et al., 2003). In transformed cervical cells, c-Myc binds to the promoters of tRNA and 5S rRNA genes through its interaction with TFIIIB, leading to an elevation in the transcription of these genes (Felton-Edkins et al., 2003b; Gomez-Roman et al., 2003).

Kinases CK2 and ERK, both regulators of pol III, are known to display a level of deregulation in many tumours. For instance, CK2 is abnormally active in a variety of cancers, including leukemias and solid tumours (Faust et al., 1996a; Munstermann et al., 1990; Notterman et al., 2001). Additionally, oncogenic Ras is known to cooperate with CK2 in the transformation of primary fibroblasts (Orlandini et al., 1998). Ras is upstream of ERK in the MAP kinase-signalling pathway. Therefore, it may also be the case that a deregulation of Ras results in the deregulation of ERK, which alters pol III activity.

Pol III transcriptional activity can be increased by raising the levels of limiting transcription factors which coordinate its recruitment to gene promoters. Overexpression of transcription factors is observed in a number of transformed cells types. For example, levels of all five subunits of TFIIIC are elevated (at the mRNA and protein level) in fibroblasts transformed by SV40 or polyomavirus (Felton-Edkins and White, 2002; Larminie et al., 1999). In ovarian tumors, there is an increased abundance of TFIIIC (Winter et al., 2000). This is thought to promote the transcription of pol III templates (Winter et al., 2000). TFIIIC110, a subunit of the TFIIIC complex, is thought to play an important role in pol III's transcriptional activity. Two TFIIIC complexes have been identified, with or without TFIIIC110, dubbed TFIIIC2a and TFIIIC2b, respectively (Kovelman and Roeder, 1992; Sinn et al., 1995). Both TFIIIC complexes have similar DNA-binding affinities (Hoeffler et al., 1988; Kovelman and Roeder, 1992). However, the complex without TFIIIC110 is unable to support transcription (Hoeffler et al., 1988; Kovelman and Roeder, 1992). HeLa cells infected with the E1A oncoprotein have an elevated level of the active TFIIIC2a compared to the inactive TFIIIC2b (Hoeffler et al., 1988). This is thought to increase the transcriptional activity of pol III. Overexpression of pol III transcription factors is not restricted to TFIIIC, with both Brf1 and Bdp1 being elevated in a subset of cervical carcinomas (Daly et al., 2005).

1.8.4 Coordinated regulation of pol I and pol III

Organisms must coordinate their translational capacity to relate in response need for protein synthesis. Both pol III and pol I synthesise molecules involved in translation. Pol I synthesizes 28S, 18S and 5.8S ribosomal RNAs, whereas pol III synthesizes 5S

rRNA and tRNA. Therefore, it is of no surprise that pol I and pol III share similar modes of regulation, so that they can act in a coordinated manner (White, 2005). Although pol I and pol III have different components in their transcriptional machinery, regulators such as Erk, Myc, RB and the over expression of component of the transcriptional machinery regulate both pol I and pol III by similar mechanisms (White, 2005). Therefore, when a difference is observed in the regulation of pol I and III, this is seen as an exception and not the rule.

1.9 Aims of PhD

Previous work in yeast and in a mammalian system has demonstrated that use of rapamycin, the specific mTOR inhibitor reduced the expression of pol III-transcribed genes (Graham, Data not published; Zaragoza et al., 1998). However, little is known about the way in which mTOR control the activity of pol III. Therefore, work in this study will focus on the mechanism by which mTOR controls the expression of pol III-transcribed genes. A number of studies have shown that mTOR controls pol I transcription through the kinase S6K (Hannan et al., 2003; James and Zomerdijk, 2004; Mayer et al., 2004; Zhang et al., 2005). Consequently, initial experiments will be carried out to see if pol I and pol III are regulated in a similar manner by mTOR.

Chapter 2-Materials and Methods

2.1 Cell Culture

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

2.1.1 Growth conditions

HeLa, MEF and A31 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).

For rapamycin (Calbiochem) treatment, cells were grown to 70% confluency and treated with 100 nM final concentration of rapamycin for specific times. Rapamycin stock was diluted in DMSO.

HeLa TET-ON cells were cultured in 10% FBS (tetracycline free), 100 µg/ml streptomycin, 100 µg/ml G418, 100 µg/ml hygromycin and 100 U/ml penicillin. Expression of HA-TFIIIC110 was induced by the addition of 1 µg/ml doxycycline for 48 hrs. Cells were passaged when 80-90% confluent (3-4 days between passages) using buffered trypsin (Sigma).

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). Cells were passaged when 80-90% confluent (3-4 days between passages) using buffered trypsin (Sigma).

2.1.2 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.

2.2 Preparation of Extracts

2.2.1 Preparation of extracts for western blots, co-immunoprecipitations and in vitro transcription assays

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 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.

2.2.2 Preparation of nuclear and cytoplasmic fractions

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 1 ml of hypertonic buffer (20 mM HEPES pH 7.0, 10 mM KCl, 1 mM DTT, 0.1 % triton X-100, 20 % glycerol, 2 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin). The cell suspension was then subjected to 10 slow strokes in a Dounce homogenizer and centrifuged at 800 g for 5 min. The resultant supernatant is the cytoplasmic fraction. The supernatant was removed, aliquoted and snap frozen on dry ice. The cytoplasmic extracts were stored at -80°C. The pellet was resuspended in 100 μl cold extraction buffer (20 mM HEPES pH 7.0, 10 mM KCl, 1 mM DTT, 0.1 % Triton X-100, 20 % glycerol, 2 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 420 mM NaCl). The suspension was then placed on a

spinning wheel for 20 min at 4 °C and then centrifuged at 16,000 g for 10 min. The supernatant contains the nuclear extract and the pellet is the nuclear matrix. The supernatant was removed, aliquoted and snap frozen on dry ice. The extracts were stored at -80°C.

2.2.3 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. 2μl 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.

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

2.3.1 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.

2.3.2 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 2.1. 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).

Antibodies used in western blot analysis

Protein	Antibody	Type	Dilution	Source
Brf1	128	Serum	1:1000	In house
TFIIIC 90	1898	Serum	1:1000	In house
TFIIIC 102	3238	Serum	1:1000	In house
TFIIIC 110	3208	Serum	1:1000	In house
TFIIIC 220	Ab 7	Serum	1:1000	In house
RPC 155	1900	Serum	1:1000	In house
S6K1	9202	Polyclonal	1:1000	Santa Cruz
Phospho-S6K1	9202	Polyclonal	1:1000	Cell signalling technologies
c-Jun	Sc-44	Polyclonal	1:1000	Santa Cruz
HA-tag	Sc-7392	Monoclonal	1:2000	Santa Cruz
Acetyl Histone H4	06-866	Polyclonal	1:5000	Upstate
Acetyl Histone H3	06-599	Polyclonal	1:5000	Upstate
Raptor	Ab5454	Polyclonal	1:1000	Abcam
Actin	C-11	Polyclonal	1:5000	Santa Cruz

Table 2.1

2.4 Co-immunoprecipitation

Anti-TFIIIC110 (4286) 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-TFIIIC110 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.

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

2.5.1 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 into 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 13,000 rpm at 4°C. The pellet was washed with 75% ethanol and centrifuged again for 10min at 13,000 rpm 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 formula1 $A_{260} = 40$ µg/µl RNA.

2.5.2 cDNA production

3µg of RNA, prepared as outlined in the 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) were added. Reverse transcription reaction was incubated at 42°C for 1 hour. The reaction was terminated by incubating tubes at 70°C for 15 min to denaturing the enzyme.

2.5.3 Polymerase Chain Reaction (PCR)

Each PCR was performed using 2µl of cDNA or 2µl of ChIP DNA. 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 2.2. 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.

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 mRNA	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
Pre Pol I-rRNA	5' GCTTGGGTCTGTCGCGGT 3' 5' CACCTCGGGGAAATCGGGA 3'	15-20	151 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
TFIIIC110 mRNA	5' CCAGAAGGGGTCTCAAAAGTCC 3' 5' CTTTCTTCAGAGATGTCAAAGG 3'	25-30	300 bp	95°C for 3 min; 95°C for 1 min, 62°C for 40 s, 72°C for 40 s, 72°C for 5 min
P21 mRNA	5' GCCTTAGCCCTCACTCTGTG 3' 5' AGGGCCCTACCGTCCTACTA 3'	25-30	257 bp	95°C for 3 min; 95°C for 30 s, 58°C for 30 s, 72°C for 30 s; 72°C for 5 min
B2 RNA	5' GGGGCTGGAGAGATGGCT 3' 5' CCATGTGGTTGCTGGGAT 3'	12-15	90 bp	95°C for 3 min; 95°C for 30 s, 58°C for 30 s, 72°C for 30 s; 72°C for 5 min

2.6 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 Tris HCl, 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 2.4), 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 Tris HCl, pH 8.0, 150mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40) buffer, twice with 10ml LiCl buffer (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 2.3)

Primers used in PCR analysis of ChIP samples

Gene	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 Regiou	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
Alu Chromosome 6	5' CCAGAAAAATTACCAATTAGTTC 3' 5' GGGCCTATTGACTATGCTTAC 3'	25-30	396 bp	95°C for 3 min; 95°C for 30 s, 53°C for 1 min, 72°C for 1 min; 72°C for 5 min
Alu Chromosome 8	5' GCACTCCATAAGAAATGTTTTT 3' 5' GAATTTTGGTTCAGTTGTGTTA 3'	25-30	431 bp	95°C for 3 min; 95°C for 30 s, 53°C for 1 min, 72°C for 1 min; 72°C for 5 min
Alu Chromosome 10	5' GATTCTCAACAGCAGAATTCCA 3' 5' CATGTTTGAGAATGTCTACTTC 3'	25-30	442 bp	95°C for 3 min; 95°C for 30 s, 53°C for 1 min, 72°C for 1 min; 72°C for 5 min
Alu Chromosome 19	5' CCACGTGTTTATCTGTAAGGTG 3' 5' GTTAGGAGCTAGAAGGAGCCT 3'	25-30	381 bp	95°C for 3 min; 95°C for 30 s, 58°C for 1 min, 72°C for 1 mn; 72°C for 5 min
Alu Chromosome 20	5' GTTTATTTAGAGAAGCAAATGC 3' 5' CCAGATAATTTTATCATGTCCT 3'	25-30	456 bp	95°C for 3 min; 95°C for 30 s, 58°C for 1 min, 72°C for 1 min; 72°C for 5 min

Alu Chromosome 22	5' GTTCTGACACACTTGGAGAAA 3' 5' GTTGTTGTTATTGCACAACTCA 3'	25-30	370 bp	95°C for 3 min; 95°C for 30 s, 58°C for 1 min, 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
$tRNA^{Arg}$	5' GGCTCTGTGGCGCAATGGATA 3' 5' TTCGAACCCACAACCTTTGAATT 3'	25-30	74 bp	94°C for 2 min; 95°C for 30 s, 66°C for 30 s, 72°C for 15 s; 72°C for 5 min
MRP	5' CGTGCTGAAGGCCTGTATC 3' 5' GGTGCGCGGACACGCAC 3'	25-30	232 bp	95°C for 3 min; 95°C for 30 s, 58°C for 30 s, 72°C for 30 s; 72°C for 5 min
B2	5' GGGGCTGGAGAGATGGCT 3' 5' CCATGTGGTTGCTGGGAT 3'	12-15	90 bp	95°C for 3 min; 95°C for 30 s, 58°C for 30 s, 72°C for 30 s; 72°C for 5 min
7SL	5' GTGCCGCACTAAGTTCGGCATC 3' 5' TATTCACAGGCGCGATCCCACTACTGAGA TC 3'	150 bp	10-20	94°C for 2 min; 94°C for 20 s, 62°C for 30 s, 72°C for 30 s, 72°C for 10 min
TFIIIC220	5' TCCAGCGAGACCTTCACAAA 3' 5' GGATTGAGTGTTGCTGGGCT 3'	25-30	144 bp	95°C for 3 min; 94°C for 20 s, 62°C for 30 s, 72°C for 30 s; 72°C for 10 min

Antibodies for ChIP analysis

Protein	Antibody	Type	Source	Quantity used per IP
Brf1	128	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
TFIIIC110	3208	Serum	In house	20 µl
MTOR	T2949	Polyclonal	Sigma	4 µg
Acetyl Histone H4	06-866	Polyclonal	Upstate	4 µg
Acetyl Histone H3	06-599	Polyclonal	Upstate	4 µg
Trimethyl-histone H3 (Lys 4)	07-473	Polyclonal	Upstate	4 µg
Trimethyl-histone H3 (Lys 9)	07-523	Polyclonal	Upstate	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

Table 2.4

2.7 Pol III in vitro transcription assay

In vitro transcription of class III genes was reconstituted using 15μg of HeLa nuclear extract (Computer Cell Culture Center, Mons, Belgium) or 15μg of A31 mouse fibroblast microextract. This was supplemented with the addition of 250ng of plasmid DNA to supply specific pol III templates and reactions were carried out in a 25μl volume with a final concentration of 12 mM HEPES pH 7.9, 60 mM KCl, 7.2 mM $MgCl_2$, 0.28 mM EDTA, 1.2 mM DTT, 10% (v/v) glycerol, 1 mM creatine phosphate, 0.5 mM each of rATP, rCTP and rGTP and 10 μCi [α-^{32}P] UTP (400 mCi/mmol) (Amersham). Transcription components were assembled on ice and the reaction was performed at 30°C for 1 hour. Transcription was terminated by the addition of 250 μl of 1 M ammonium acetate/0.1% SDS containing 20 μg of yeast tRNA, which acts as a carrier for synthesised RNA. Phenol-chloroform extraction of the samples was performed to remove protein and DNA by adding 250 μl of a 25:24:1 ratio of PhOH/$CHCl_3$/IAA. The samples were vortexed, microcentrifuged at 13,000g for 5 minutes, and 200 μl of the upper aqueous layer was then transferred to a fresh epppendorf tube containing 750 μl of 96% ethanol in order to precipitate the RNA. The samples were thoroughly mixed by repeated inversion, left at -20 °C overnight before being microcentrifuged at 13,000g for 30 minutes to pellet the precipitated RNA. The supernantant was carefully removed and 750 μl of 70% ethanol was added to each sample to wash the pellet. This was also carefully removed to avoid dislodging the pellet and the samples were heated at 50 °C for 5-10 minutes to dry. 4μl of formamide loading buffer (98% formamide, 10 mM EDTA pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol FF) was added to each sample, which was then

vortexed for 1 hour to ensure the RNA was fully re-dissolved. 1.5 µl of each sample was loaded on a pre-run 7% polyacrylamide sequencing gel containing 7 M urea and 0.5 x TBE after being boiled at 95 °C for 2 minutes and quenched on ice. Electrophoresis was performed at 40 W for 1 hour in 0.5 x TBE before being dried and exposed to autoradiography film in order to detect the radiolabelled transcripts.

2.7.1 Peptide substrate inhibitors and drugs used in *in vitro* transcription assays

The peptide substrate inhibitors used for *in vitro* transcription were PKB substrate peptide inhibitor GRPRTSSFAEG (Biomol) or PKA phospho-acceptor peptide LRRASLG (Upstate). The phosphatase inhibitors used in *in vitro* transcription assay were Okadaic acid (Sigma) and Calyculin A (Sigma).

2.8 Plasmid Preparation

2.8.1 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

were then plated onto LB-agar plates (LB-broth, 2% agar, 100 µg/ml ampicillin) and then incubated at 37°C overnight.

2.8.2 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 in a quartz cuvette, using the equation 1 A_{260} = 50 µg/µl dsDNA.

Description of plasmids used in *in vitro* transcription assays

Plasmid	Description
$tRNA^{Leu}$	pLeu is a 240 bp *EcoR*I-*Hind*III fragment of human genomic DNA carrying a $tRNA^{Leu}$ gene, subcloned into pAT153 (McLaren and Goddard, 1986).
7SL	P7L30.1 contains a *Hind*III-*Eco*R1 fragment carrying a human 7SL gene subcloned into pUC13 (Ullu and Weiner, 1985).
5S rRNA	Phu5S3.1 is a 638bp *Bam*HI-*Sac*I fragment of human genomic DNA containing a 5S rRNA gene, subcloned into pBluescript SK+.

Table 2.5

All experiments were carried out three times unless otherwise stated.

Chapter 3-The mTOR pathway regulates expression of pol III-transcribed genes

3.1 Introduction

3.1.1 TOR

Yeast TOR1 and TOR2 were originally identified as targets of Rapamycin (Kunz et al., 1993). Rapamycin is an antifungal agent originally purified from *Streptomyces hygroscopicus* (Abraham and Wiederrecht, 1996). Analogues of Rapamycin have been used clinically to inhibit host rejection following organ transplantation (Garaza et al., 2002). More recent studies have highlighted its potent growth inhibitory activity, giving it the potential for use in anticancer treatment (Inoki et al., 2005)

Mutations in TOR1 and TOR2 genes show similar growth inhibitory properties to those seen with rapamycin treatment (Kunz et al., 1993). In yeast and mammalian cells, TOR proteins can regulate cell growth by controlling transcription, translation and ribosome biogenesis (Inoki et al., 2005). Inhibition of TOR by rapamycin is not a direct effect as another protein, FK506-binding protein (FKBP12), is required for repression to take place. Rapamycin forms a complex with FKBP12, which binds to TOR and inhibits its function (Abraham and Wiederrecht, 1996). Mammalian TOR or mTOR was subsequently identified due to its ability to bind to FKBP12 (Chiu et al., 1994). Mammals contain a single TOR gene (mTOR) compared to two found in yeast. mTOR is a 289kDa protein that belongs to the phophoinositide 3-kinase related kinase (PIKKs) family. PIKKs are involved in a diverse array of cellular functions including gene expression, cell cycle control, DNA damage checkpoint regulation and cell growth control.

The best-studied targets of the mTOR pathway are proteins that regulate the translational machinery. One such protein is the translational repressor 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1). 4E-BP1 inhibits translation by binding to eukaryotic translation factor 4E (eIF4E). eIF4E recognises the 5'-end cap of the majority of eukaryotic mRNAs. Repression is relieved when mTOR phosphorylates 4E-BP1, releasing eIF4E, which is then free to associate with eIF4G and initiate translation (Wullschleger et al., 2006).

S6K1 is another prominent example of a protein that is directly phosphorylated by mTOR. S6K1, when active, phosphorylates the 40S ribosomal protein S6. This was thought to promote the translation of a subset of mRNAs that contain a 5' tract of oligopyrimidine (TOP). However, recent studies have shown that although phosphorylation of S6 is essential for regulating cell size, it is dispensable for translational control of TOP mRNAs (Ruvinsky et al., 2006). An alternative explanation for S6K's ability to control translation can be found in its regulation of eukaryotic elongation factor 2 kinase (eEF2K). S6K phosphorylates and inactivates eEF2K, a factor that mediates the translocation step of translational elongation. Therefore, S6K phosphorylates and inactivates eEF2K, thus promoting translational elongation (Wang et al., 2001).

3.1.2 TSC1/2 and Rheb

TSC1/2 and Rheb form two key components of the mTOR-signalling pathway. Both are found upstream of mTOR and are regulated by a number of different pathways that feed into the mTOR pathway.

3.1.2.1 TSC1/TSC2

TSC1 and TSC2 genes encode the proteins hamartin and tuberin. Mutations within TSC1 and TSC2 are responsible for the disease Tuberous sclerosis. Tuberous sclerosis is a relatively common autosomal dominant disorder. It occurs with a frequency of 1 in 6,000 to 10,000 of the population. It is characterised by the development of benign tumours called hamartomas. Common symptoms include seizures, autism, mental retardation, kidney failure, facial angiofibromas, and cardial rhabdomyomas (Gomez, 1991). Hamartin and tuberin interact to form a functional tumour suppressor (TSC1-2) (Morry and Harding, 1986; van Slegtenhorst et al., 1998). TSC2 encodes a putative GTPase-activating protein (GAP), whereas TSC1 encodes a protein that contains two coiled-coil domains. Studies in Drosophila have shown that the loss-of-function of either TSC gene leads to an increase in cell size and proliferation (Potter et al., 2001). Homozygous inactivation of either TSC1 or TSC2 in mice is lethal at the embryo stage (Kobayashi et al., 2001), while heterozygous animals are tumour-prone (Onda et al., 1999).

3.1.2.2 Rheb

Ras homology enriched in brain (Rheb) is a direct target of TSC2. Rheb is a small GTPase and is a member of the Ras superfamily of GTP-binding proteins. Active TSC2

converts Rheb from a GTP to a GDP-bound state, thereby inactivating it. Point mutations within the GAP domain of TSC2 disrupt its ability to regulate Rheb. This occurs without affecting TSC2's ability to form a complex with TSC1 (Zhang et al., 2003b). Thus, Rheb is found downstream of the TSC1-TSC2 complex. In relation to mTOR, biochemical analysis would indicate that Rheb functions upstream of mTOR (Saucedo et al., 2003). Recent studies have indicated this is through a direct interaction, where Rheb binds directly to the amino-terminal lobe of the mTOR catalytic domain (Long et al., 2005a; Long et al., 2005b). This interaction is not attributed to changes in Rheb-GTP charging (Long et al., 2005a; Long et al., 2005b).

3.1.3 Upstream regulators of mTOR signalling

TSC1/2 and Rheb form targets for a number of signalling pathways. This allows mTOR to respond to cellular energy levels, the presence of nutrients and growth factors.

3.1.3.1 Energy sensing

Cell growth is dependent on the rate of protein synthesis, a process that requires a large amount of energy. Therefore, mTOR must be regulated in response to cellular energy levels. mTOR senses cellular energy levels through AMP-activated protein kinase (AMPK). AMPK is a kinase that is activated by a high AMP/ATP ratio. The tumour suppressor LKB1 also regulates AMPK. Individuals who have mutations within LKB1 develop Peutz-Jeghers syndrome, characterized by the formation of hamartomas in the gastrointestinal tract (Boudeau et al., 2003). Under low ATP conditions, LKB1 phosphorylates AMPK, contributing to AMPKs activation (Shaw et al., 2004). Active

AMPK represses mTOR, resulting in a decrease in mTOR-dependent phosphorylation of 4E-BP1 and S6K (Inoki et al., 2003b). Repression of mTOR is mediated by AMPK's phosphorylation of TSC2, which enhances TSC2 GAP activity (Inoki et al., 2003b). Thus, when cellular energy levels are low, cell growth is inhibited through AMPK's repression of the mTOR pathway.

3.1.3.2 Growth factors

mTOR senses the presence of growth factors through the PI3K pathway. Many growth factors, including insulin, bind to their receptors and promote recruitment and phosphorylation of insulin receptor substrate (IRS). Following the phosphorylation of IRS, PI3K binds to IRS, stimulating PI3K's ability to covert phosphatidylinositol-4,5-phosphate (PIP2) to phophatidylinositol-3,4,5-phosphate (PIP3). PIP3 recruits 3-phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (PKB, also known as Akt) through their pleckstrin homology domains. This results in the phosphorylation and activation of PKB by PDK1.

Both PI3K and PKB have been shown to regulate mTOR activity. Overexpression of PKB promotes the phosphorylation of 4E-BP1 in a rapamycin-sensitive manner (Gingras et al., 1998). Furthermore, the phosphorylation of 4E-BP1 is sensitive to wortmannin, an inhibitor of PI3K (Gingras et al., 1998). These findings place the PI3K pathway upstream of mTOR. The bridge between these two pathways is mediated by PKB kinase activity (Cai et al., 2006). Hypophosphorylated TSC2 is associated with TSC1 at the membrane where it represses Rheb through its GAP activity. Active PKB phosphorylates

TSC2, which promotes TSC1/TSC2 interaction with 14-3-3 (Cai et al., 2006). 14-3-3 sequesters TSC2 in the cytosol, preventing its repression of its membrane bound target Rheb (Cai et al., 2006). Thus, growth factors activate PKB, which activates mTOR by its repression of TSC2.

3.1.3.3 Nutrients

Nutrients such as glucose and amino acids play an important role in the regulation of mTOR signalling. In mammalian cells, amino acid deprivation results in 4E-BP1 and S6K become rapidly dephosphorylated, whereas amino acid supplements stimulate 4E-BP1 and S6K phosphorylation (Hara et al., 1998). Rheb has been shown to play a role in regulating mTOR activity in response to the presence or absence of amino acids (Long et al., 2005b). Rhebs interaction with mTOR is stimulated by the presence of amino acids, most notably leucine (Long et al., 2005b). This interaction is independent of Rheb GTP charging (Long et al., 2005a; Long et al., 2005b). Furthermore, inactive TSC1/TSC2 renders cells resistant to amino acid starvation (Gao et al., 2003), demonstrating that the TSC1/TSC2 complex is also important in amino acid sensing. However, a number of other studies have proposed a TSC1/TSC2-independent mechanism whereby amino acids regulate the mTOR pathway (Kim et al., 2002; Smith et al., 2004). One such mechanism involves mTOR directly sensing the presence of amino acids (Kim et al., 2002). Therefore, the role of nutrients is important in the regulation of mTOR, though a clear mechanism has yet to be defined. For a summary of the mTOR pathway and the various pathways that feed into it see figure. 3.1.

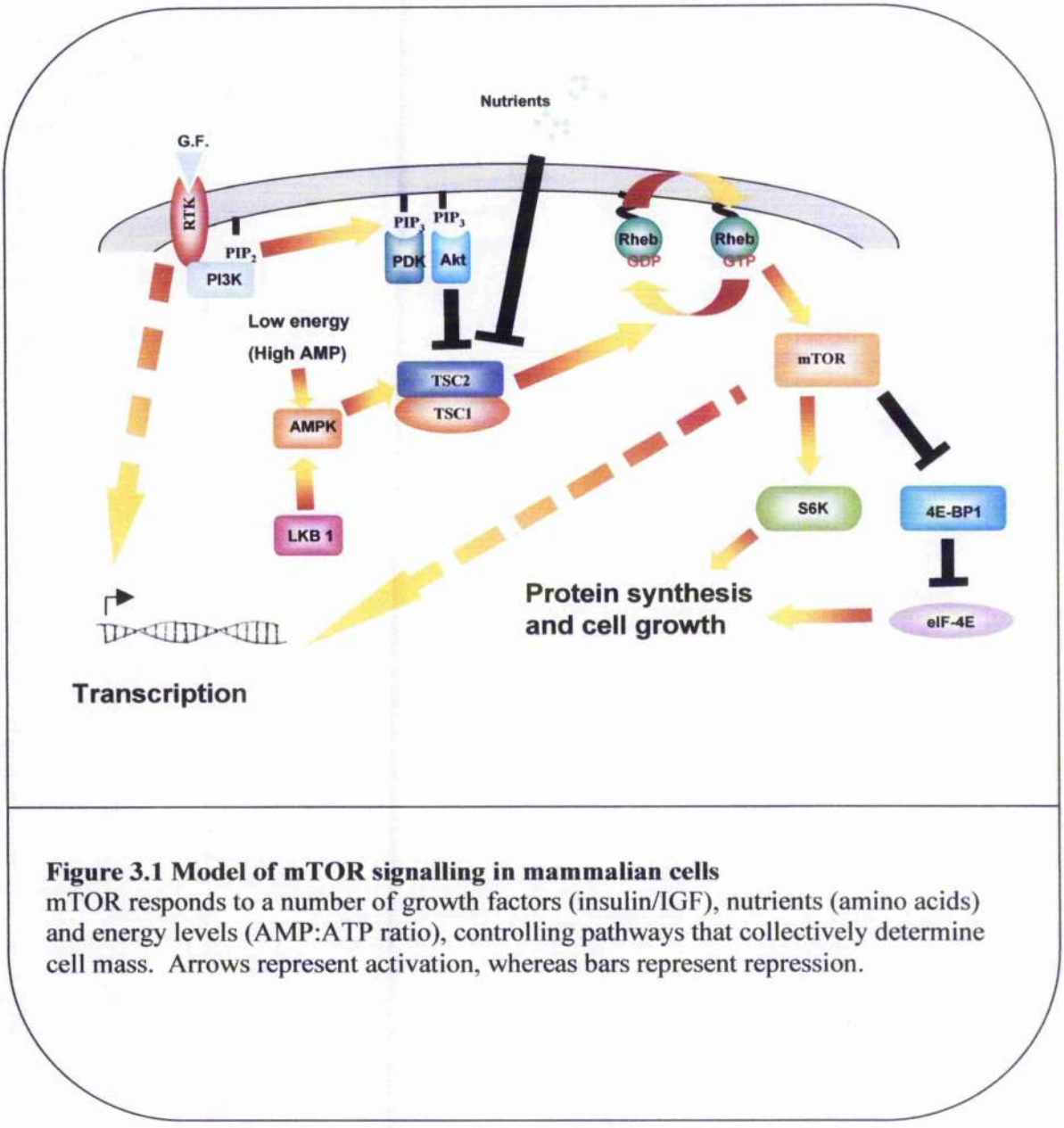


Figure 3.1 Model of mTOR signalling in mammalian cells
mTOR responds to a number of growth factors (insulin/IGF), nutrients (amino acids) and energy levels (AMP:ATP ratio), controlling pathways that collectively determine cell mass. Arrows represent activation, whereas bars represent repression.

As discussed previously, rapamycin was shown to have a repressive effect on pol III transcription in *S. cerevisiae* (Zaragoza et al., 1998). Therefore, rapamycin was used to investigate the role of mTOR on the transcription of pol III transcribed genes in mammals. Further to this, various components of the mTOR-signalling pathway were investigated to determine their regulatory effect on pol III activity.

3.2 Results

3.2.1 Inhibition of mTOR by rapamycin reduces pol III transcriptional activity

Initial experiments were performed to see if the effect of rapamycin on pol III in *S. cerevisiae* (Zaragoza et al., 1998) was mirrored in mammalian cells. Asynchronously grown mouse fibroblasts were treated with rapamycin for the times indicated. An *in vitro* transcription assay was performed using extracts derived from these cells to measure pol III transcriptional activity (figure 3.2). After one hour of rapamycin treatment, there is a decrease in the level of pol III transcriptional activity. This effect is seen on both 7SL and $tRNA^{Leu}$ genes.

In a second experiment, RNA was harvested from asynchronous grown cells either treated with a vehicle or rapamycin for 4 hours. RT-PCR analysis revealed that levels of $tRNA^{Leu}$ and B2 transcripts were reduced after the addition of rapamycin (figure 3.3). This effect is specific, as the level of the pol II transcript encoding ARPP P0 remains unchanged. Type 3 promoters were ignored as previous data from Emma Graham had shown that rapamycin had no effect on the transcription of U6.

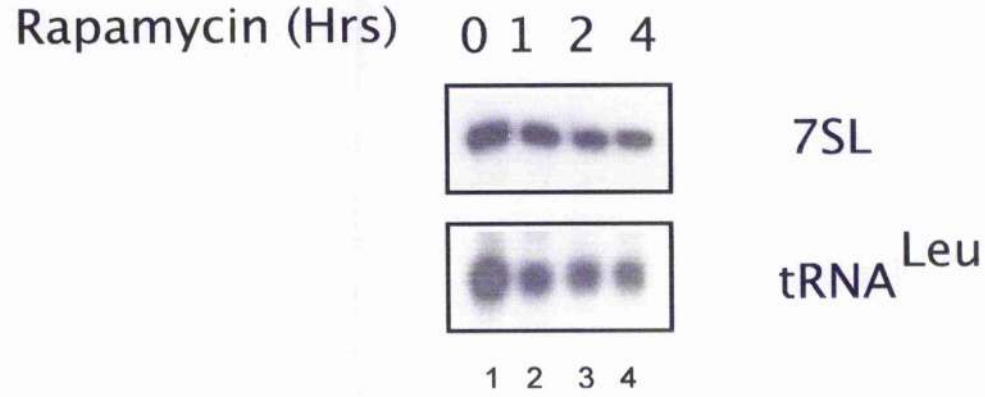


Figure 3.2 mTOR inhibition by rapamycin reduces pol III transcriptional activity.
Whole cell extracts were made from A31 mouse fibroblasts treated with rapamycin (100nM) for 1, 2 and 4 hours (lanes 2-4) or with vehicle (lane 1). 15μg of extract was used in an in vitro transcription assay using a $tRNA^{Leu}$ (250ng) and 7SL (250ng) template.

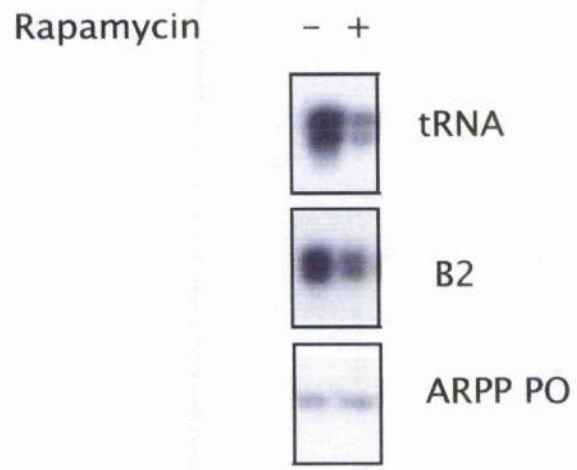


Figure 3.3 Inhibition of mTOR by rapamycin reduces the levels of pol III transcripts.
RNA was harvested from A31 mouse fibrobroblasts treated with rapamycin (100nM) or vehicle control for 4 hours. RNA was analysed by RT-PCR for the expression of B2, $tRNA^{Leu}$, and ARPP P0 mRNA(control).

3.2.2 Inhibition of mTOR by rapamycin reduces the promoter occupancy of pol III and TFIIIB

mTOR has been shown to regulate gene expression by controlling the promoter occupancy of transcription factors (James and Zomerdijk, 2004; Zhang et al., 2005). Therefore, to determine the effects of rapamycin on the promoter occupancy of the pol III transcriptional machinery a ChIP assay was performed (figure 3.4). Formaldehyde cross-linked chromatin was prepared from asynchronously growing cells either treated for 4hrs with 100nM rapamycin or a vehicle control. Antibodies against RPC155 (a component of pol III), TFIIIC110 and the TFIIIB subunit Brf1 were used to determine their occupancy. TFIIA was used as a negative control as it is not present on pol III-transcribed genes. PCR analysis showed that the promoter occupancy of pol III and TFIIIB (Brf1) were diminished after the addition of rapamycin. In contrast, the levels of TFIIIC remain comparable in both rapamycin-treated and vehicle-treated cells.

3.2.3 Knockdown of endogenous Rheb in MEF cells by siRNA decreases the levels of pol III transcripts

Use of the mTOR specific inhibitor rapamycin has highlighted a new role for mTOR in regulation of pol III. As previously stated, Rheb is the direct upstream regulator of mTOR (Long et al., 2005a; Long et al., 2005b). To examine the role of Rheb in the regulation of pol III, Rheb was knocked down using small interfering RNA (siRNA). RNA was obtained from Richard Lamb at The Institute of Cancer Research, London (figure 3.5). RT-PCR analysis of RNA derived from the Rheb knockdown experiments showed that the levels of pol III transcribed $tRNA^{Leu}$ and B2 RNA are down compared to

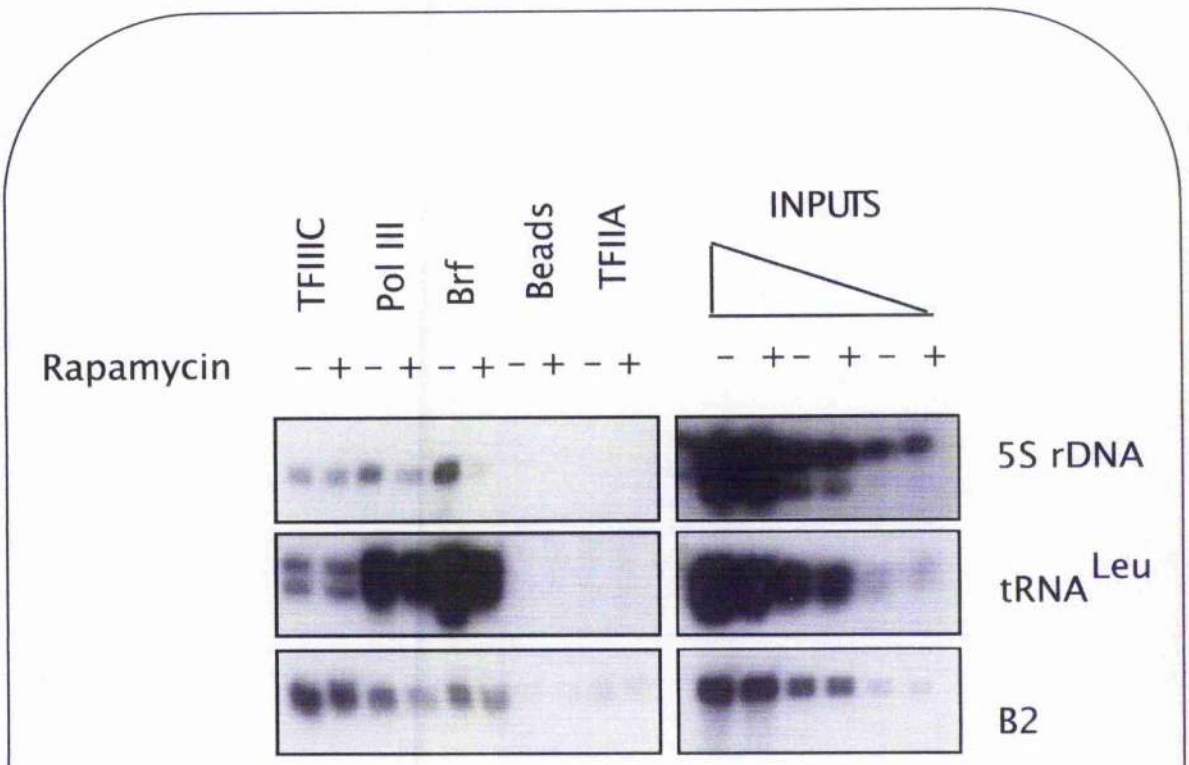


Figure 3.4 mTOR inhibition by rapamycin reduces promoter occupancy by pol III and TFIIIB

Promoter occupancy of the pol III transcriptional machinery was measured in A31 mouse fibroblasts treated with either rapamycin (100nM) or vehicle control for 4 hours. ChIPs were performed using antibodies against TFIIIC110, Brf1 (TFIIIB), RPC155 (pol III) and TFIIA (negative control). Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4%), using 5S rRNA, $tRNA^{Leu}$ and B2 primers.

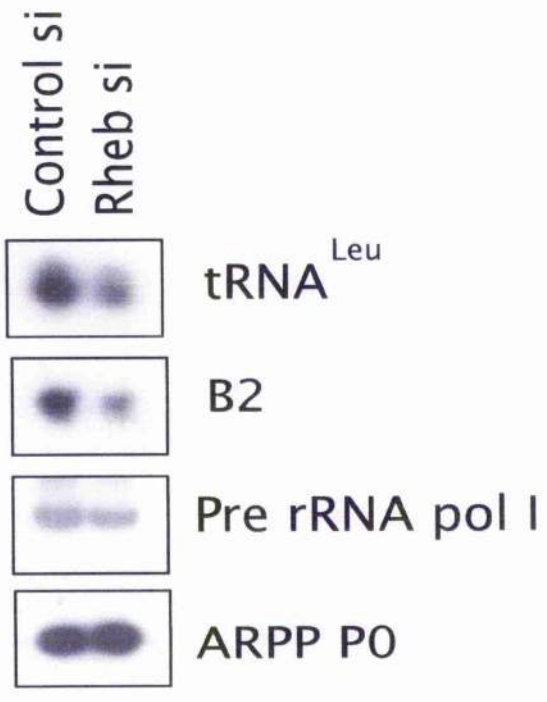


Figure 3.5 Knockdown of endogenous Rheb in MEF cells by siRNA decreased the levels of pol III transcripts.
Knockdown of Rheb in MEF cells was performed by Richard Lamb's group at the Institue of Cancer Research, London (data unpublished). MEFs were transfected with Rheb (lane 2) or scrambled siRNA (control; lane 1). RNA was harvested and analysed by RT-PCR for the expression of 5S rRNA, tRNALeu, B2, pre rRNA and ARPP P0 mRNA(control) (n=1).

the control siRNA transfection. The effect is specific, as the pol II transcribed ARPP PO mRNA does not change in response to Rheb knock down. The levels of the pol I-transcribed, pre-rRNA was analysed, as previous work has shown that pol I is controlled by various components of the mTOR pathway (Hannan et al., 2003; James and Zomerdijk, 2004; Mayer et al., 2004; Zang et al., 2005). As expected, the level of pre-rRNA is reduced when Rheb is knocked-down. Thus, Rheb regulates the levels of both pol I and pol III transcripts.

3.2.4 TSC2 negatively regulates the levels of pol III transcripts

Rheb is the direct target for TSC2 GAP activity, which converts Rheb from an active GTP bound form to an inactive GDP form. To determine whether TSC2 regulates the levels of pol III transcripts, control $TSC2^{+/+}$ mouse embryonic fibroblasts (MEFs) were compared to $TSC2^{-/-}$ MEF knockouts (figure 3.6). RT-PCR analysis of these cells revealed that both 5S rRNA and $tRNA^{Leu}$ are elevated in the $TSC2^{-/-}$ MEFs compared to the control $TSC2^{+/+}$ MEFs (lanes 1 and 2, respectively). This effect is reversed when TSC2 is reintroduced via transient transfection in the knockout cells (lane 3). A TSC2 mutant with compromised GTPase activity partially reversed the effect of TSC2 knockout (lane 4). These effects are specific, as the levels of the pol II-transcribed ARPP P0 transcript remain constant.

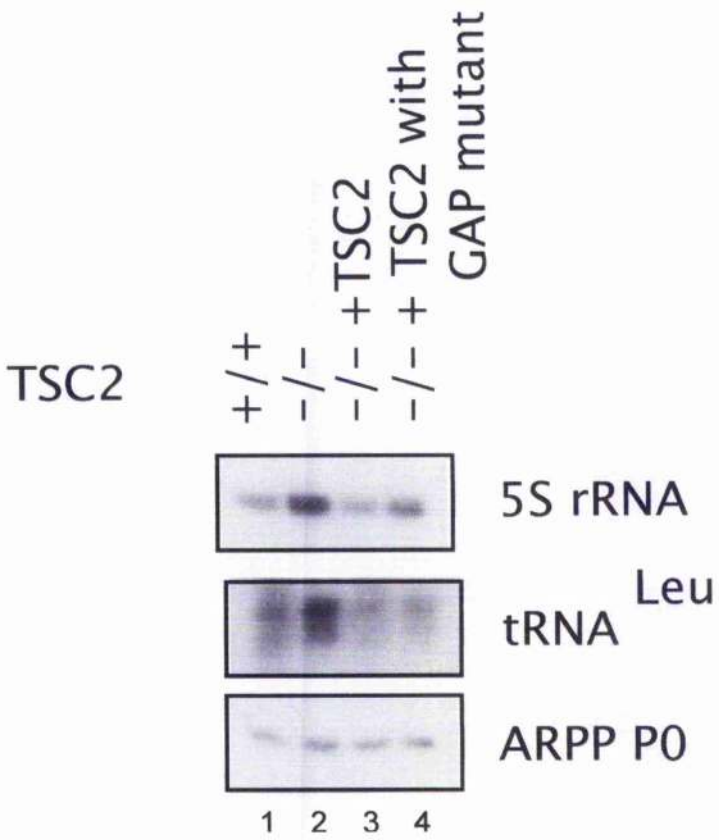


Figure 3.6 TSC2 negative regulates the levels of pol III transcripts
RNA was harvested from wild type MEFs (lane 1), TSC2-/- MEF knockouts (lane 2), TSC2-/- MEF knockouts transiently transfected with wild type TSC2 (lane 3) and TSC2-/- knockout MEFs transiently transfected with TSC2 with GAP mutant (lane 4). RNA was analysed by RT-PCR for the expression of 5S rRNA, $tRNA^{Leu}$, and ARPP P0 (control) (n=1).

3.2.5 Knockout of TSC2 increases the promoter occupancy of TFIIIC, pol III and TFIIIB

The role of TSC2 in the regulation of pol III is accordant with that of mTOR function in this study. Therefore, a ChIP assay was performed to determine if there are any changes in the promoter occupancy of the pol III transcriptional machinery in response to the knockout of TSC2 (figure 3.7). To do this, control $TSC2^{+/+}$ MEFs were compared to $TSC2^{-/-}$ MEF knockouts using antibodies against TFIIIC110, RPC155 (a component of pol III) and Brf1 (a component of TFIIIB). In agreement with the rapamycin ChIP, promoter occupancy of TFIIIB and pol III is greater in the $TSC2^{-/-}$ MEF knockouts compared to the wild type cell line. However, promoter occupancy of TFIIIC is also elevated; this is in contrast to the rapamycin ChIP, where no changes in the promoter occupancy of TFIIIC were observed.

A problem with ChIP assays is that conformational changes or the recruitment of other factors could block the binding of antibodies to their intended target. Thus, the result gained could be a result of epitope masking and not changes in the promoter occupancy on the target protein. In an attempt to overcome this, three antibodies, two against TFIIIC110 and one against TFIIIC220, were used to determine if the change in TFIIIC detection was as a result of its promoter occupancy (figure 3.8). This proved to be the case, as all three antibodies detected a greater presence of TFIIIC in $TSC2^{-/-}$ MEF knockouts compared to the wild type cell line.

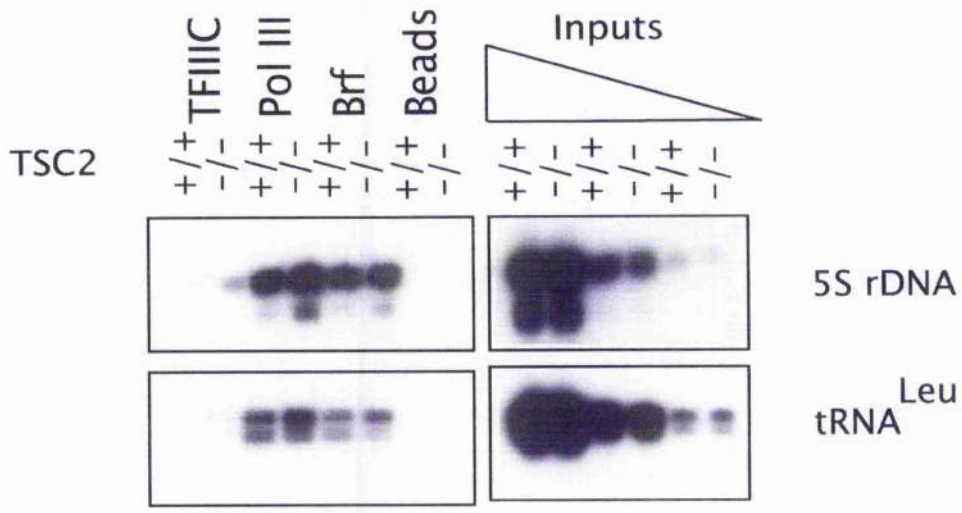


Figure 3.7 TSC2 negatively regulates the promoter occupancy of TFIIIC, pol III and TFIIIB on pol III transcribed genes.
Promoter occupancy of the pol III transcriptional machinery was measured in TSC2-/- MEF knockouts compared to TSC2+/+ MEF wild types. ChIPs were performed using antibodies against TFIIIC110, Brf1 (TFIIIB) and RPC155 (pol III). Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4%), using 5S rRNA and $tRNA^{Leu}$ (n=2).

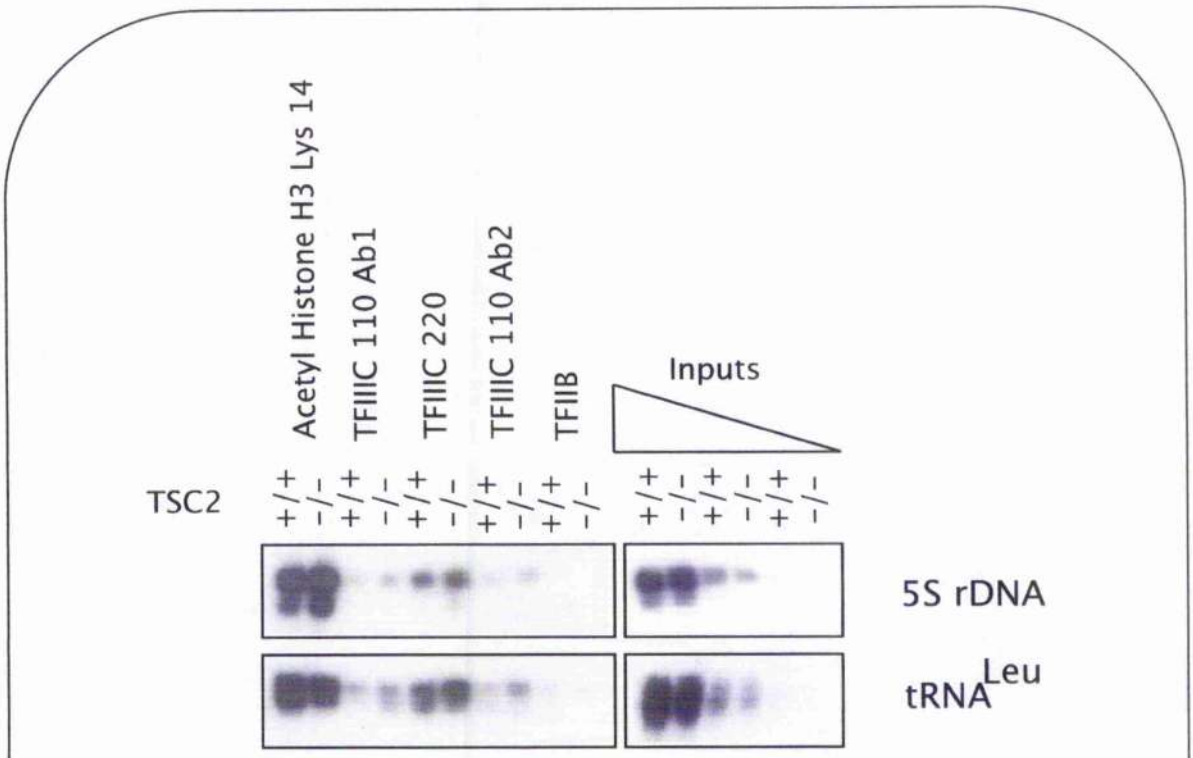


Figure 3.8 Knockout of TSC2 increases the promoter occupancy of TFIIIC on pol III-transcribed genes.

Promoter occupancy of TFIIIC was measure in TSC2-/- MEF knockouts compared to TSC2+/+ MEF wild types. ChIPs were performed using antibodies against acetylated histone H3 Lys14, TFIIIC110, TFIIIC220, TFIIIC110 (recognises different epitope to the first TFIIIC110 antibody) and TFIIA (negative control). Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4%), using 5S rRNA and $tRNA^{Leu}$ (n=2).

3.2.6 Pol III transcriptional activity is blocked specifically by a substrate inhibitor for PKB

PKB phosphorylates and represses TSC2 in response to the presence of growth factors (Cai et al., 2006). To test if PKB has an effect on pol III activity, a substrate peptide inhibitor of PKB was used in an *in vitro* transcription assay (figure 3.9). $tRNA^{Leu}$ transcription was reduced in a dose-dependent manner by the substrate peptide containing the PKB consensus phospho-acceptor site. This response was deemed specific, since a substrate peptide for protein kinase A (PKA) had no effect on $tRNA^{Leu}$ transcription.

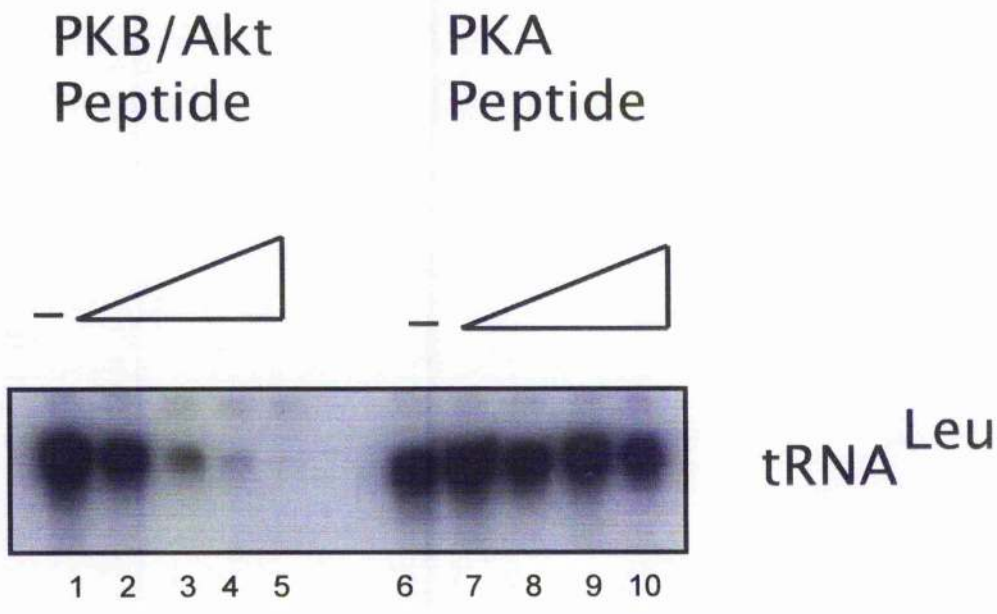


Figure 3.9 Pol III transcription is blocked by a substrate inhibitor for PKB.
In vitro transcription assay was performed using 15μg of HeLa nuclear extract. Pre incubation was carried out with buffer (lanes 1 and 6) or with 10, 20, 30, 40μg of PKB substrate peptide inhibitor (lanes 2-5) or with 10, 20, 30, 40μg of PKA phospho-acceptor peptide (lanes 7-10). 250ng $tRNA^{Leu}$ template was used to transcribe.

3.3 Discussion

Use of rapamycin demonstrates that mTOR is a positive regulator of pol III activity. Further to this, Rheb and TSC2, components of the mTOR-signalling pathway, have been shown to regulate the transcription of pol III-transcribed genes.

mTOR coordinates nutrient availability with cellular growth. This is achieved through its ability to regulate the activity of various components of the cell's translational machinery. Both pol I and pol III are involved in the transcription of genes encoding components of the translational machinery and are often co-ordinately regulated (White, 2005). Previous studies in mammals have shown that pol I is regulated by the mTOR pathway (Hannan et al., 2003; James and Zomerdijk, 2004; Mayer et al., 2004; Zhang et al., 2005). TOR also regulates pol III transcription in yeast (Zaragoza et al., 1998). Evidence provided here shows for the first time that in mammalian cells pol III transcription is regulated by mTOR. This highlights the coordinated regulation of both pol I and pol III in mammalian cells, reinforcing the link between growth factor signalling and ribosome biogenesis.

TSC2 and Rheb are two prominent examples of upstream regulators of mTOR. Rheb in its GTP bound form activates mTOR, whereas TSC2 represses mTOR (Gao et al., 2003). TSC2 represses mTOR through its GAP activity towards Rheb, converting Rheb from an active GTP-bound form into an inactive GDP-bound form (Zhang et al., 2003b). In this study, knockouts of TSC2 compared to wild type $TSC2^{+/+}$ MEF, demonstrated that TSC2 negatively regulates the levels of pol III transcripts, highlighting a novel role for this

tumour suppressor. This is consistent with a study that identified TSC1 and TSC2 as negative regulators of pol I transcription (Zhang et al., 2005), again reinforcing the link between pol I and pol III regulation. The ability of TSC2 to repress pol III transcripts is in part mediated by TSC2 GAP activity. Transient transfection of wild type TSC2 into $TSC2^{-/-}$ MEFs resulted in a recovery of TSC2 function in these cells, as the levels of pol III transcripts were similar to those of the wild type $TSC2^{+/+}$ cells. However, transient transfection of a mutant TSC2 with compromised GAP activity was unable to regain the full activity of TSC2 in repressing the level of pol III transcripts. This highlights the importance of TSC2 GAP activity in the regulation of pol III.

Consistent with TSC2 inhibitory effect, knockdown of Rheb reduces the levels of pol III transcripts. TSC2 inhibits the activity of mTOR through its repression of Rheb, which is a positive regulator of mTOR (Li et al., 2004). TSC2's ability to inhibit mTOR is lost when its GAP activity is lost (Li et al., 2004). The only target identified for TSC2 GAP activity thus far is the GTPase Rheb. Therefore, knockdown of Rheb produces a response similar to that when TSC2 is active, resulting in a decrease in mTOR activity and a repression of mTOR's downstream targets. In this study, knockdown of Rheb resulted in a decrease in the levels of pol III transcripts, demonstrating that the small GTPase Rheb positively regulates pol III. Furthermore, pol I was also shown to be regulated by Rheb, again highlighting the similarity by which both pol I and pol III are regulated. Although previous studies have highlighted the role of mTOR in the regulation of pol I (Hannan et al., 2003; James and Zomerdijk, 2004; Mayer et al., 2004;

Zhang et al., 2005), this is the first time that Rheb has been shown to regulate the level of pol I transcripts.

Use of rapamycin and a TSC2 knockout cell line have shown that both mTOR and TSC2 control the promoter occupancy of the pol III transcriptional machinery. Repression of mTOR results in a decrease in the promoter occupancy of TFIIIB and pol III, whereas TFIIIC remains unaffected. In contrast, promoter occupancy of TFIIIC, along with TFIIIB and pol III, are altered in response to the presence or absence of TSC2. This may reflect the two conditions observed, demonstrating the short and the long-term effects of mTOR regulation. Rapamycin was used to inhibit mTOR over a period of 4 hours, whereas the knockout of TSC2 is a permanent effect. Therefore, further work is needed to determine the long-term mechanism by which the promoter occupancy of TFIIIC is regulated via TSC2.

PKB phosphorylates and inactivates TSC2 in response to the presence of growth factors (Cai et al., 2006). Inactivation of TSC2 promotes the accumulation of active GTP bound Rheb, which in turn activates mTOR (Cai et al., 2006). Manipulation of PKB has shown that it plays an important role in the regulation of the phosphorylation of 4E-BP1, a downstream target of mTOR (Gingras et al., 1998). When a competitive substrate inhibitor for PKB was used in an *in vitro* transcription assay, $tRNA^{Leu}$ transcription was reduced in a dose-dependent manner. This suggests that PKB can regulate the transcription of a $tRNA^{Leu}$ gene *in vitro*. Whether this is through the mTOR pathway or through an alternative mechanism is yet to be determined.

Addition of rapamycin resulted in substantial changes in the promoter occupancy of both TFIIIB and pol III on pol III transcribed genes. In comparison, only mild changes in the promoter occupancy of TFIIIB and pol III were observed when TSC2 knockout cells were compared to the wild type. A potential explanation is that tuberous sclerosis is a largely benign tumour syndrome, with progression to malignancy being very rare. Loss of TSC2 results in the inappropriate activation of the Rheb/mTOR/S6K pathway (Shah et al., 2004). However, the unrestrained activation of S6K results in a negative feedback loop, which inactivates IRS-1, via repression of gene expression and the direct phosphorylation of IRS-1 (Harrington et al., 2006; Manning et al., 2006; Shah et al., 2004; Zhang et al., 2003a). The inactivation of IRS-1 results in the reduced activity of PI3K and its downstream effectors such as PKB (Harrington et al., 2006; Manning et al., 2006; Shah et al., 2004; Zhang et al., 2003a). Repression of the PI3K-PKB pathway in tumours lacking TSC2 contributes to their benign nature (Manning et al., 2006). Previous studies have shown that $TSC2^{-/-}$ MEF knockouts display reduced PKB activity compared to the $TSC2^{+/+}$ MEF wild types (Harrington et al., 2006; Shah et al., 2004; Zhang et al., 2003a). Furthermore, heterozygous $TSC2^{+/-}$ mice also have diminished PKB activity (Manning et al., 2006). Regulation of pol III by PKB was suggested by an *in vitro* transcription assay, where a competitive substrate inhibitor of PKB reduced $tRNA^{Leu}$ transcription in a dose-dependent manner. Therefore, PKB's ability to activate pol III transcription may be compromised in the TSC2 knockout cell line. This may have effects on the expression of pol III transcribed genes and the promoter occupancy of the transcriptional complex.

Chapter 4-Mechanisms of pol III regulation by mTOR

4.1 Introduction

The previous chapter demonstrated that various regulators of mTOR control the expression of pol III-transcribed genes. This is achieved through alterations in promoter occupancy of pol III, TFIIIB and TFIIIC. Therefore, this chapter will focus on the mechanisms that control the recruitment of these transcription factors.

4.1.2 The mTOR complex

mTOR regulates a number of proteins through a direct protein-protein interaction. Phosphorylation of mTOR targets is optimised via scaffold proteins that help facilitate its binding to its target protein. Two such proteins, Raptor (regulatory associated protein of mTOR) and mLst8/GβL (Hara et al., 2002; Kim et al., 2003), positively regulate mTOR and target mTOR to its substrates. The mTOR-Raptor-GβL complex contains a total of 14 WD40 motifs and 23 HEAT motifs (Inoki et al., 2005). Both WD40 and HEAT domains are important for protein-protein interactions, suggesting that this complex serves as a central nexus for TOR signaling. Raptor interacts with its target protein via TOR signalling (TOS) motifs (Nojima et al., 2003). The integrity of TOS motifs is vital for mTOR phosphorylation (Nojima et al., 2003). A single mutation within this motif can result in a dramatic decrease in S6K1 and 4E-BP1 phosphorylation by mTOR in vitro (Nojima et al., 2003).

4.1.2 mTOR regulates transcription and ribosome biogenesis

Work in both yeast and mammals has identified a number of transcription factors that are regulated by TOR/mTOR in a nutrient- and stress-responsive manner (Wullschleger et

al., 2006). Two such examples can be found within the pol I transcriptional machinery; UBF and TIF-1A (Hannan et al., 2003; Mayer et al., 2004). mTOR inhibition by rapamycin results in the dephosphorylation of these two proteins, leading to a decrease in the expression of 45S ribosomal genes (rRNA transcription) (Hannan et al., 2003; Mayer et al., 2004). Phosphorylation controls TIF-1A cytoplasmic and nuclear localisation (Mayer et al., 2004). Under favourable conditions, mTOR promotes the Cdk-mediated phosphorylation of TIF-1A (Mayer et al., 2004). This modification tethers TIF-1A to pol I and retains TIF-1A in the nucleus, promoting rRNA synthesis (Mayer et al., 2004). During unfavourable conditions or after the addition of rapamycin, mTOR is inactive, resulting in a decrease in TIF-1A phosphorylation and sequestration of TIF-1A in the cytoplasm (Mayer et al., 2004). Dephosphorylation is mediated by Protein phosphatase 2A (PP2A), which is active when mTOR is in a repressed state (Mayer et al., 2004). In a different study, UBF was also shown to be a downstream target of mTOR regulation (Hannan et al., 2003). This is mediated by the kinase S6K1, which promotes the transcriptional activity of pol I (Hannan et al., 2003). Other studies have highlighted the importance of other signalling components upstream of mTOR involved in the regulation of pol I. Examples include PTEN, Akt/PKB and PI(3)K (James and Zomerdijk, 2004; Zang et al., 2005). One such study demonstrated that overexpression of PTEN results in changes in the promoter occupancy of a number of SL1 subunits on the promoters of genes transcribed by pol I (Zhang et al., 2005). For a summary of pol I regulation by mTOR, see figure 4.1.

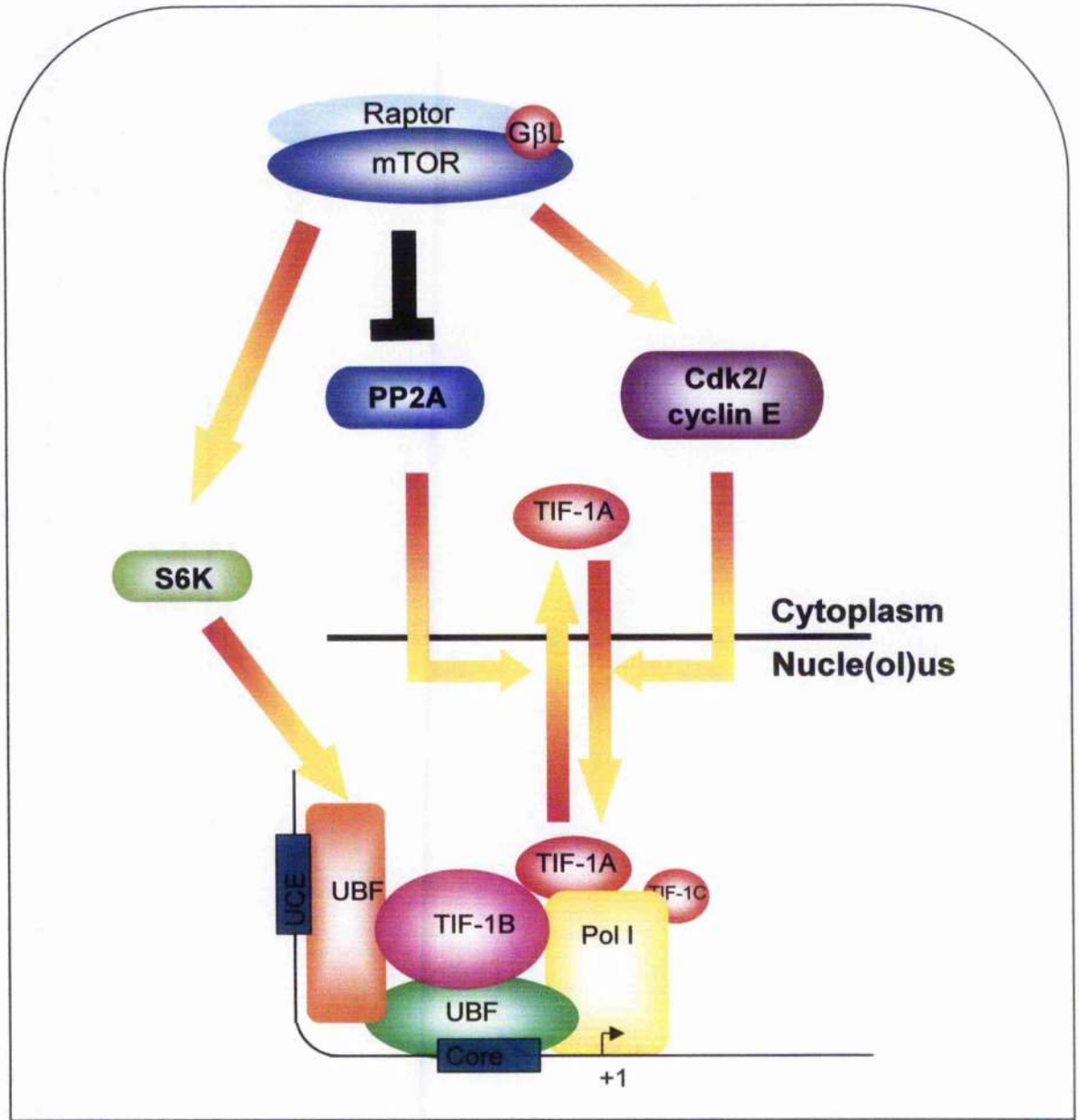


Figure 4.1 mTOR controls the transcription of pol I transcribed genes through the regulation of UBF and TIF-1A.
mTOR promotes phosphorylation of TIF-1A through the activation of cdk2/cyclin E and the repression of PP2A. When TIF-1A is hyperphosphorylated, it is present in the nucleus. When hypophosphorylated it is sequestered in the cytoplasm. mTOR also promotes UBF phosphorylation through the kinase S6K. Phosphorylation of both UBF and TIF-1A promote pol I activity.

Studies in yeast have highlighted a further mechanism by which TOR controls the expression of pol I-transcribed genes. Inhibition of TOR changes the acetylation pattern of histones found on pol I-transcribed genes, identifying a chromatin-mediated mechanism by which TOR regulates transcription (Tsang et al., 2003). The nucleosome is the basic unit of chromatin, consisting of two copies of H2A, H2B, H3 and H4 core histones that bind to 146 base pairs of DNA (Luger et al., 1997). Transcription is inhibited by the nucleosome's ability to hide DNA sequences required for the binding of transcription factors (Edmondson and Roth, 1996). Chromatin can be altered by covalently modifying histone proteins. One such covalent modification, acetylation, is catalysed by histone acetyltransferase (HAT) enzymes (Eberharter and Becker, 2002). Acetylation is a reversible process and is catalysed by histone deacetylases (HDAC)(Eberharter and Becker, 2002). Acetylation of histones is generally associated with transcriptional activation, whereas a lack of acetylation tends to correlate with repression (Eberharter and Becker, 2002).

Addition of rapamycin decreases histone H4 acetylation at rRNA genes through the activation of the HDAC complex Rpd3-Sin3 (Tsang et al., 2003). Further to this, transcription of pol II transcribed ribosomal protein genes is regulated in a similar manner. Again, H4 acetylation decreases in response to rapamycin and the effect is mediated by Rpd3-Sin3 (Rohde and Cardenas, 2003). Therefore, components of the ribosome, either untranslated RNAs or proteins, are regulated in a similar chromatin-mediated fashion. However, in mammals a link between mTOR and alterations in histone acetylation has yet to be determined.

mTOR/TOR controls pol I transcription through phosphorylation and alterations in histone acetylation (Hannan et al., 2003; James and Zomerdijk, 2004; Mayer et al., 2004; Zhang et al., 2005; Zhang et al., 2003a). Such modification can alter promoter occupancy and cellular localization of transcription factors that are required for pol I transcription (Mayer et al., 2004; Tsang et al., 2003; Zhang et al., 2005). For pol III, work in yeast suggested that TFIIIB might be the target of TOR regulation. PP2A was speculated to be the mediator of TOR's pol III regulation (Zaragoza et al., 1998). This chapter will examine these mechanisms to see if pol III in mammalian cells is regulated in a similar manner.

4.2 Results

4.2.1 Levels of pol III, TFIIIC110 and TFIIIB are unaffected by rapamycin treatment

Experiments in the previous chapter have shown that promoter occupancy of TFIIIB and pol III change in response to rapamycin. To see if this is a direct result of changes in abundance of pol III and TFIIIB, western blot analysis was used to measure these factors at the protein level (figure 4.2). No change in the abundance of TFIIIC110, TFIIIB (Brf1) and pol III is detected between rapamycin- and vehicle control- treated cells.

4.2.2 Levels of TFIIIC110 are lower in $TSC2^{-/-}$ MEF knockouts

In contrast to rapamycin treatment, TSC2 was shown to regulate the promoter occupancy of TFIIIC, along with TFIIIB and pol III. Again, western blot analysis was used to measure the presence of these factors at the protein level (figure 4.3). The abundance of both Pol III and TFIIIB (Brf1) are unaffected by the presence or absence of TSC2. However, TFIIIC110 is elevated in the $TSC2^{-/-}$ MEF knockouts compared to the $TSC2^{+/+}$ MEF wild types. To determine if this effect is specific to the 110 subunit of TFIIIC, the abundance of both TFIIIC220 and TFIIIC102 was also measured. As with pol III and TFIIIB, there was no change in the abundance of TFIIIC220 and TFIIIC102 in the $TSC2^{-/-}$ MEF knockouts compared to the $TSC2^{+/+}$ MEF wild types. Therefore, TSC2 specifically regulates the abundance of the TFIIIC110 subunit of TFIIIC.

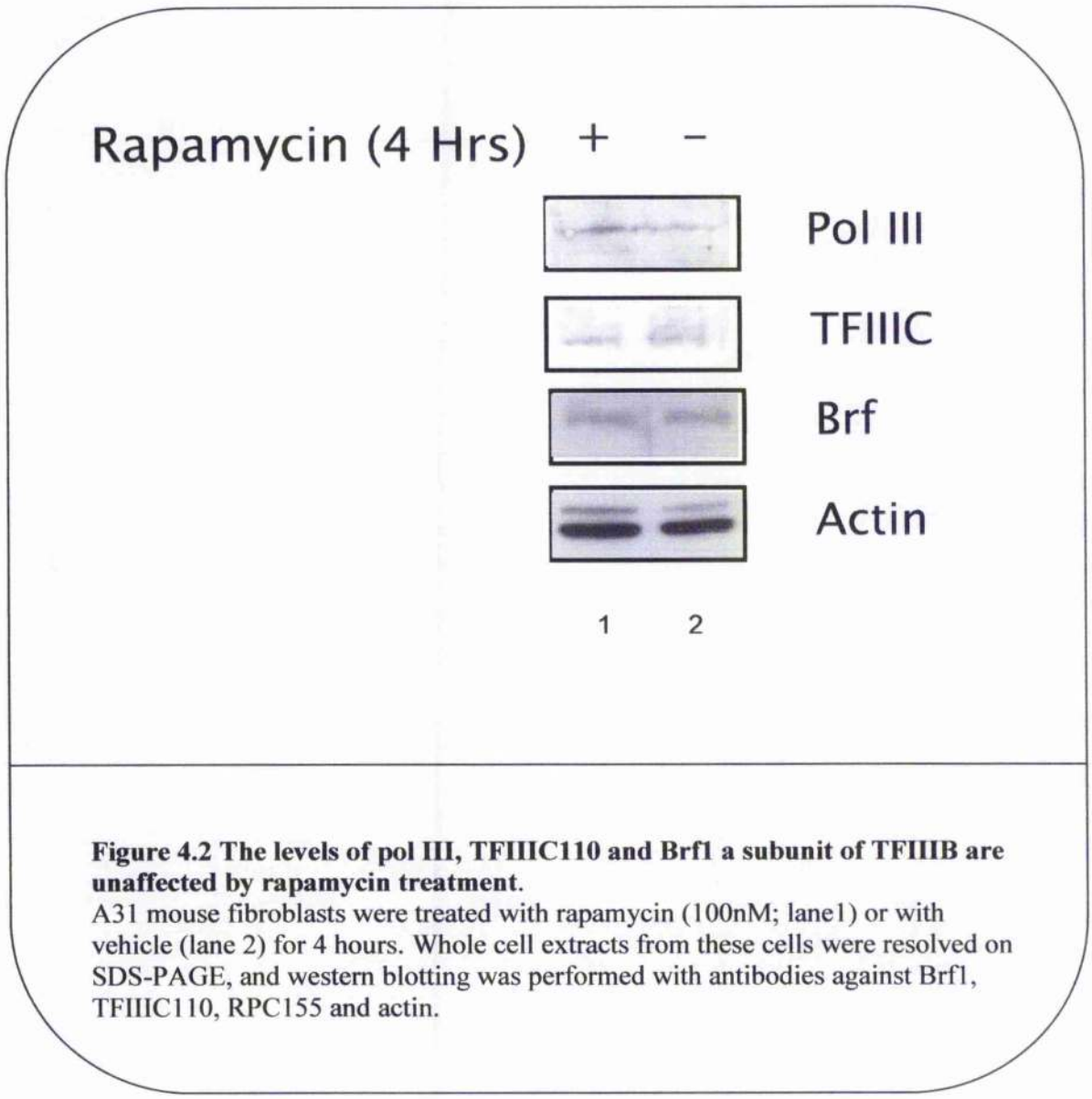


Figure 4.2 The levels of pol III, TFIIIC110 and Brf1 a subunit of TFIIIB are unaffected by rapamycin treatment.
A31 mouse fibroblasts were treated with rapamycin (100nM; lane1) or with vehicle (lane 2) for 4 hours. Whole cell extracts from these cells were resolved on SDS-PAGE, and western blotting was performed with antibodies against Brf1, TFIIIC110, RPC155 and actin.

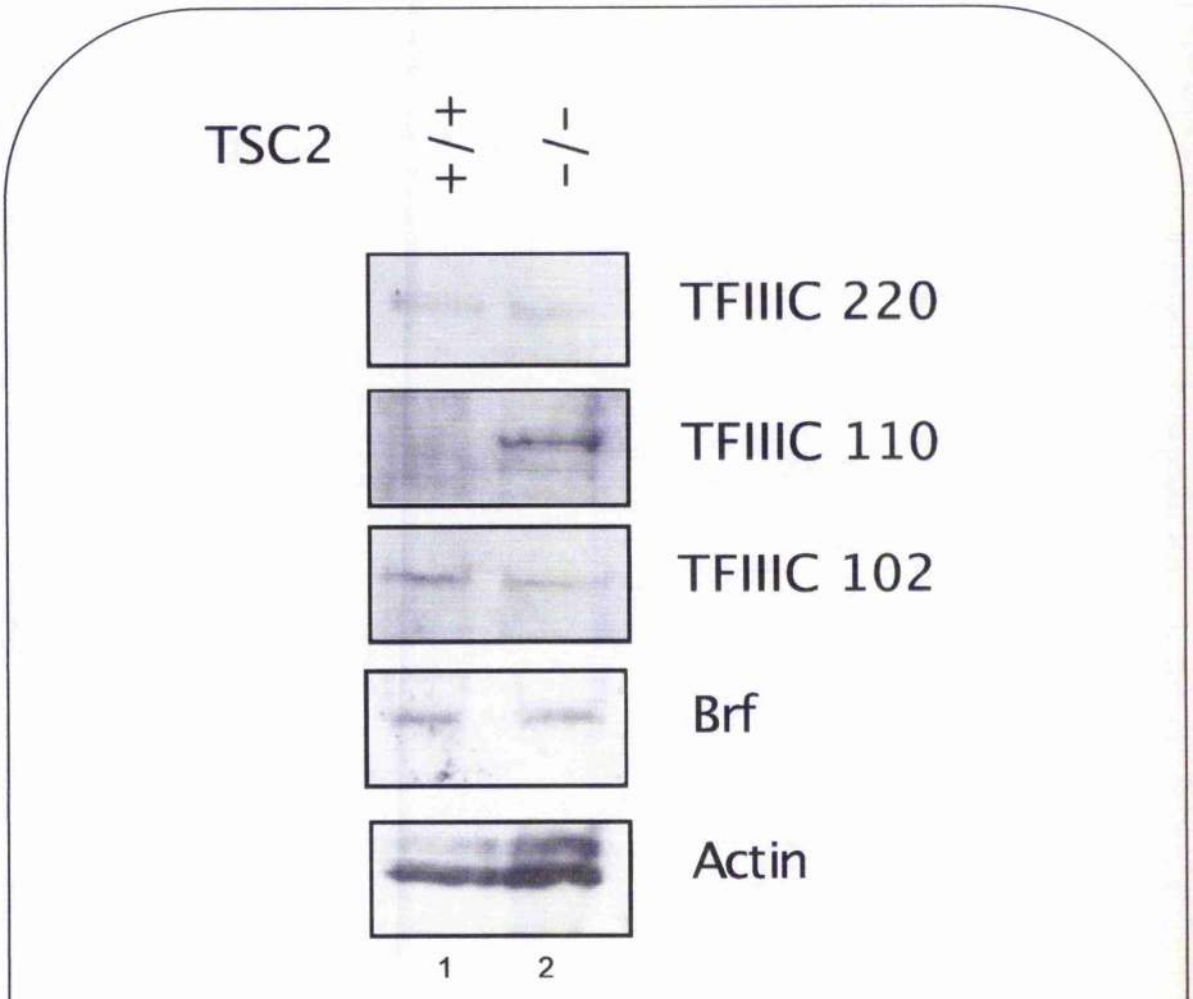


Figure 4.3 The level TFIIIC110 are higher in TSC2$^{-/-}$ MEF knockouts.
Whole cell extracts were harvested from TSC2$^{-/-}$ MEF knockouts (lane 2) and wild type MEFs (lane1). Protein extracts were resolved on SDS-PAGE, and western blotting was performed with antibodies Brf1, TFIIIC110, TFIIIC220, TFIIIC102, RPC155 and actin.

4.2.3 Cellular localization of the pol III transcriptional machinery is unaltered by rapamycin

mTOR has been shown to regulate the cellular localization of a number of transcription factors. One example of that is the pol I transcription factor TIF-1A. TIF-1A is sequestered in the cytoplasm when mTOR is repressed by rapamycin (Mayer et al., 2004). To investigate whether pol III was regulated in a similar fashion, nuclear and cytoplasmic extracts were made from asynchronous mouse fibroblasts treated with either rapamycin or a vehicle-control (figure 4.4). Cellular localization of pol III and its associated transcription factors were looked at via western analysis. The fractionation efficiency of nuclear and cytoplasmic fractions was assessed by the presence of c-Jun and p70/S6k, as they are nuclear and cytoplasmic markers, respectively. As expected, S6K1 was found within the cytoplasmic fraction, whereas c-Jun was found within the nuclear fraction. Addition of rapamycin resulted in a decrease in phosphorylation of the mTOR target S6K, demonstrating the activity of rapamycin in this assay. This is seen with both the phospho-specific S6K antibody and the electromobility shift seen when probing for total S6K. However, predominant nuclear localization of pol III and its associated transcription factors remains unchanged after the addition of rapamycin.

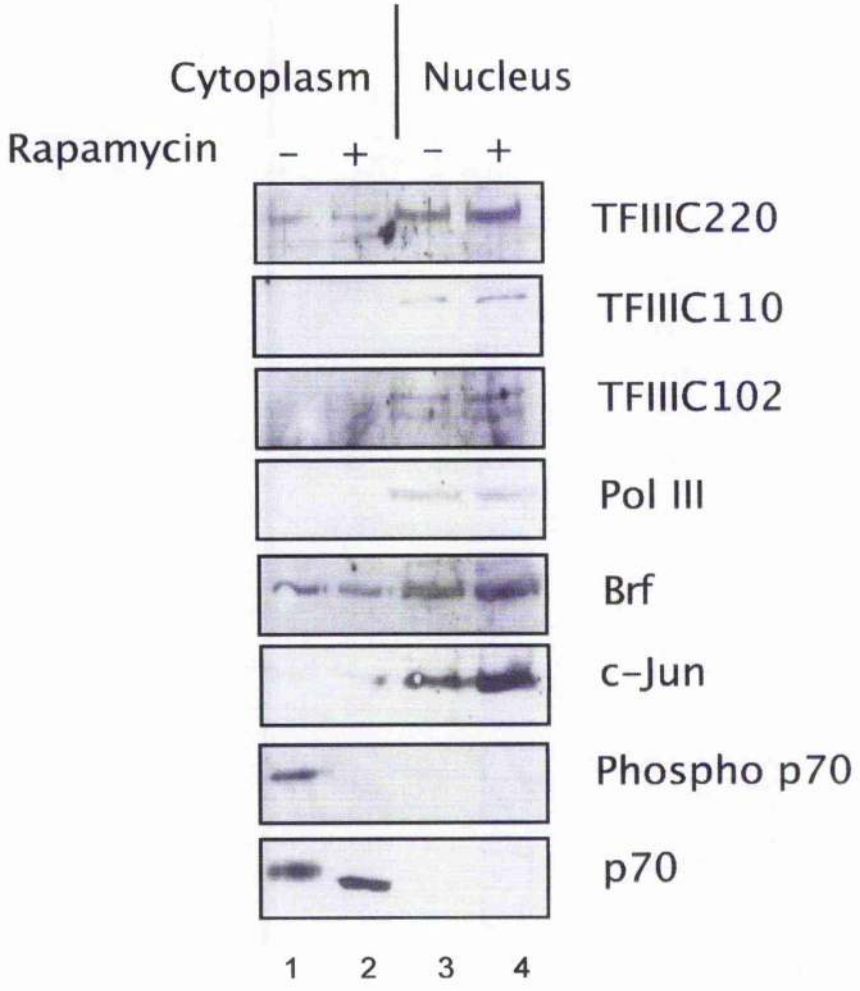


Figure 4.4 mTOR inhibition by rapamycin did not affect the cellular localization of the pol III transcriptional machinery.
Nuclear (lanes 3 and 4) and cytoplasmic (lanes 1 and 2) extracts were harvested from rapamycin (100nM; lanes 2 and 4) or vehicle-treated (lanes 1 and 3) A31 mouse fibroblasts. Protein extracts were resolved on SDS-PAGE, and western blotting was performed with antibodies to Brf1, TFIIIC110, TFIIIC220, TFIIIC102, RPC155, c-Jun, p70/S6K and phosphos p70/S6K (n=2).

4.2.4 Inhibition of PP2A does not relieve inhibition of pol III transcription by rapamycin

There are a number of possible mechanisms that could regulate the transcriptional activity of pol III in response to mTOR. One such mechanism is via protein phosphatase 2A (PP2A), which has been shown to play a role in mTOR regulation of pol I (Mayer et al., 2004) and the mitotic repression of pol III (Fairley et al., 2003). To test for a potential PP2A role, asynchronous mouse fibroblasts were treated with rapamycin. Extracts from these cells were then treated with okadaic acid (100 or 0.2 nM) in an *in vitro* transcription assay. Okadaic acid is known to inhibit a range of protein phosphatases, but displays a greater efficiency for the protein phosphatase 1 (PP1) and PP2A at the nanomolar range (Cohen et al., 1990). Addition of okadaic acid had no effect on $tRNA^{Leu}$ synthesis in both vehicle-treated control cells and rapamycin-treated cells. Calyculin A was also tested, which is another potent inhibitor of PP1 and PP2A (Cohen et al., 1990). Again, the addition of calyculin A (100 or 0.2 nM), as with okadaic acid, had no effect on $tRNA^{Leu}$ transcription in both the vehicle-treated control cells and the rapamycin-treated cells (figure 4.5).

Okadaic acid and calyculin A have both previously been shown to inhibit pol III transcription in mitotic extracts (Fairley et al., 2003). Therefore, a control experiment was performed to demonstrate that okadaic acid and calyculin A are active at the concentrations used in this assay (figure 4.5). Both phosphatase inhibitors repressed the transcription of $tRNA^{Leu}$ in mitotic extracts, as previously reported (Fairley et al., 2003).

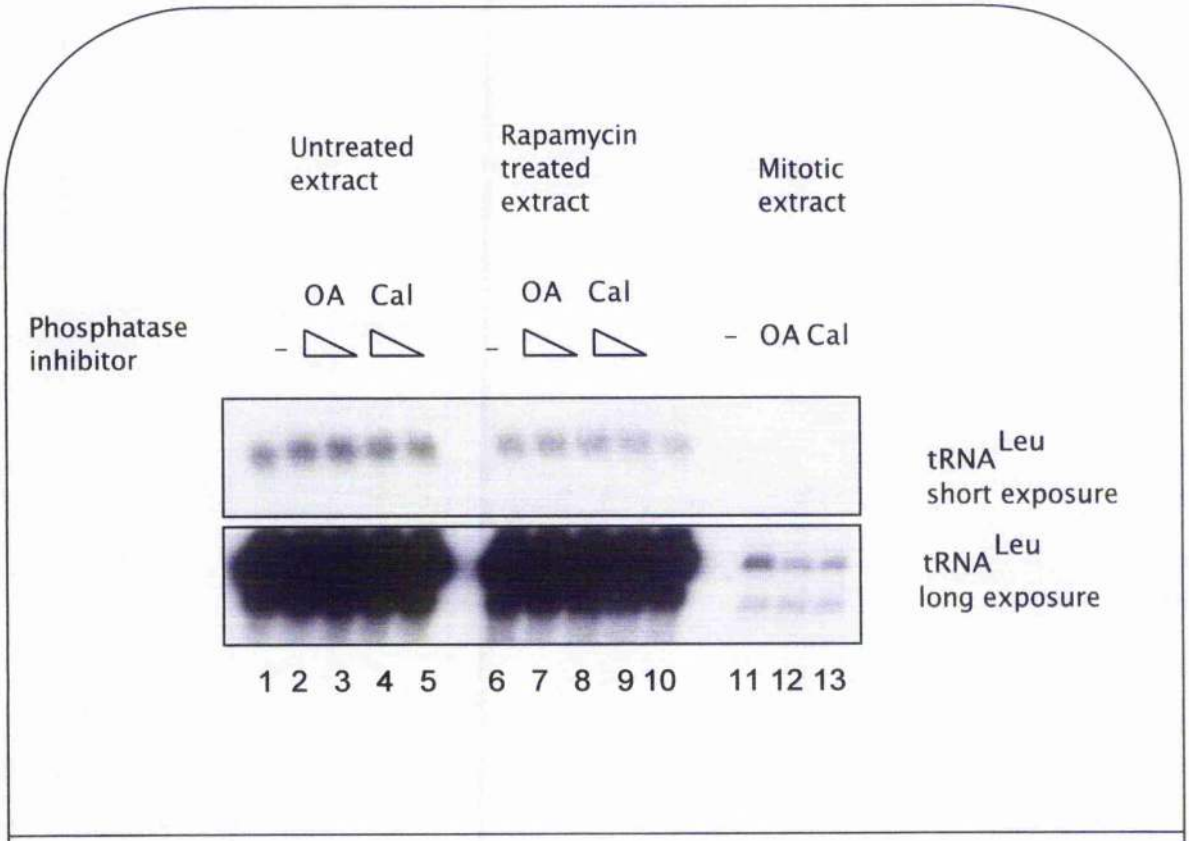


Figure 4.5 Inhibition of PP2A does not relieve inhibition of pol III transcription by rapamycin.
A31 mouse fibroblasts were treated with rapamycin (100nM; lanes 1-5) or with vehicle (lanes 6-10) for 4 hours. Whole cell extracts derived from these cells were pre-incubated for 10 minutes at 30°C in the presence of buffer alone (lanes 1 and 6), 100 (lanes 2 and 7) or 0.2nM (lanes 3 and 8) okadaic acid and 100 (4 and 9) or 0.2 nM (lanes 5 and 10) calyculin A. Mitotic HeLa nuclear extracts were pre-incubated for 10 minutes at 30°C with buffer alone (lane 11), 100nM okadaic acid (lane 12) or 100nM calyculin A (lane13).

4.2.5 Knockdown of endogenous S6K1 and S6K2 had no effect on the level of pol III transcripts

Small interfering RNA (siRNA) was used to determine if either S6K1or S6K2 have a role in the expression of pol III-transcribed genes. RNA was obtained from a previous study carried out by Richard Lamb's group at The Institute of Cancer Research, London (Harrington et al., 2006). siRNA directed against S6K1 and S6K2 results in a near complete knock-down of S6K1 and a 70-80% knock-down of S6K2 (Harrington et al., 2006). RT-PCR analysis revealed that knockdown of both S6K1 and S6K2 had no effect on the levels of 5S rRNA, $tRNA^{Leu}$ and B2 transcripts compared to the control transfection (figure 4.6). As there is no effect on the levels of pol III transcripts, two positive controls were used to demonstrate that the knockdown of both S6K1 and S6K2 could have an effect on transcription. Knockdown of S6K1 results in a decrease in the levels of p21 mRNA. This is consistent with previous reports that have shown that S6K1 is a known regulator of p21 expression (Gu et al., 2000). The knock-down of both S6K1 and S6K2 resulted in a decrease in the levels of the pol I-transcribed, pre-rRNA. Again, this is in agreement with previous reports that show that pol I transcriptional activity is regulated by S6K (Hannan et al., 2003; Zang et al., 2005).

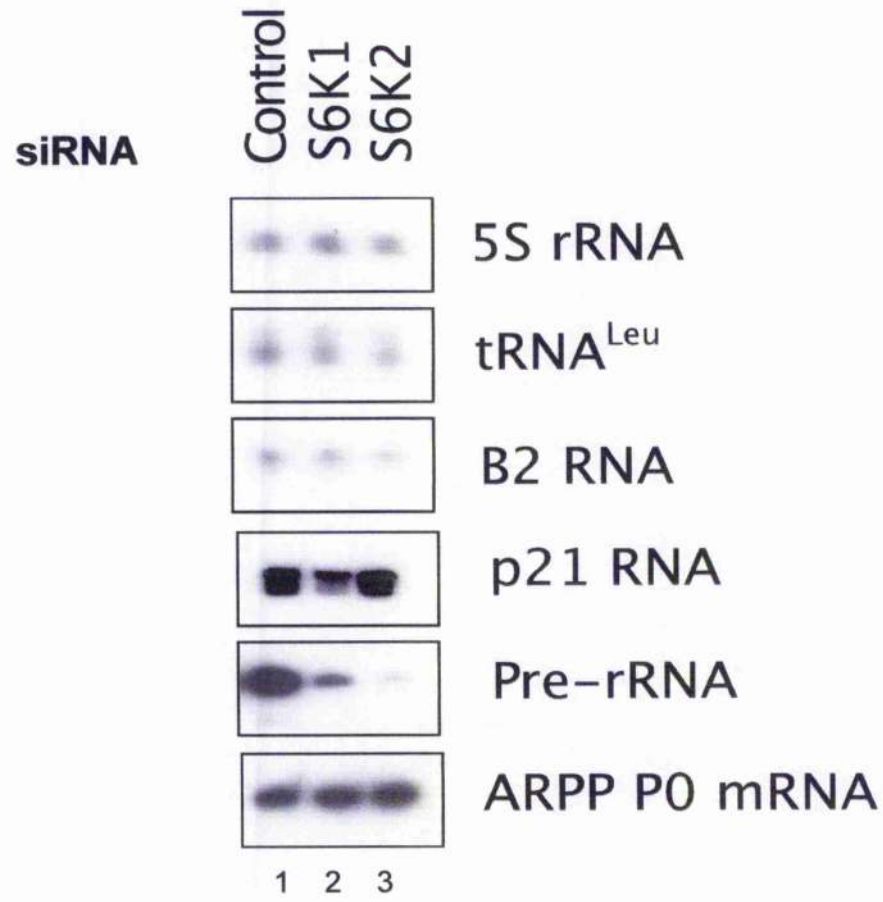


Figure 4.6 Knockdown of endogenous S6K1 and S6K2 in MEF cells by siRNA had no effect on the levels of pol III transcripts.
Knockdown of S6K1 and S6K2 in MEF cells was performed in a previous study (Harrington et al., 2006). MEFs were transfected with S6K1 (lane 2), S6K2 (lane 3) and scrambled siRNA (control; lane 1). RNA was harvested and analysed by RT-PCR for the expression of 5S rRNA, $tRNA^{Leu}$, B2, p21, pre rRNA and ARPP P0 (control) (n=2).

4.2.6 mTOR inhibition by rapamycin decreases the level of acetylated histone H3 on pol III-transcribed genes

As previously stated, work in yeast has shown that TOR can control the pattern of histone acetylation, regulating gene expression by a chromatin-mediated process (Rohde and Cardenas, 2003; Tsang et al., 2003). However the link between mammalian mTOR and changes in histone acetylation has not been made. Therefore, ChIP assays were performed to determine if this was a potential mechanism for the regulation of pol III in response to rapamycin (figure 4.7). Formaldehyde cross-linked chromatin was prepared from asynchronously grown fibroblasts treated with a vehicle or rapamycin for 4 hours. To assess changes in histone acetylation, an anti-acetyl H3 and an anti-acetyl H4 antibody were used. A pol III antibody was used as a positive control. PCR analysis showed that pol III promoter occupancy was reduced in the rapamycin-treated cells compared to the vehicle-treated cells. In parallel to this, there is a decrease in the levels of histone H3 acetylation. This is in contrast to histone H4, which maintains a similar level of acetylation in both the rapamycin and vehicle-treated cells.

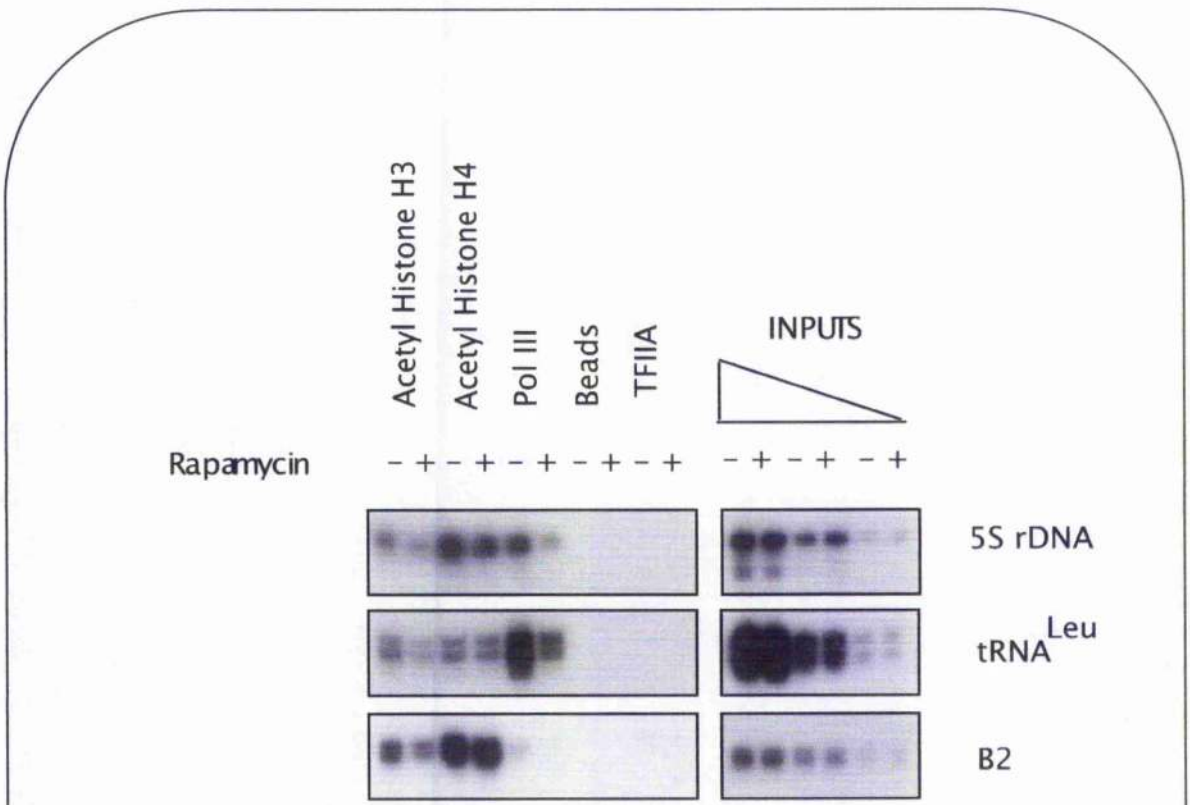


Figure 4.7 mTOR inhibition by rapamycin decreases the level of acetylated histone H3 on pol III-transcribed genes

Levels of histone acetylation at pol III-transcribed genes were measured in A31 mouse fibroblasts treated with either rapamycin (100nM) or vehicle control for 4 hours. ChIPs were performed using antibodies against acetylated histone H3, acetylated histone H4, RPC155 (pol III) and TFIIA (negative control). Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4% of input), using 5S rRNA, $tRNA^{Leu}$ and B2 primers.

Similar experiments were performed with the $TSC2^{-/-}$ MEF knockouts compared to the $TSC2^{+/+}$ wild type MEFs to see if a similar pattern of histone acetylation is observed (figure 4.8). Again, to assess changes in histone acetylation an anti-acetyl H3 and an anti-acetyl H4 antibody were used. Two additional antibodies were used that specifically recognise acetylation of histone H4 Lys5 and histone H3 Lys14, to see if these specific lysine residues were affected by the presence or absence of TSC2. Consistent with rapamycin treatment, acetylation of histone H3 was elevated when TSC2 is not present, whereas acetylation of histone H4 is unaffected. In parallel, acetylation of histone H4 Lys5 and histone H3 Lys14 appear to be unaffected by the presence or absence of TSC2. However, the effect on histone acetylation is only mild and is not pronounced as the effect observed after rapamycin treatment.

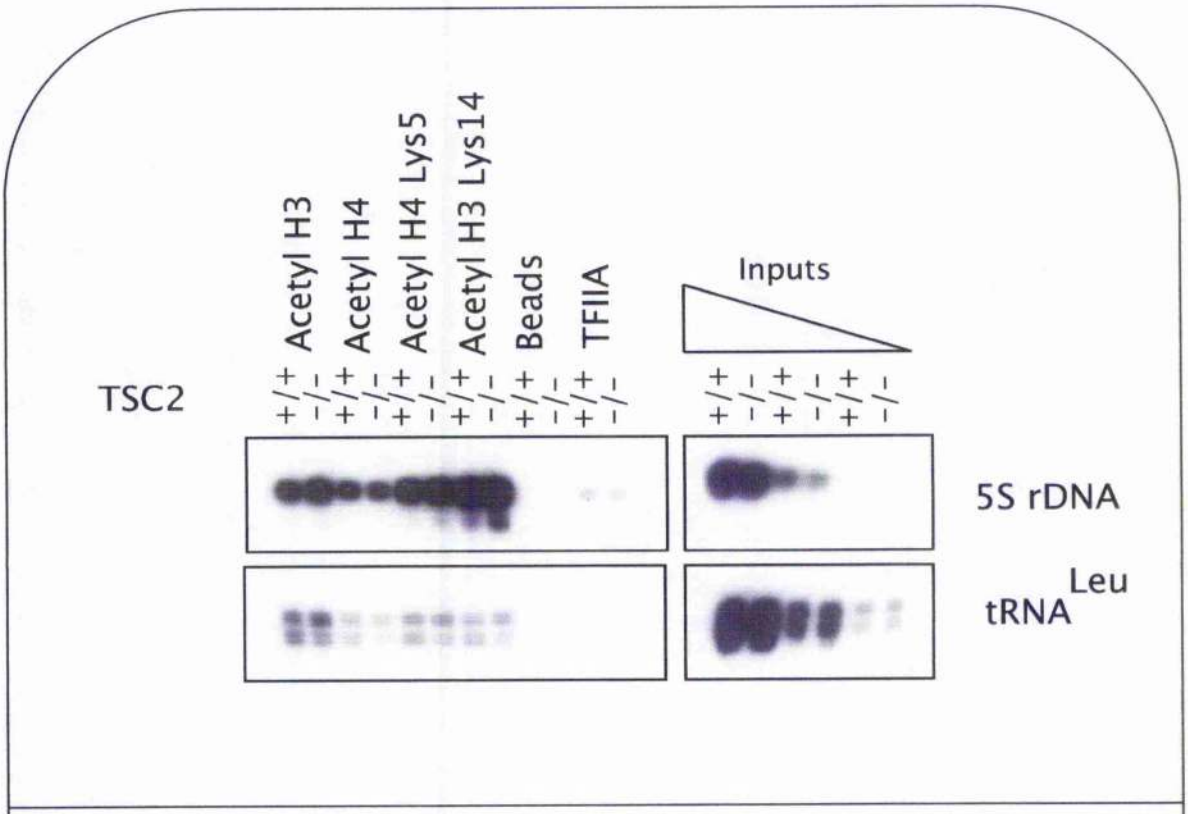


Figure 4.8 Knockout of TSC2 increases the level of acetylated histone H3 on pol III-transcribed genes

The level of histone acetylation was measured in TSC2-/- knockouts MEF compared to TSC2+/+ wildtypes MEF. ChIPs were performed using antibodies against acetylated histone H3, acetylated histone H4, acetylated histone H4 lysine 5, acetylated histone H3 lysine 14 and TFIIA (negative control). Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4% of inputs), using 5S rRNA and $tRNA^{Leu}$ primers.

4.2.7 Raptor co-immunoprecipitates with TFIIIC

Previous work has shown that phosphorylation of TFIIIC110 decreases after the addition of rapamycin (Emma Graham, personal communication). This led to the hypothesis that phosphorylation of TFIIIC110, by an as yet unidentified kinase, regulated pol III's transcriptional activity in an mTOR-dependent fashion. I found that pol III transcription is not regulated by S6K, as the knockdown of both S6K1 and S6K2 had no effect on the expression of pol III-transcribed genes. This led to the hypothesis that mTOR might itself be directly phosphorylating TFIIIC110. To test this idea, TFIIIC110 was immunoprecipitated from extracts derived from asynchronous mouse fibroblasts, treated with either a vehicle or rapamycin. Western analysis of the immunoprecipitation was performed to test whether any factors from the mTOR complex co-immunoprecipitated with TFIIIC110 (figure 4.9). Raptor co-immunoprecipitates with TFIIIC110, whereas in immunoprecipitaions carried out with pre-immune serum no Raptor was detected.

4.2.8 mTOR is associated with pol III transcribed genes

To determine if mTOR is present on pol III-transcribed genes, ChIP assays were performed (figure 4.10). Formaldehyde cross-linked chromatin was prepared from asynchronously growing cells. An antibody against mTOR was used to detect its presence. TFIIA and beads were used as negative controls. mTOR is present on both 5S rRNA and $tRNA^{Leu}$ genes, but not associated with the pol II-transcribed gene encoding cyclin D2. Conversely, as expected, TFIIA is associated with the cyclin D2 gene, whereas it is not associated with either 5S rRNA or $tRNA^{Leu}$ genes. Therefore, the association of mTOR with pol III-transcribed genes is specific.

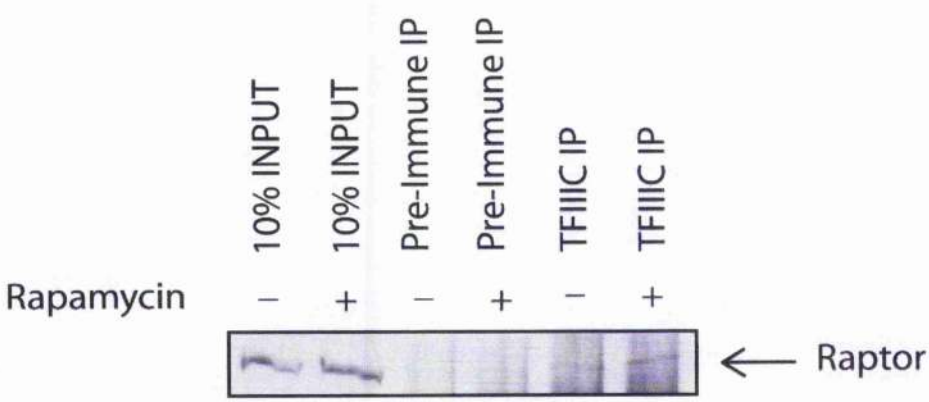


Figure 4.9 Rapator co-immunoprecipiates with TFIIIC.
A31 mouse fibroblasts were treated with Rapamycin (100nM) or vehicle control for 4 hours. Whole cell extracts from these cells were immunoprecipitated with anti-TFIIIC110 antibody or pre-immune (control). Precipitates were resolved by SDS-PAGE and immunoblotted with anti-raptor antibody.

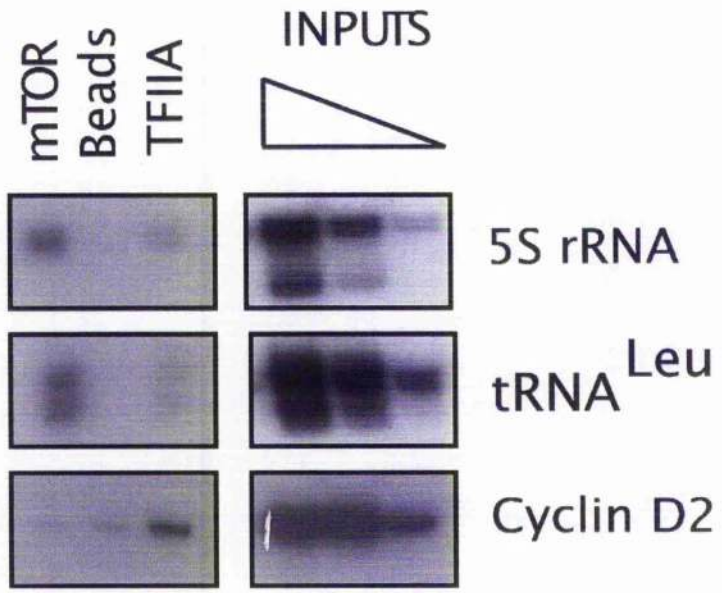


Figure 4.10 mTOR is associated with pol III-transcribed genes.
ChIPs were performed on asynchronous A31 mouse fibroblast cells using antibodies against acetylated mTOR and TFIIA (negative control). The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4% of inputs), using 5S rRNA and $tRNA^{Leu}$ gene primers.

4.3 Discussion

These results suggest that mTOR directly regulates the expression of pol III-transcribed genes through a direct interaction with pol III's transcriptional machinery. The data also suggest that there are a number of differences in the regulation of pol I and pol III by mTOR.

Repression of mTOR by rapamycin resulted in no change in the abundance of TFIIIC110, TFIIIB and pol III at the protein level. Consistent with this, knockout of TSC resulted in no change in the abundance of pol III, TFIIIB, TFIIIC220 and TFIIIC102. However, TFIIIC110 was elevated in the $TSC2^{-/-}$ MEF knockouts, compared to the wild type $TSC2^{+/+}$ MEFs. As previously suggested, this may reflect the two conditions observed, demonstrating a short and a long-term effect of mTOR regulation. Rapamycin was used to inhibit mTOR over a period of 4 hours, whereas the knockout of TSC2 is a permanent effect. This would be consistent with a previous study where 24 hours of rapamycin treatment resulted in a decrease in abundance of UBF (a pol I transcription factor). By comparison, rapamycin treatment for 3 hours had no effect on the abundance of UBF (Hannan et al., 2003). Therefore, a prolonged period where mTOR activity is altered can change the abundance of specific transcription factors.

The abundance of pol III transcription factors is elevated in a number of transformed cells. More specifically, TFIIIC has been shown to be elevated in fibroblasts that were transformed by SV40 and the polyomavirus (Felton-Edkins and White, 2002). In ovarian tumours there is also an increase in the abundance of TFIIIC, which is thought to promote the expression of pol III-transcribed genes (Winter et al., 2000). Both studies

demonstrated that all five components of TFIIIC were overexpressed in the conditions observed (Felton-Edkins and White, 2002; Winter et al., 2000). However, the results gained here are more consistent with the model where TFIIIC activity is regulated by the relative abundance of just one TFIIIC subunit, TFIIIC110 (Kovelman and Roeder, 1992; Sinn et al., 1995). As previously stated, TFIIIC was reported to be present in two forms, with or without TFIIIC110, dubbed TFIIIC2a and TFIIIC2b, respectively (Hoeffler et al., 1988; Kovelman and Roeder, 1992). TFIIIC2a is able to support transcription whereas TFIIIC2b is not (Hoeffler et al., 1988; Kovelman and Roeder, 1992). When TSC2 is knocked out you see an increase in the abundance of TFIIIC110. This could promote a rise in the ratio of active TFIIIC2a compared to inactive TFIIIC2b, which increases the expression of pol III-transcribed genes.

TFIIIC2a and TFIIIC2b have similar DNA-binding affinities (Hoeffler et al., 1988; Kovelman and Roeder, 1992). Therefore, loss of TFIIIC110, should not affect the promoter occupancy of the remaining subunits of TFIIIC (TFIIIC220, TFIIIC102, TFIIIC90 and TFIIIC63). However, as shown in the previous chapter, the promoter occupancy of both TFIIIC220 and TFIIIC110 change in response to the presence or absence of TSC2. This may suggest an alternative mode of TFIIIC regulation, whereby the promoter occupancy of more than one TFIIIC subunit is affected by the presence of TSC2.

Previous studies in yeast have established that TOR can regulate the activity of transcription factors through their cellular localization (Beck and Hall, 1999; Mayer et

al., 2004). For example, TOR controls the transcription factor Gln3 through its cellular localization (Beck and Hall, 1999). Under nutrient rich conditions, TOR phosphorylates Ure2p, a cytoplasmic protein that binds and sequesters Gln3 in the cytoplasm. Rapamycin induces the dephosphorylation of Ure2p which disrupts its interaction with Gln3, allowing Gln3 to translocate into the nucleus (Beck and Hall, 1999). In mammalian cells, the cellular localization of TIF-1A is controlled by mTOR (Mayer et al., 2004). During rapamycin repression, TIF-1A translocates to the cytoplasm, thus inhibiting the expression of pol I-transcribed genes (Mayer et al., 2004). In this study, the cellular localization of pol III and its transcription factors appear to be unaffected by the presence or absence of rapamycin. Therefore, mTOR probably does not control the expression of pol III transcripts genes via the cellular localization of its transcriptional machinery.

Zaragoza *et al.* found that pol III and probably TFIIIB are under control of TOR in yeast (Zaragoza et al., 1998). These authors speculated that TOR regulates TFIIIB and pol III through PP2A (Zaragoza et al., 1998). This was based on a previous yeast study, which found that mutations in the regulatory subunit of PP2A reduced the transcription of tRNA through inhibition of TFIIIB and pol III at the non-permissive temperature (Van Zyl et al., 1992). Furthermore, PP2A is very rapidly activated by rapamycin (Hartley and Cooper, 20002). Therefore, Zaragoza *et al.* proposed that inhibition of TOR activates PP2A, which dephosphorylates and activates a repressor of TFIIIB and/or pol III (Zaragoza et al., 1998). Other reports in yeast have highlighted the importance of Maf1 (a repressor of pol III-transcribed genes) as a key component for rapamycin repression of

pol III-transcribed genes (Oficjalska-Pham et al., 2006; Roberts et al., 2006; Upadhya et al., 2002a). Maf1 becomes dephosphorylated after the addition of rapamycin (Oficjalska-Pham et al., 2006; Roberts et al., 2006). This allows Maf1 to accumulate in the nucleus where it binds to pol III-transcribed genes and inhibits their expression (Oficjalska-Pham et al., 2006; Roberts et al., 2006). Dephosphorylation is carried out by PP2A (Oficjalska-Pham et al., 2006), which reinforces the claims made by Zaragoza et al.. However, I found that repression of pol III activity by rapamycin was not reversed by the PP2A inhibitors okadaic acid and calyculin A. Pol III activity in asynchronously grown cells, which are not treated with rapamycin, are not affected by repression of PP2A. This is in contrast to mitotic cells, where inhibition of PP2A represses pol III (Fairley et al., 2003).

mTOR regulation of rRNA gene transcription requires S6K (Hannan et al., 2003). Expression of a constitutively active, rapamycin-insensitive mutant of S6K stimulated rRNA gene expression and rescued rapamycin repression of rRNA gene transcription (Hannan et al., 2003). As pol I and pol III are often regulated by similar mechanisms (White, 2005), regulation of pol III by mTOR was expected to require S6K. However, knockdown of S6K1 and S6K2 had no effect on the presence of pol III transcripts. Consistent with previous studies (Hannan et al., 2003; Mayer et al., 2004; Zhang et al., 2005), knockdown of S6K1 and S6K2 resulted in a decrease in the expression of the pol I transcript. Therefore, knockdown of S6K1 and S6K2 represses pol I but not pol III activity, highlighting a difference in the regulation of these two polymerases. This does not rule out S6K as a regulator of pol III, as there is a high level of redundancy between S6K1 and S6K2 (Pende et al., 2004; Shima et al., 1998). For example, deletion of the

S6K1 gene leads to a compensatory increase in the expression of S6K2 (Shima et al., 1998). Thus, future studies will require simultaneous knockdown of both S6K1 and S6K2 to determine if redundancy is masking the effect of S6K1 and S6K2 on pol III activity.

Histone acetylation plays an important role in the regulation of gene expression and maintenance of chromatin structure. An increase in histone acetylation is often correlated with the transcriptional activation of the gene it is associated with. A decrease in acetylation is often correlated with transcriptional repression. Previous studies in yeast have shown that the expression of both pol I and pol II transcribed ribosomal genes are regulated in a chromatin-mediated manner by TOR (Rohde and Cardenas, 2003; Tsang et al., 2003). Both studies highlighted the importance of histone H4 acetylation (Rohde and Cardenas, 2003; Tsang et al., 2003). However, in this present study, acetylation of histone H3 changed in response to the inhibition of mTOR or when TSC2 was knocked out. The change in histone H3 acetylation is consistent with that of the transcriptional response. For example, inhibition of mTOR causes a decrease in the presence of pol III transcripts, which is correlated with a decrease in histone H3 acetylation. Knockout of TSC2, results in an increase in pol III activity that is seen in parallel with an increase in histone H3 acetylation. Therefore, histone H3 acetylation appears to change in parallel to the expression of pol III-transcribed genes.

Although histone acetylation may provide a potential mechanism for the control of pol III by mTOR, it cannot be the whole story. This is demonstrated by the *in vitro* transcription

assay performed in the previous chapter (figure 3.2). This assay is performed on a naked DNA template, where no histones are incorporated into the template. Extracts from cells pre-treated with rapamycin showed that the inhibition of mTOR reduced pol III's transcriptional activity on this naked DNA template. Therefore, mTOR can control pol III through a chromatin-independent mechanism.

Phosphorylation plays an important role in mTOR's ability to regulate transcription factors. As stated previously, phosphorylation of pol I transcription factors by mTOR is in part mediated by the kinase S6K (Hannan et al., 2003). mTOR can also directly phosphorylate other transcription factors. An example of this is the transcription factor STAT1 (signal transducer and activator of transcription-1), which binds directly to mTOR (Kristof et al., 2003). STAT1 belongs to a family of transcription factors that remain in a latent form in the cytoplasm. They become active following phosphorylation, leaving the cytoplasm and entering the nucleus where they regulate a number of target genes (White, 2001a). This is consistent with mTOR being predominantly localized in the cytoplasm. However, a small fraction of mTOR is found within the nucleus at steady state (Kim and Chen, 2000). Therefore, mTOR could regulate pol III activity through direct interaction with components of the pol III transcriptional machinery. Indeed, Raptor, a component of the mTOR complex, has been found to co-immunoprecipitate with TFIIIC in this study. Raptor acts as a scaffold protein that facilitates mTOR's interaction with its substrates. Oshiro *et al.* demonstrated that raptor is required absolutely for the mTOR-catalysed phosphorylation of 4E-BP1 *in vitro* (Hara et al., 2002). Thus, raptor could mediate an interaction of TFIIIC with mTOR. Although

mTOR itself has not been co-immunoprecipitated with TFIIIC as yet, mTOR was found to be associated with pol III transcribed genes in a ChIP assay. This creates the possibility that mTOR regulation of pol III could be through a direct interaction with a component of the pol III transcriptional machinery.

Chapter 5-The role of acetylation in the regulation of pol III-transcribed genes

5.1 Introduction

The previous chapter highlighted a potential role for histone acetylation in the regulation of pol III. More specifically, acetylation of histone H3 was shown to change in response to mTOR activity. Therefore, this chapter will look more closely at acetylation and the role that it plays in the regulation of pol III transcription.

5.1.1 Chromatin

Chromatin structure can be altered so that it produces a more open or closed state, whereby it is more or less permissive to transcription. Regulation of chromatin structure is mediated through a number of different covalent modifications that exit on histone tails; modifications include phosphorylation, ubiquitination, ADP-ribosylation, methylation and acetylation. Two domains have been identified that bind to either acetylated or methylated histone tails marks; these are known as bromodomains and chromodomains, respectively (Cruz et al., 2005). Proteins containing bromo- or chromodomains can alter nucleosomal structure by utilising energy from ATP hydrolysis or via the addition of new covalent modifications. This can be achieved through their direct activity or the recruitment of other proteins that catalyse these processes. These observations led to the histone code hypothesis, where different combinations of covalent modifications on histone tails provide binding sites for a variety of proteins that convert chromatin to either a repressed or active state (Strahl and Allis, 2000).

The most characterized modification is acetylation, catalysed by histone acetyltransferase (HAT) enzymes. This modification is a reversible process as a number of histone

deacetylases (HDAC) have been characterized (DeRuijter et al., 2003). Acetylation is generally associated with activation, whereas a lack of acetylation tends to correlate with repression. The action of HDACs and HATs is not restricted to histones, as a large number of other proteins are acetylated; many of these proteins are transcription factors. For example, the HAT TIP60 acetylates UBF, a component of the pol I transcriptional machinery (Halkidou et al., 2004). TIP60 is localized to sites of newly synthesized rRNA (Halkidou et al., 2004). Its presence is correlated with an increase in the levels of histone H4 acetylation (Halkidou et al., 2004), a marker of transcriptional activation. Thus, acetylation can control expression of genes through acetylation of both histone tails and the acetylation of transcription factors.

5.1.2 Chromatin and pol III

Effects of histones on pol III transcription cannot easily be defined, as they vary considerably between its various templates. Middle repetitive genes such as B2 and Alu are strongly repressed by chromatin, whereas the opposite can be said for tRNA, where chromatin appears to have a negligible effect (Russanova et al., 1995). 5S rRNA templates have been shown to respond to alterations in levels of the linker histone H1 (Bouvet et al., 1994; Kandolf, 1994). During Xenopus embryo development, the injection of mRNA encoding histone H1 results in considerable repression of oocyte 5S rRNA gene expression (Bouvet et al., 1994). The opposite effect is observed when histone H1 is depleted using ribozymes, resulting in an increase in the expression of these genes (Bouvet et al., 1994; Kandolf, 1994). Neither of these histone manipulations has

an effect on transcription of tRNA and somatic rRNA genes (Bouvet et al., 1994; Kandolf, 1994).

Alus are repetitive elements, constituting a major part of the human genome, numbering 1,090,000. This potentially creates an extensive sink for transcription factors and a tremendous transcriptional potential. However, this potential is not realised, as Alu transcripts are usually very low in tissue and cultured cells (Liu et al., 1994; Sinnett et al., 1992). Much of this can be attributed to chromatin-mediated repression. Histone octamers in both reconstituted systems and in native chromatin are positioned over Alu transcriptional start site and A block regions of their promoters (Englander and Howard, 1995; Englander et al., 1993). This decreases the accessibility of DNA to transcription factors, diminishing the expression of Alus. In HeLa cells ~99% of potentially active Alu repeats are silenced by chromatin, whereas the same HeLa chromatin preparations showed that the vast majority of tRNA and 5S rRNA genes remain unaffected by chromatin (Russanova et al., 1995). The B2 middle repetitive family (a murine tRNA derived gene) is also transcriptionally repressed by chromatin (Russanova et al., 1995). Depletion of H1 increases B2 transcription by ~17-fold in 3T3 cells (Russanova et al., 1995). By comparison, in HeLa cells removal of H1 only gives a ~2 fold increase in Alu transcription (Russanova et al., 1995). This demonstrates that between families chromatin-mediated repression of pol III-transcribed genes can vary.

In comparison to Alus, tRNA gene transcription seem to be relatively unaffected by chromatin-mediated repression. Expression of the SUP4 $tRNA^{Tyr}$ gene in yeast can even

take place when nucleosome-positioning signals are fused to its promoter, so that the predicted nucleosome would incorporate the transcriptional start site and the A block (Morse et al., 1992). This is the nucleosome positioning observed in Alu repeats. However, nucleosomes were only observed at these predicted points when the SUP4 $tRNA^{Tyr}$ gene is transcriptionally inactivated by mutations in the B box of the internal promoter (Morse et al., 1992). Thus, transcriptionally active tRNA genes are able to override nucleosome positioning *in vivo*. Furthermore, an in vitro reconstituted human tRNA chromatin template was relieved of chromatin-mediated repression by highly purified TFIIIC (Kundu et al., 1999). Other studies have changed histone composition by removing histone H1 from murine chromatin, or histone H4 from yeast. Neither of these manipulations had a significant effect on tRNA transcription (Han and Grunstein, 1988; Russanova et al., 1995).

Promoter integrity of the U6 gene is important in its ability to overcome nucleosome-mediated repression. Deletion of the histone H4 gene in yeast does not promote the expression of U6 transcription (Marsolier et al., 1995). However, when the same deletion was made with a mutant U6 promoter, there is an increase in the level of U6 transcription (Marsolier et al., 1995). Characterization of the promoter has revealed that a functional B block is required to overcome nucleosome repression, due to its ability to bind TFIIIC (Burnol et al., 1993b). This interaction allows TFIIIC to displace nucleosomes from the template, allowing transcription (Burnol et al., 1993a). Furthermore, binding of TFIIIC to the promoter leads to remodelling of chromatin found on U6 genes in an ATP-

dependent fashion (Shivaswamy et al., 2004). Chromatin remodelling facilitates a more open state allowing transcription to take place.

TFIIIC has been proposed to have a histone-specific acetyltransferase activity. Previous studies have demonstrated that TFIIIC can acetylate both free and nucleosomal H3 and H4, as well as nucleosomal H2A. This activity was attributed to the subunits TFIIIC110, TFIIIC220 (Kundu et al., 1999) and TFIIIC90 (Hsieh et al., 1999a; Kundu et al., 1999) of the TFIIIC complex. TFIIIC90 predominately acetylates both free and nucleosomal H3, preferentially acetylating lysine 14 on the N-terminal tail (Hsieh et al., 1999a). Therefore, recruitment of TFIIIC promotes transcription of its target genes through the acetylation of histone tails.

TFIIIC plays an important role in relieving chromatin-mediated repression of pol III-transcribed genes. This is perhaps of no surprise, as in most cases TFIIIC is the first component of the pol III transcriptional machinery to make contact with DNA. This initial step may therefore convert pol III-transcribed genes from closed to an open state, allowing expression of the gene to take place.

To look at the role of TFIIIC as a HAT, a TFIIIC110 inducible cell line will be used. As previously stated, TFIIIC exits in two forms, with or without the TFIIIC110 subunit, dubbed TFIIIC2a and TFIIIC2b, resectively (Kovelman and Roeder, 1992; Sinn et al., 1995). Although both forms produced identical footprints, the complex without TFIIIC110 was unable to support transcription (Hoeffler et al., 1988; Kovelman and

Roeder, 1992). This led to the model where the activity of TFIIIC could be regulated by its interaction with TFIIIC110. Interaction might be controlled by the abundance of TFIIIC110. This potential mechanism was suggested as expression of TFIIIC110 is diminished under low serum conditions in HeLa cells, whereas TFIIIC220 levels are unchanged (Sinn et al., 1995). In parallel, low serum conditions also led to a decrease in the ratio of active TFIIIC2a to inactive TFIIIC2b (Sinn et al., 1995). Experiments in chapter 4 have shown that expression of TFIIIC110 is elevated in $TSC2^{-/-}$ MEF knockouts, whereas the levels of TFIIIC220 and TFIIIC102 are unaltered. This was correlated with a rise in the expression of pol III-transcribed genes and an increase in histone H3 acetylation. Thus, selective expression of TFIIIC110 may provide a mechanism for controlling TFIIIC activity. Furthermore, TFIIIC110 along with TFIIIC220 and TFIIIC90 have been proposed to have HAT activity (Hsieh et al., 1999a; Kundu et al., 1999).

Experiments in this chapter will focus on the role of acetylation in the regulation of pol III transcription. This will be done using the drug trichostatin A (TSA), a potent HDAC inhibitor (Jung, 2001). Leading on from this, a TFIIIC110-inducible cell line was used to see if overexpression of TFIIIC led to an increase in the expression of pol III-transcribed genes. In parallel, changes in histone acetylation were observed on these genes.

5.2 Results

5.2.1 TSA promotes pol III transcriptional activity

To determine the general effect of acetylation, the HDAC inhibitor trichostatin A (TSA) was used. TSA is thought to mimic the acetyl-lysine side chain and thereby inhibit HDACs (Jung, 2001). Asynchronous A31 mouse fibroblasts treated with TSA for 6, 12, 24 and 48 hours. RT-PCR analysis revealed a bi-phasic response, where transcript levels of $tRNA^{Leu}$ and B2 had two peaks at 6 and 24 hours compared to the vehicle treated control cells (figure 5.1). At 12 hours the levels of both $tRNA^{Leu}$ and B2 were similar to that of the vehicle control treated cells. 5S rRNA transcripts do not display an increase after 6 hours of TSA treatment. Instead, expression is only elevated after 24 hours of TSA treatment. This is probably due to the fact that the primers are designed for the mature 5S rRNA, and the half-life of 5S rRNA is relatively long. By comparison, B2 has a relatively short half-life (Bladon et al., 1990). The primers for $tRNA^{Leu}$ are designed to detect the unspliced $tRNA^{Leu}$ precursor; this allows the detection of newly synthesised $tRNA^{Leu}$ (Gomez-Roman et al., 2003). Thus, B2 and $tRNA^{Leu}$ provide a more direct indication of transcriptional output compared to 5S rRNA. These effects are specific, as levels of the pol II transcribed control ARPP P0 mRNA remain unaltered by TSA.

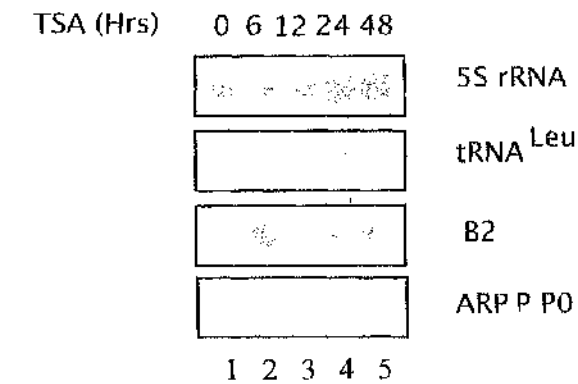


Figure 5.1. TSA produces two peaks of pol III transcripts.
RNA was harvested from A31 mouse fibrobroblasts treated with TSA (100nM) for 6, 12, 24 and 48 hours (lanes 2-5) or vehicle control hours (lane 1). RNA was analysed by RT-PCR for the expression of 5S rRNA, B2, $tRNA^{Leu}$, and ARPP P0 mRNA(control).

As previously stated, *in vitro* transcription assays are performed on naked DNA, where no histones are incorporated onto the template. Therefore, performing this assay with extracts treated with TSA should demonstrate if TSA has an effect independent of histone acetylation. Asynchronously grown A31 mouse fibroblasts were treated with TSA for 6, 12 and 24 hours. An *in vitro* transcription assay was performed using extracts derived from these cells and extracts from vehicle-treated control cells to measure pol III's activity in response to TSA. After 6 hours of TSA treatment there was an increase in pol III transcriptional activity (figure 5.2). However, the activity of pol III after 12 and 24 hours of TSA treatment was comparable to the vehicle-treated control.

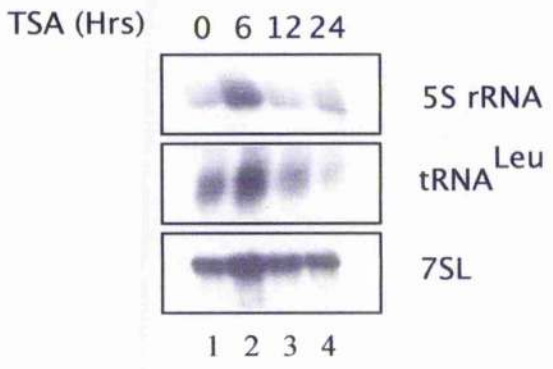


Figure 5.2. TSA increases pol III's transcriptional activity only after 6 hours of treatment.

Whole cell extracts were made from A31 mouse fibroblasts treated with vehicle (lane 1) or TSA (200nM) for 6, 12 and 24 hours (lanes 2-4). 15μg of extract was used in an in vitro transcription assay using a $tRNA^{Leu}$ (250ng), 5S rRNA (250ng) and 7SL (250ng) gene template.

5.2.2 TSA promotes the recruitment of TFIIIB and pol III to the promoter

The addition of TSA caused an increase in the expression of pol III-transcribed genes. Past studies have shown that the promoter occupancy of transcription factors is elevated during TSA treatment (Kwon et al., 2006). Therefore, a ChIP assay was used to determine the effects of acetylation on the promoter occupancy of the pol III transcriptional machinery. Formaldehyde cross-linked chromatin was prepared from asynchronously growing A31 mouse fibroblasts treated for 0, 6, 12 and 24hrs with 200nM TSA. Antibodies against RPC155 (a component of pol III), TFIIIC110 and the TFIIIB subunit Brf1 were used to determine their occupancy. To assess changes in histone acetylation, an anti-acetyl H3 and an anti-acetyl H4 antibody were used. TFIIA was used as a negative control, as it is not present on pol III-transcribed genes. PCR analysis showed that the promoter occupancy of pol III and TFIIIB (Brf1) were elevated after 6 hours of TSA treatment on $tRNA^{Leu}$ and 5S rRNA genes (figure 5.3). In contrast, the promoter occupancy of TFIIIC remains unchanged after the addition of TSA. In parallel to changes in the promoter occupancy of TFIIIB and pol III, an increase in the acetylation of histone H3 and H4 were observed. Thus, TSA increases the promoter occupancy of TFIIIB and pol III and the acetylation of histone H3 and H4 on pol III-transcribed genes.

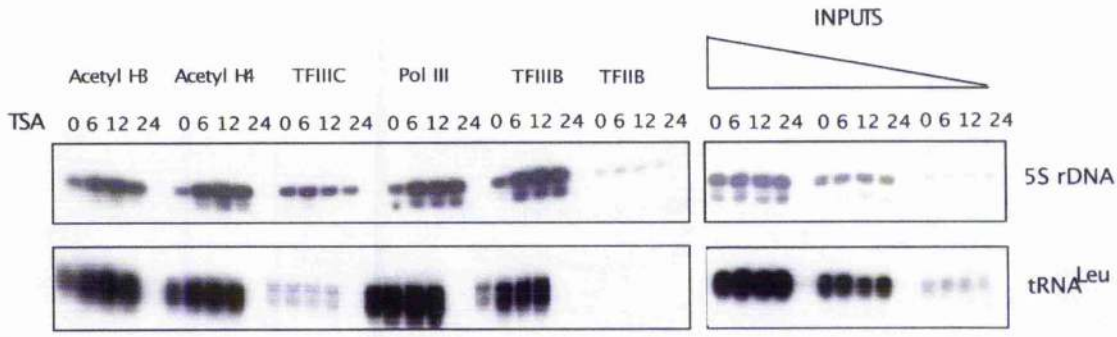


Figure 5.3 TSA promotes the promoter ooccupancy of TFIIIB, pol III, but not TFIIIC

Promoter occupancy of pol III, TFIIIB and TFIIIC, along with histone acetylation was measured in TSA-treated A31 mouse fibroblasts. Cells were treated with TSA (200nM) for 6, 12 and 24 or with vehicle. ChIPs were performed using antibodies against acetylated histone H3, acetylated histone H4, TFIIIC110, Brf1 (a component of TFIIIB), RPC155 (pol III) and TFIIIB (negative control). Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4% of input), using 5S rRNA and tRNA^{Leu} primers (n=2).

5.2.3 TSA does not affect the level of the pol III transcriptional machinery

The increase in promoter occupancy of TFIIIB and pol III in response to TSA might be as a direct result of changes in abundance of pol III and TFIIIB. Therefore, western blot analysis of extracts treated for 0, 6, 12 and 24hrs with 200nM TSA was used to measure the levels of these transcription factors. No change in the abundance of TFIIIC110, TFIIIB (Brf1) and pol III (RPC155) is detected after the addition of TSA (figure 5.4). By comparison, levels of acetylated histone H3 and acetylated histone H4 are elevated after 6 hours of TSA treatment.

5.2.4 P300 and TIP60 mildly promote pol III activity

TSA promotes the accumulation of acetylated proteins through the repression of HDACs. However, HATs are required for acetylation to take place in the first instance. To investigate the role of HATs in the expression of pol III-transcribed genes, recombinant HATs PCAF, p300 and TIP60 were used in an *in vitro* transcription assay. Recombinant p300, TIP60 and PCAF were pre-incubated for 15 minutes with A31 whole cell extract. An *in vitro* transcription assay was then subsequently performed. p300 and TIP60 mildly promoted the expression of 7SL and $tRNA^{Leu}$ genes in a dose-dependent manner, whereas PCAF if anything had a repressive effect on pol III activity (figure 5.5). This would suggest that p300 and TIP60 stimulate the activity of pol III through the acetylation of non-histone targets, as *in vitro* transcription assays are performed on naked DNA.

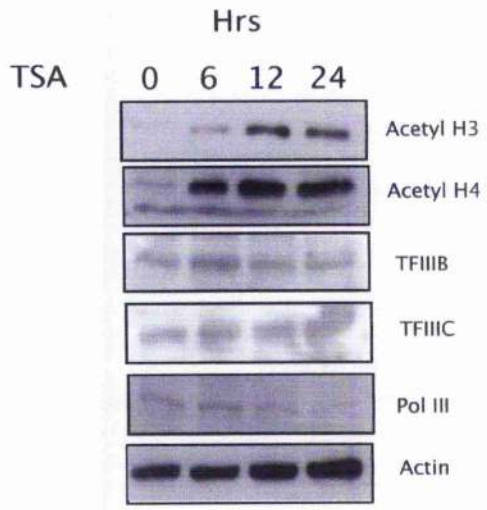


Figure 5.4. Levels of pol III, TFIIIC110 and the Brf1 subunit of TFIIIB are unaffected by TSA treatment.
A31 mouse fibroblasts were treated with TSA (200nM) for 6, 12, and 24 hours (lanes 2-4) or with vehicle (lane 1) for 4 hours. Whole cell extracts from these cells was resolved on SDS-PAGE. Western blotting was performed with antibodies against Brf1, TFIIIC110, RPC155, acetyl H3, acetyl H4 and actin.

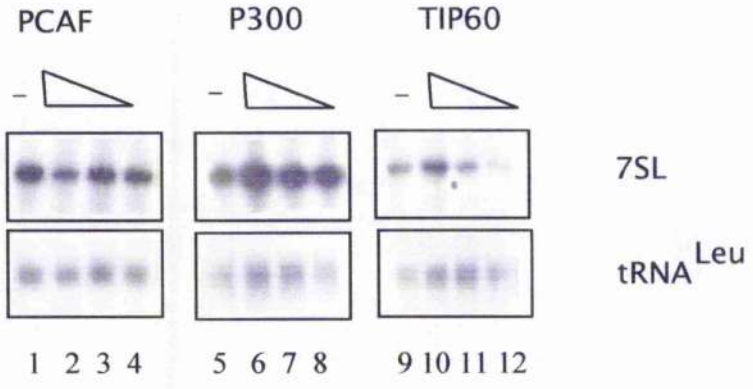


Figure 5.5. HATs p300 and TIP60 have a mild stimulatory effect on pol III's transcriptional activity 15µg of whole cell extracts were made from A31 mouse fibroblasts were pre-treated with 2, 1, and 0.5µl of HATs PCAF (lanes 2-4), TIP60 (lanes 10-12) and p300 (lanes 6-8) or with vehicle control (lanes 1, 5 and 9). An *in vitro* transcription assay was performed using a $tRNA^{Leu}$ (250ng) and 7SL (250ng) template to measure the effects of these HATs.

5.2.5 Induction of HA-TFIIIC110 had no effect on pol III transcriptional activity

To look at the role of TFIIIC as a HAT, a TFIIIC110 inducible cell line was used. This was obtain from co-worker Fiona Innes (Innes et al., 2006). TFIIIC110 cDNA was sub-cloned into pTRE2hyg, which carries a doxycycline-responsive promoter. This was stably transfected into HeLa cells, where addition of doxycyline induces the expression of HA-TFIIIC110. Expression of HA-TFIIIC110 was confirmed through western blot analysis (figure 5.6). An empty vector control cell line was used to demonstrate that the band detected is HA-TFIIIC110 and not a non-specific effect of doxycyline.

An *in vitro* transcription assay was performed to see if the overexpression of HA-TFIIIC110 had an effect on the activity of pol III. Extract made from doxycyline-treated HA-TFIIIC110-inducible and control cell lines were used in an *in vitro* transcription assay. No change in the transcription of 7SL and $tRNA^{Leu}$ genes was observed using cell extracts from the inducible cell line before or after the addition of doxycyline (figure 5.7).

In vitro transcription assays are performed on naked DNA templates, where no histones are incorporated into the template. Therefore, an effect of histone acetylation on transcription would be lost in such an assay. RT-PCR analysis was performed to see the effect of HA-TFIIIC110 induction *in vivo*. The levels of both 5S rRNA and $tRNA^{Leu}$ transcripts are unaffected by the overexpression of HA-TFIIIC110 (figure 5.8). However, TFIIIC110 transcripts are elevated when HA-TFIIIC110 is induced.

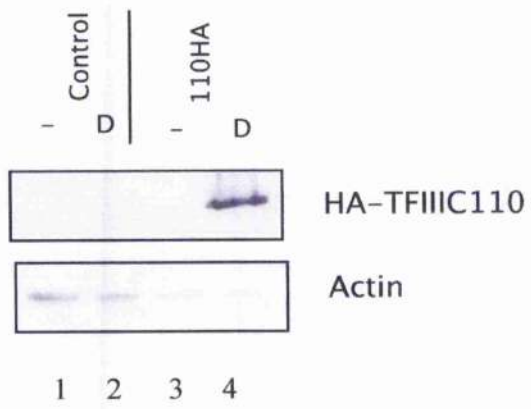


Figure 5.6. HA-TFIIIC110 induction results in an increase of HA-TFIIIC110 at the protein level

The abundance of HA-TFIIIC110 was measured using a HA-TFIIIC110 inducible cell line (lanes 3 and 4). Cells were either untreated (-) (lanes 1 and 3) or induced with doxycyclin (+) (lanes 2 and 4). A control cell line containing an empty vector was also used (lanes 1 and 2). Protein extracts from these cells were resolved on SDS-PAGE, and western blotting was performed with antibodies to the HA tag and actin.

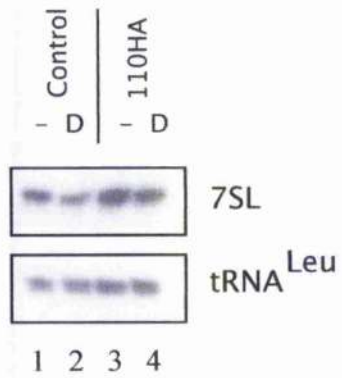


Figure 5.7. Induced expression of HA-TFIIIC110 had no effect on pol III transcriptional activity.

The effect of TFIIIC110 on the level of pol III transcription was measured using a HA-TFIIIC110 inducible cell line (lanes 3 and 4). Cells were either untreated (-) (lanes 1 and 3) or induced with doxycyclin (+) (lanes 2 and 4). A control cell line containing an empty vector was also used (lanes 1 and 2). Whole cell extracts were made from these treated cell lines. 15µg of extract was used in an in vitro transcription assay using a $tRNA^{Leu}$ (250ng) and 7SL (250ng) template.

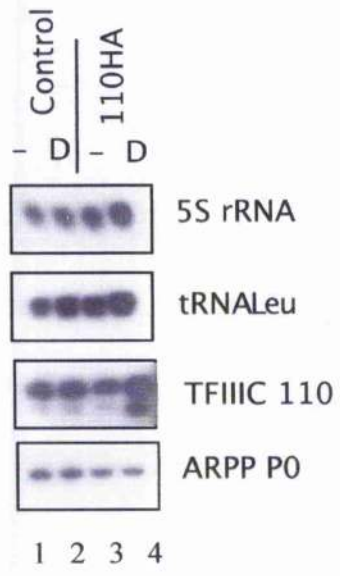


Figure 5.8. Induced expression of HA-TFIIIC110 had no effect on the levels of pol III transcripts

The effect of TFIIIC110 on the level of pol III transcripts was measured using a HA-TFIIIC110 inducible cell line (lanes 3 and 4). Cells were either untreated (-) (lanes 1 and 3) or induced with doxycycline (+) (lanes 2 and 4). A control cell line containing an empty vector was also used (lanes 1 and 2). RNA was analysed by RT-PCR for the expression of 5S rRNA, $tRNA^{Leu}$, TFIIIC110 and ARPP P0 mRNA(control).

5.2.6 Induction of HA-TFIIIC110 had no effect on the recruitment of the pol III transcriptional machinery

ChIP analysis was used to verify that exogenous HA-TFIIIC110 is present on pol III-transcribed genes. Formaldehyde cross-linked chromatin was prepared from the HA-TFIIIC110 inducible cell line along with the control cell line grown either in the presence or absence of doxycyline. Antibodies against TFIIIC110, TBP and the HA epitope were used. A TFIIB antibody was used as a negative control. HA-TFIIIC110 is detected on $tRNA^{Leu}$ and 5S rRNA, but not at the gene encoding TFIIIC220, which was used as a pol II-transcribed negative control (figure 5.9). However, the promoter occupancy of total TFIIIC110 (both HA-TFIIIC110 and endogenous TFIIIC110 combined) remains unaffected by the induction of HA-TFIIIC110 on 5S rRNA and $tRNA^{Leu}$ genes.

ChIP experiments were also performed to determine if the induction of HA-TFIIIC110 had any effect on the promoter occupancy of pol III and TFIIIB. Antibodies against RPC155 (a subunit of pol III), TFIIIC110 and Brf (a component of TFIIIB) were used. Promoter occupancy of pol III and TFIIIB are unaffected by the induction of HA-TFIIIC110 on 5S rRNA and $tRNA^{Leu}$ genes (figure 5.10). Consistent with the previous ChIP, promoter occupancy of total TFIIIC110 remained unchanged after the induction of HA-TFIIIC110.

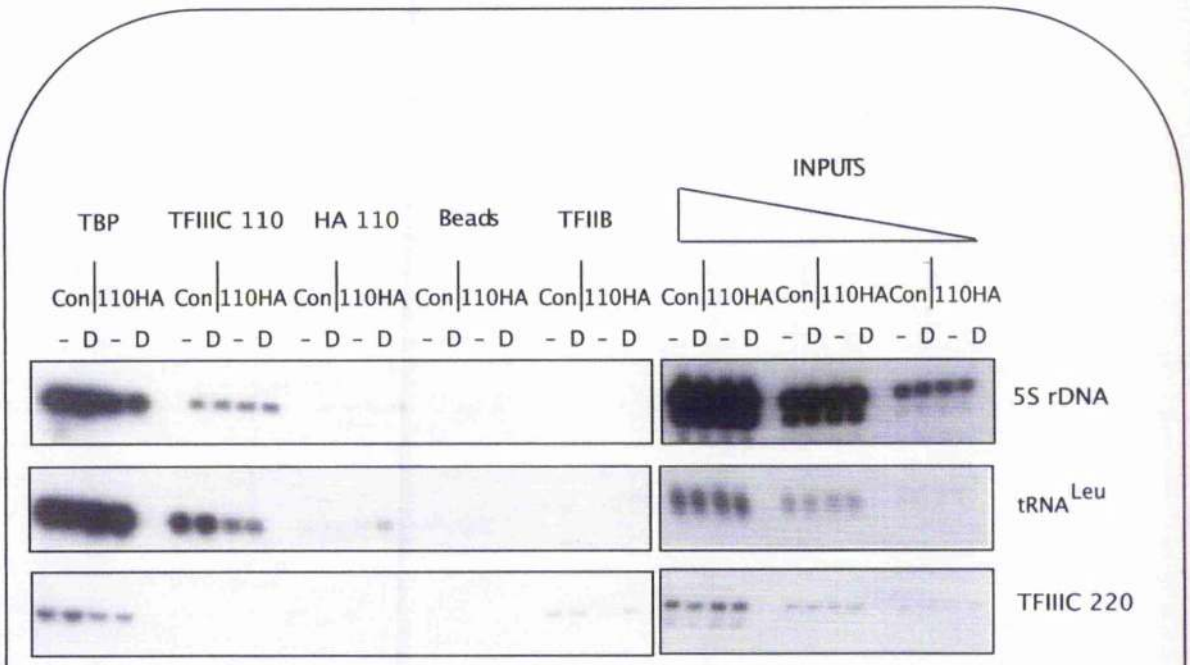


Figure 5.9. HA-TFIIIC110 is found at the promoter of pol III-transcribed genes

The level of TFIIIC/HA-TFIIIC110 promoter occupancy was measured in a HA-TFIIIC110 inducible cell line. Cells were either untreated (-) or induced with doxycycline (+).A control cell line containing an empty vector was also used. ChIPs were performed using antibodies against TFIIIC110, HA, TBP and TFIIA (negative control). Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4% of input), using 5S rRNA, $tRNA^{Leu}$ and TFIIIC220 primers.

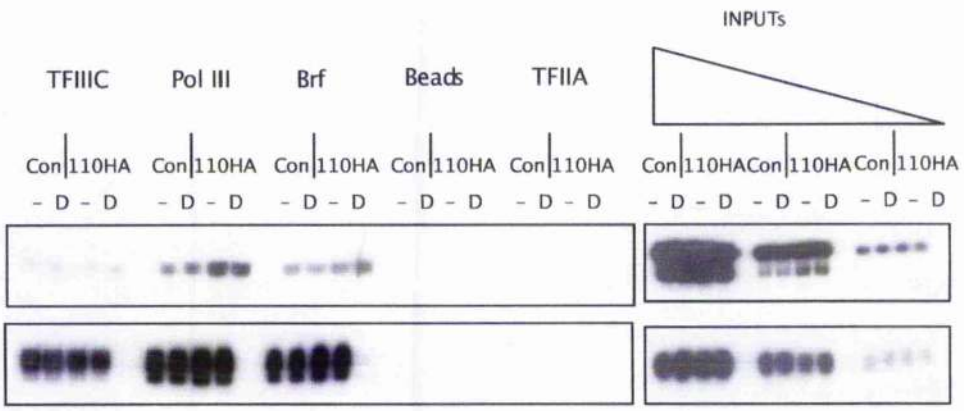


Figure 5.10. Induction of HA-TFIIIC110 had no effect on the promoter occupancy of pol III and TFIIIB

The level of histone acetylation was measured in a HA-TFIIIC110 inducible cell line. Cells were either untreated (-) or induced with doxycycline (+). A control cell line containing an empty vector was also used. ChIPs were performed using antibodies against acetylated histone H3, acetylated histone H4, RPC155 (pol III) and TFIIA (negative control). Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4%of input), using 5S rRNA and $tRNA^{Leu}$ primers.

5.2.7 Induction of HA-TFIIIC110 had no effect on the acetylation of histone H3 and H4 found on pol III-transcribed genes

A third ChIP using the inducible cell line was used to see if the overexpression of HA-TFIIIC110 had an effect on the acetylation of histone tails found on pol III-transcribed genes. To assess changes in histone acetylation, an anti-acetyl H3 and an anti-acetyl H4 antibody were used. In addition, an antibody against acetyl histone H3 Lys14 was used to see if this specific lysine residue was acetylated by the induction of HA-TFIIIC110. Lysine 14 on histone H3 tails is reported to be a specific target of TFIIIC's HAT activity (Hsieh et al., 1999a). PCR analysis revealed that acetylation of histone H3 is unaffected by the induction of HA-TFIIIC110 (figure 5.11). Acetylation of histone H4 was shown to increase when HA-TFIIIC110 was induced. However, the same effect was observed when doxycycline was added to the control cell line. Therefore, this may reflect a doxycycline effect. Consistent with the previous ChIP, the promoter occupancy of pol III was also unaffected by this induction.

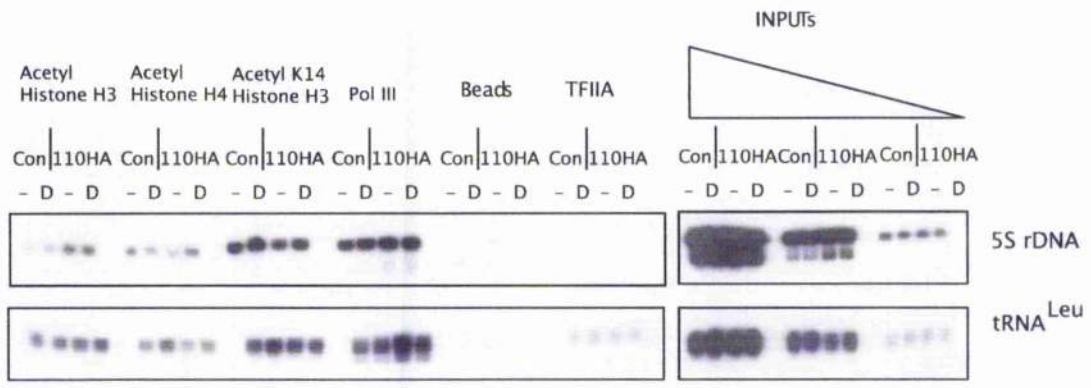


Figure 5.11. Induction of HA-TFIIIC110 had no effect on the acetylation of histones H3 and H4 on pol III-transcribed genes

The level of histone acetylation was measured in a HA-TFIIIC110-inducible cell line. Cells were either untreated (-) or induced with doxycycline (+). A control cell line containing an empty vector was also used. ChIPs were performed using antibodies against acetylated histone H3, acetylated histone H4, acetylated histone H3 lysine 14, RPC155 (pol III) and TFIIA (negative control). Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4% of input), using 5S rRNA and $tRNA^{Leu}$.

5.3 Discussion

Data from this chapter suggest that pol III is regulated by acetylation. Treatment of cells with the HDAC inhibitor TSA resulted in an increase in the acetylation of histone found on pol III-transcribed genes. Furthermore, a histone-free *in vitro* transcription assays showed that pol III activity was elevated in extracts treated with TSA, demonstrating that acetylation of a non-histone target is important.

TSA produced different effects in *in vitro* transcription assays and RT-PCRs. Both assays show an increase in the expression of pol III-transcribed genes after 6 hours of TSA treatment. However, RT-PCR analysis reveals a second peak at 24 hours, which is not observed in the *in vitro* transcription assay. It would be easy to suggest that this is a histone effect, as *in vitro* transcription assays are performed on naked DNA. However, TSA is a potent drug and over a 24-hour time a number of secondary effects will take place. Therefore, these results should be viewed with caution and only suggest that acetylation is important for pol III activity in both a histone and histone-free environment.

Previous studies have shown that both histone and factor acetylation play important roles in the regulation of gene transcription (Glozak et al., 2005; Strahl and Allis, 2000). In this study, addition of TSA increased the promoter occupancy of both pol III and TFIIIB. This was seen in parallel with an increase in the level of histone H3 and histone H4 acetylation. Much of the previous work on histone acetylation and transcription has been purely correlative and has not identified a direct link. A number of studies have

developed *in vitro* models where the effect of histone acetylation on transcription can be measured (An et al., 2004; An et al., 2002). In this case there is no direct evidence to say that histone acetylation is directly regulating the expression of pol III-transcribed genes. However, it can be viewed as a marker for transcriptional activation. This is reinforced by the results from chapter 4 where rapamycin caused a decrease in the levels of histone H3 acetylation was observed.

TSA promotes the accumulation of acetylated histone through the repression of HDACs. However, HATs are needed in the first instance to facilitate this acetylation. Both histone H3 and histone H4 have elevated levels of acetylation in response to TSA. This response would suggest that HATs capable of acetylating histone H3 and histone H4 are recruited onto pol III-transcribed genes. HATs often show a level of specificity for lysines on histone tails *in vivo* (Lachner et al., 2003). Therefore, further work is needed to characterize which HATs and specific lysines are involved in this process.

There are two HDAC protein families: the SIR2 family of NAD^+-dependent HDACs, and the classical HDAC family (which includes HDAC 1-10) (DeRuijter et al., 2003). TSA inhibits the latter of these two families by blocking access to their active site (DeRuijter et al., 2003). As pol III activity is increased by TSA, the classical HDAC family must therefore repress the expression of pol III-transcribed genes either directly or indirectly. This is in contrast to pol I transcription which is also regulated by acetylation but is not affected by the addition of TSA (Muth et al., 2001). The SIR2 family of HDACs

regulates pol I, whereas the classical HDAC family regulates the expression of pol III-transcribed genes.

Initial studies concerning the role of acetylation focused on its effects on core histones. In addition to histone, a number of other targets have been identified including transcription factors. The recombinant HATs used in this study, p300, TIP60 and PCAF, have all been shown to acetylate transcription factors (Halkidou et al., 2004; Muth et al., 2001; Perrot and Rechler, 2005). Furthermore, TIP60 and PCAF have been implicated in the acetylation and regulation of pol I transcription factors (Halkidou et al., 2004; Muth et al., 2001). This present study demonstrates that both p300 and TIP60 mildly promote the expression of pol III-transcribed genes. This was demonstrated in an *in vitro* transcription assay where no histones are present on the DNA template. Therefore, p300 and TIP60 regulate the activity of pol III through the acetylation of a non-histone target. Targets could include components of the pol III transcriptional machinery, although further work is needed to address this suggestion. The results of this experiment do not rule out the possibility that PCAF still may regulate the expression of pol III-transcribed genes. PCAF could potentially regulate pol III activity through its classical target of histone tails.

Previous reports have proposed TFIIIC110 as the limiting component for transcription by pol III. Its relative abundance was thought to control the ratio of active TFIIIC2a to inactive TFIIIC2b in HeLa cells (Sinn et al., 1995). Therefore, specific induction of TFIIIC110 in HeLa cells should be sufficient to stimulate the expression of pol III-

transcribed genes. However, data in this chapter do not support this idea. Induction of HA-TFIIIC110 does not increase the expression of pol III-transcribed genes, seen in both RT-PCR analysis and *in vitro* transcription assays. Furthermore, overexpression of HA-TFIIIC110 had not effect on the promoter occupancy of TFIIIC, pol III and TFIIIB. Work from this study on the induction of HA-TFIIIC110 combined with that of a co-worker Fiona Innes has now been published (Innes et al., 2006).

Experiments carried out by Kovelman *et al.* demonstrated that purified TFIIIC was only able to support transcription when TFIIIC110 was part of this complex (Kovelman and Roeder, 1992), highlighting TFIIIC110 as an essential component for transcription by pol III. This present study does not disprove this idea; instead it demonstrates that in HeLa cells, TFIIIC110 is not the rate-limiting component for pol III transcription. Hoffler *et al.* showed that treating TFIIIC2a with a phosphatase could generate TFIIIC2b (Hoeffler et al., 1988). Therefore, phosphorylation could be regulating the interaction of TFIIIC110 with the remainder of the TFIIIC complex.

The idea that TFIIIC110 is a limiting factor for the expression of pol III-transcribed genes is based on correlative data. When HeLa cells are grown in the presence of E1A or high serum, transcription by pol III is elevated. In parallel, relatively high ratios of TFIIIC2a to TFIIIC2b and of TFIIIC110 to TFIIIC220 are observed (Hoeffler et al., 1988; Kovelman and Roeder, 1992; Sinn et al., 1995). As TFIIIC110 is not the rate-limiting component for transcription by pol III in HeLa cells, other mechanisms may account for pol III regulation under these circumstances. For example, E1A can overcome RB-

mediated repression of pol III transcription, both *in vivo* and *in vitro* (White et al., 1996). Serum leads to the phosphorylation and inactivation RB, which promotes pol III activity (Scott et al., 2001). Furthermore, pol III is directly activated by c-Myc and Erk (Felton-Edkins et al., 2003a; Gomez-Roman et al., 2003), both of which are serum-inducible. These alternative mechanisms may go some way to explain pol III's regulation by E1A and serum.

Chapter 6-The role of histone methylation in the expression of pol III-transcribed genes

6.1 Introduction

The previous chapter focused on the role of histone and factor acetylation and how this covalent modification contributes to the regulation of pol III-transcribed genes. A number of other covalent modifications exist on histone tails, which are also thought to regulate gene expression. One such modification, methylation, has recently become the focus of intense research due to the characterization of a number of proteins that regulate its presence on histone tails (Huang et al., 2006; Klose et al., 2006; Tsukada et al., 2006). This chapter will look at the presence of this covalent modification on pol III-transcribed genes.

6.1.2 Histone methylation

Histone methylation occurs on both lysine and arginine residues (Lachner et al., 2003). Methylation of these two amino acids correlates with the formation of heterochromatin, x-chromosome inactivation and transcriptional regulation. Lysine methylation can either signal for a repressed or active transcriptional state (Lachner et al., 2003). This is in contrast to acetylation, which generally correlates with transcriptional activation. Furthermore, there is an additional level of regulation, lysine can either be mono-, di-, or trimethylated (Lachner et al., 2003). Methylation is also seen as a more stable modification, frequently having a half-life in a time-scale of hours. It is thought that this may contribute to histone memory, where inheritance of this epigenetic mark takes place following replication (Bannister et al., 2002).

Five lysine residues on histone H3 can be methylated (K4, K9, K27, K36 and K79) (Lachner et al., 2003). Histone H3 lysine 4 (H3 Lys^4) and histone H3 lysine 9 (H3 Lys^9)

are perhaps the best studied of these modifications and are known to have opposing roles (Liang et al., 2004; Nakayama et al., 2001). H3 Lys^4 methylation is associated with transcriptional activation, whereas H3 Lys^9 methylation is associated with transcriptional repression and the assembly of heterochromatin (Liang et al., 2004; Nakayama et al., 2001). As previously stated, chromodomain proteins bind to methylated histone tails. One such protein, HP1 binds to methylated H3 Lys^9. HP1 participates in chromatin packaging and gene silencing by directing the binding of other proteins that control chromatin structure and gene function (Lachner et al., 2001). Another prominent example of a chromodomain protein is Chd1 (chromo-ATPase/helicase-DNA binding domain 1). Chd1 is a chromatin remodelling protein that recognizes methylated H3 Lys^4 (Pray-Grant et al., 2005). It is a component of SAGA (Spt-Ada-Gcn5 acetyltransferases) and SLIK (SAGA-like) complexes, which preferentially acetylate histones H3 and H2B. The presence of Chd1 is associated with transcriptional activation (Pray-Grant et al., 2005). Thus, Chd1 and HP1 form part of the histone code hypothesis, where different covalent modifications on histone tails provide binding sites for a variety of proteins that convert chromatin to either a repressed or active state.

6.1.2 Alu histone methylation

Previous studies have highlighted a role for histone methylation on pol III-transcribed Alu genes (Hakimi et al., 2002; Kondo and Issa, 2003). Kondo and Issa demonstrated that Alus are enriched for H3 Lys^9 methylation (Kondo and Issa, 2003). This would suggest that Alus have a low level of expression, as K9 is associated with transcriptional silencing (Lachner et al., 2001). Indeed, Alu transcript levels are usually very low in

tissue and cultured cells (Liu et al., 1994; Sinnett et al., 1992), even though Alus constitute a major part of the human genome, numbering over a million copies. Chromatin is known to have a prominent role in the repression of Alus, as in HeLa cells ~99% of potentially active Alu repeats are silenced by chromatin, whereas the vast majority of tRNA remain unaffected by chromatin (Russanova et al., 1995). A direct link between H3 Lys^9 methylation and the repression of Alu transcription by chromatin has yet to be determined.

6.1.3 Histone methylation, a reversible process

Work over recent years has led to the discovery of a number of proteins that regulate histone methylation. Suv39h was the first histone methyltransferase to be identified (Rea et al., 2000). It catalyzes the methylation of H3 Lys^9, creating a high-affinity binding site for the chromodomain protein HP1 (Lachner et al., 2001). The methyltransferase activity of Suv39h is mediated through a highly conserved structure known as the SET domain. Analysis of the human genome has shown that there are a total of 73 SET-domain proteins (Kouzarides, 2002). Identification of this domain led to the discovery of a number of other histone methyltransferases, including the protein Set1 (the yeast homologue of mammalian MLL) (Milne et al., 2002). Set1 forms part of a larger complex called COMPASS (complex of proteins associated with Set1) (Schneider et al., 2005). Past studies have shown that Set1 is responsible for the methylation of H3 Lys^4 (Milne et al., 2002; Schneider et al., 2005). Thus, Suv39h and Set1 are two prominent examples of proteins that methylate specific lysines on histone tails.

For a number of years methylation has been seen as an irreversible process as previous studies reported that the half-life of methylated lysine and histones are the same (Byvoet, 1972; Duerre and Lee, 1974). This idea is consistent with heterochromatin, where histone methylation could mediate 'permanent' transcriptional silencing. However, recent work has identified a number of histone demethylases, demonstrating that this mark is reversible (Klose et al., 2006; Shi et al., 2004; Tsukada et al., 2006). LSD1 (lysine specific demethylase 1) was the first of these to be characterized (Shi et al., 2004). It specifically demethylates both mono- and dimethylated H3 Lys^4 (Shi et al., 2004). Following on from this work, a new family of histone demethylases have been identified containing the so called Jmjc domain (Klose et al., 2006; Tsukada et al., 2006; Whetstine et al., 2006; Yamane et al., 2006). A member of this family, JMJD2A, has been the first demethylase identified to remove a trimethylated lysine mark (Klose et al., 2006; Whetstine et al., 2006). Thus, histone methylation can now be viewed as a reversible process where demethylases can remove mono- di- and trimethylated histone marks.

Thus far, limited work has been carried out on the role of histone methylation on pol III-transcribed genes. Therefore, this chapter will investigate the dynamics of histone methylation on pol III-transcribed genes. Work will focus on H3 Lys^4 and H3 Lys^9 methylation and how these modifications relate to gene expression.

6.2 Results:

6.2.1 Pol III-transcribed genes contain H3 Lys^4 methylation.

Initial experiments were performed to look at the pattern of histone methylation found on pol III-transcribed genes. ChIP samples were obtained from Nicol Keith's lab, The University of Glasgow. Levels of H3 Lys^4 and H3 Lys^9 di- and trimethylation were examined in an ovarian carcinoma cell line (A2280) and a bladder cancer carcinoma cell line (5637) (figure 6.1). PCR analysis of the two cells lines revealed that all pol III-transcribed genes observed had a high level of H3 Lys^4 trimethylation. A lower level of H3 Lys^4 dimethylation was also observed in 5637 cells on 5S rRNA, 7SL, $tRNA^{Arg}$ and U6 gene, but this was not observed in A2280 cells. Neither cell line displayed much H3 Lys^9 methylation. A2280 cells had a low level of H3 Lys^9 dimethylation on 5S rRNA, 7SL and $tRNA^{Leu}$ genes, whereas 5637 cells had a faint level of H3 Lys^9 trimethylation on 5S rRNA genes.

A second ChIP looking at the same modifications was performed comparing WI-38 lung fibroblasts to transformed C-33A cervical carinoma cells (figure 6.2). Again, samples were obtained from Nicol Keith's lab, the University of Glasgow. PCR analysis showed that all genes observed had an elevated level of H3 Lys^4 di- and trimethylation in the transformed C-33A compared to the untransformed WI-38. However, no H3 Lys^9 methylation was observed in either cell line.

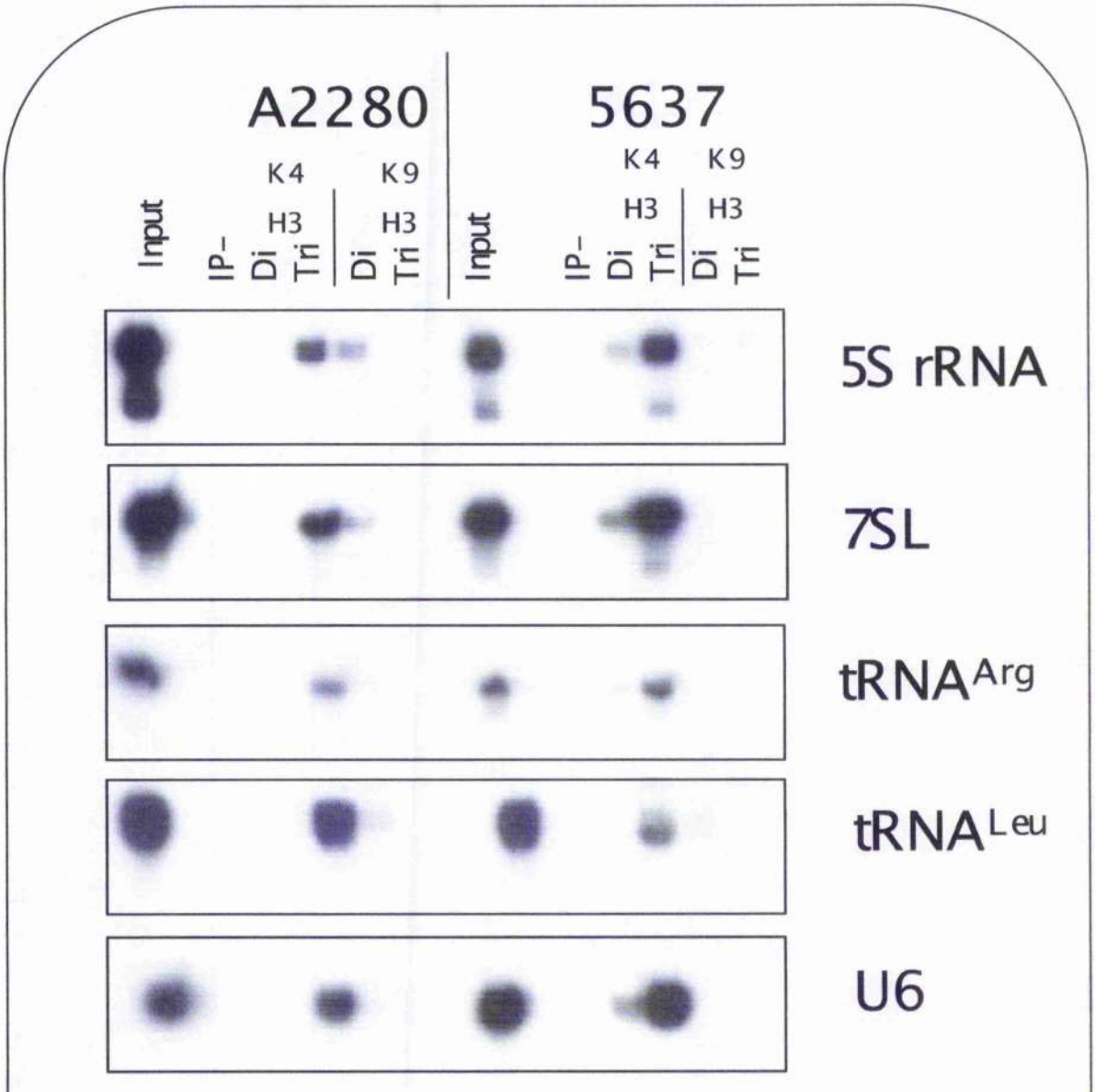


Figure 6.1 A high level of trimethylted H3 Lys^4 is found in both ovarian carcinoma and bladder carcinoma cell lines

Histone methylation on pol III-transcribed genes was observed in ovarian carcinoma (A2280) and bladder carcinoma (5637) cell lines. ChIPs were performed using antibodies against di- and trimethylted H3 Lys^4 and H3 Lys^9. Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, using 5S rRNA, tRNA^{Leu}, tRNA^{Arg}, 7SL and U6 primers (n=1).

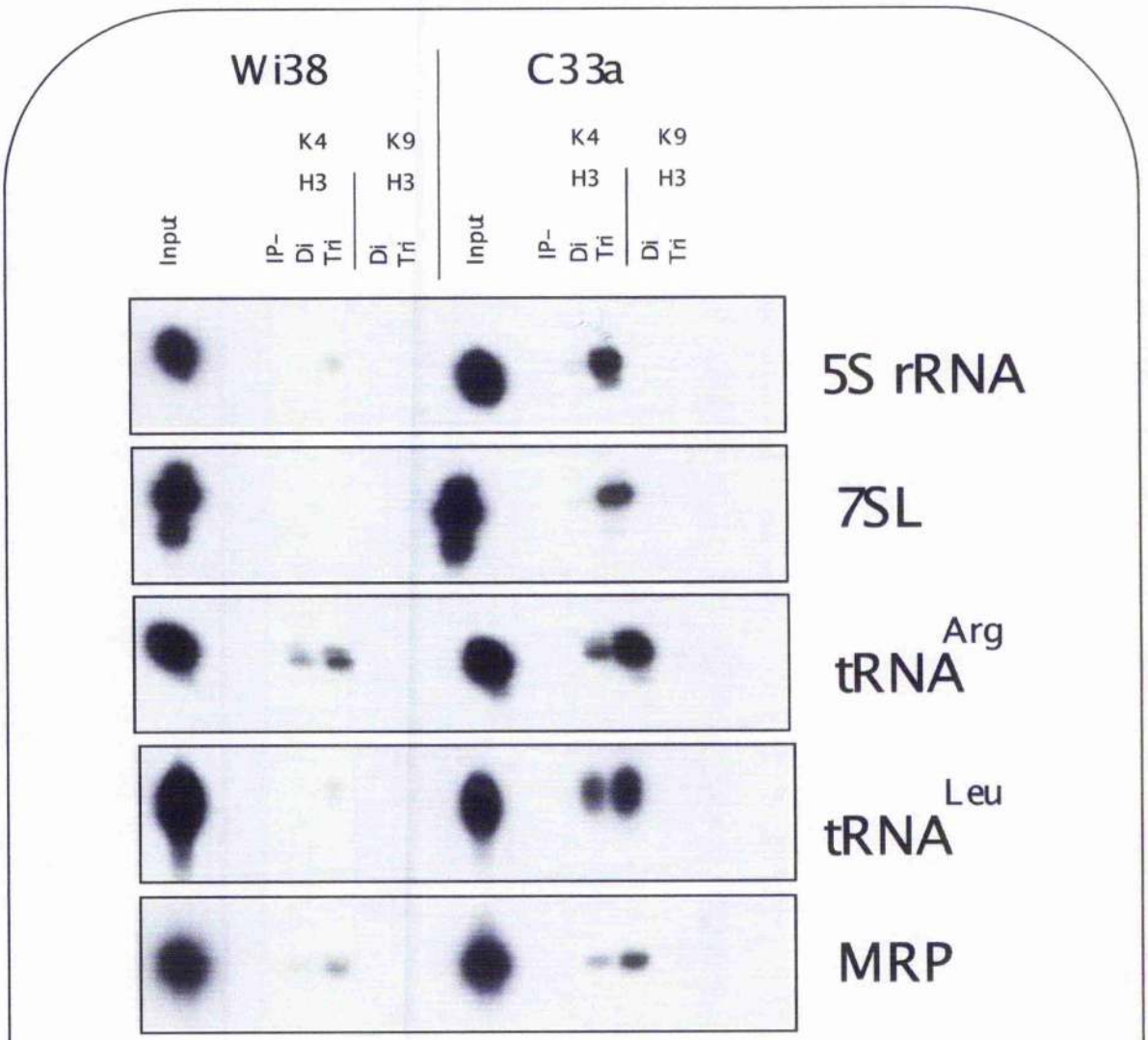


Figure 6.2 Trimethylted H3 Lys^4 is elevated in transformed cervical cancer cells compared to human lung fibroblasts

Histone methylation on pol III-transcribed genes was observed in human lung fibrioblasts (Wi38) and cervical cancer cells (C33a). ChIPs were performed using antibodies against di- and trimethylted H3 Lys^4 and H3 Lys^9. Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, using 5S rRNA, tRNA^{Leu}, tRNA^{Arg}, 7SL and MRP primers (n=1).

6.2.2 Myc has a negligible effect on histone methylation found on pol III-transcribed genes

A recent study has shown that Myc influences global chromatin structure through alteration in histone methylation and acetylation (Knoepfler et al., 2006). As previously stated, Myc has been shown to regulate the expression of pol III-transcribed genes (Gomez-Roman et al., 2003). Therefore, rat 1A c-myc-/- knockout cells were compared to wildtype rat 1A cells in a ChIP assay to see if Myc has an effect on histone methylation on pol III-transcribed genes (figure 6.3). Antibodies against K4 trimethylated histone H3 and K9 trimethylated histone H3 antibody were used to measure changes in methylation. An antibody designed against TFIIB was used as a negative control. The presence of histone H3 K4 methylation is unaltered by the presence or absence of c-Myc on the genes encoding $tRNA^{Leu}$, 5S rRNA and B2. H3 Lys^9 methylation appears to be very mildly diminished in the rat 1A c-myc-/- knockout when compared to wiltype rat 1A on the genes encoding 5S rRNA and B2. Thus, the presence or absence of Myc had very little effect on the methylation of H3 Lys^4 and H3 Lys^9 on pol III-transcribed genes.

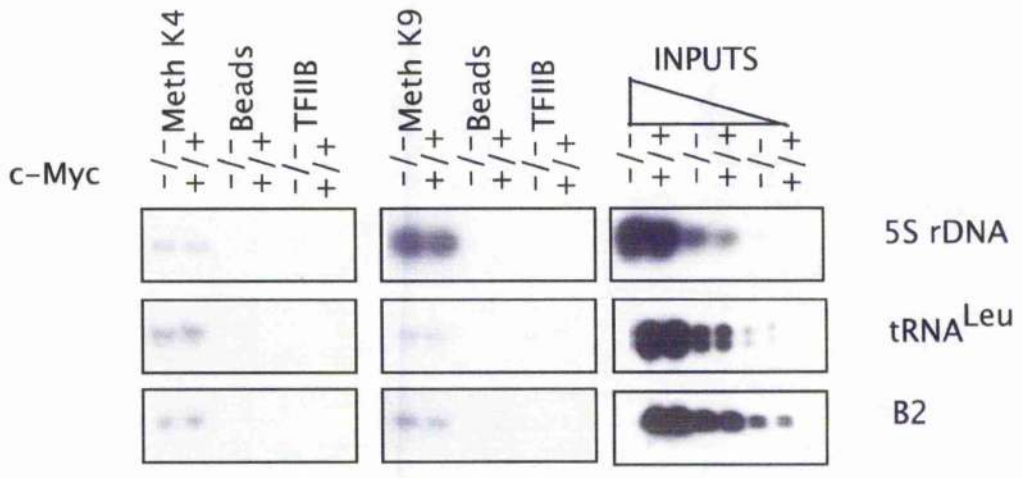


Figure 6.3 Trimethylated H3 Lys^4 and H3 Lys^9 on pol III-transcribed genes is comparable in wild type and myc null cells.
Histone methylation was compared between rat 1A Myc$^{-/-}$ knockout and rat 1A wildtype fibroblasts. A ChIP assay was performed using antibodies against trimethylated H3 Lys^4 and H3 Lys^9. TFIIB was used as a negative control. Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4%), using 5S rRNA, tRNA^{Leu} and B2 primers (n=2).

6.2.3 TSA does not alter the histone methylation pattern on pol III-transcribed genes

HATs and histone methyltransferases have been shown to cooperate together in modifying nucleosomal histones. Acetylation of lysines on a histone tail can promote the methylation of another residue (An et al., 2004). To determine if acetylation could alter the pattern of histone methylation found on pol III-transcribed genes, a ChIP assay using TSA was performed (figure 6.4). Asynchronous A31 mouse fibroblasts cells were treated with TSA (200nm) for 6, 12 and 24 hours. Antibodies against trimethylated H3 Lys^4 and H3 Lys^9 were used to measure changes in methylation. A TFIIB antibody was used as a negative control. TSA had no effect on the methylation of H3 Lys^4 and H3 Lys^9 on 5S rRNA, $tRNA^{Leu}$ and B2 genes. However, a different pattern of methylation was observed on the three genes. $tRNA^{Leu}$ genes have a higher level of H3 Lys^4 methylation compared to H3 Lys^9 methylation. The opposite can be said for B2, which have a high level of H3 Lys^9 methylation compared to H3 Lys^4 methylation. The genes encoding 5S rRNA have a similar amount of H3 Lys^4 and H3 Lys^9 histone methylation. Thus, pol III-transcribed genes display different patterns of histone methylation.

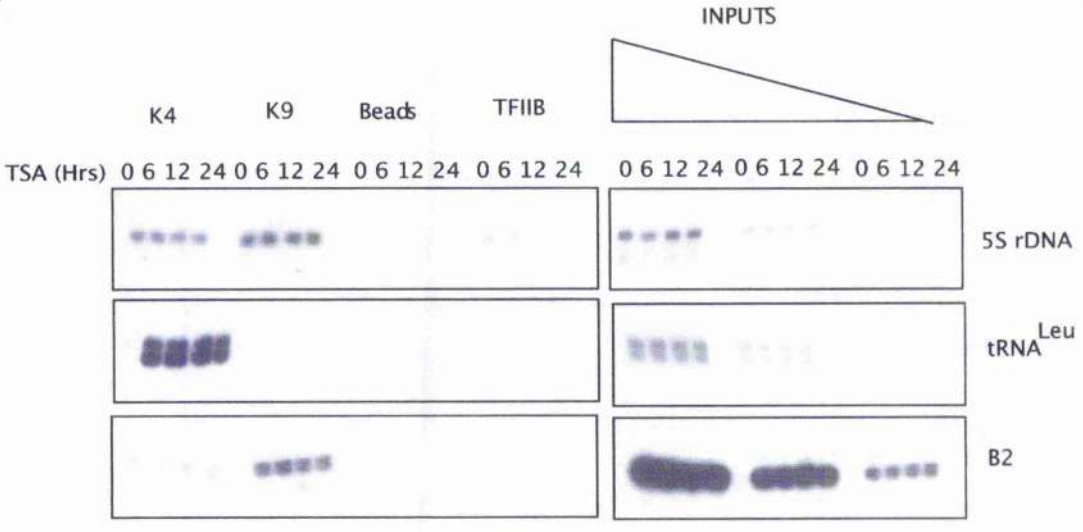


Figure 6.4 TSA does not alter the pattern of trimethylated H3 Lys^4 and H3 Lys^9 on pol III-transcribed genes.
A31 cells were treated with TSA (200nM) for 6, 12, and 24 hours (lanes 2-4) or with vehicle (lane 1). ChIPs were performed using antibodies against trimethylated H3 Lys^4 and H3 Lys^9. TFIIB was used as a negative control. Samples were normalized by comparing the genomic DNA inputs and adjusting the samples accordingly. The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4%), using 5S rRNA, tRNA^{Leu} and B2 primers.

6.2.4 Alu genes contain a high level of pol III promoter occupancy and H3 Lys^9 methylation

A previous study has shown that Alus are enriched for H3 Lys^9 methylation (Kondo and Issa, 2003). The presence of this mark was thought to be a marker for the transcriptional repression of Alus. Therefore, a high level of H3 Lys^9 methylation should correlate with a low pol III promoter occupancy. To test this idea, formaldehyde cross-linked chromatin was prepared from asynchronously growing HeLa cells (figure 6.5). Antibodies against RPC155 (a component of pol III), TFIIIC110 and the TFIIIB subunit Brf1 were used to determine the promoter occupancy of the pol III transcriptional complex. To assess the levels of histone acetylation and methylation, antibodies against acetyl H3, acetyl H4, K4 trimethylated histone H3 and K9 trimethylated histone H3 were used. A TFIIB antibody was used as a negative control, as TFIIB is not present on pol III-transcribed genes. Consistent with previous data in this chapter (figure 6.4), genes encoding $tRNA^{Leu}$ showed a greater level of H3 Lys^4 methylation compared to H3 Lys^9 methylation. Furthermore, 5S rRNA genes have a similar amount of H3 Lys^4 and H3 Lys^9 methylation. As expected, both genes have pol III, TFIIIB and TFIIIC present. Single Alu genes on different chromosomes were looked at to measure to presence or absence of H3 Lys^4 and H3 Lys^9 methylation. Of the 6 single copy Alu genes looked at, 5 had a greater level of H3 Lys^9 methylation compared to H3 Lys^4 methylation. However, apart from the Alu on chromosome 6, all had pol III present. Additionally, H3 Lys^4 methylation was found on all the Alu genes looked at.

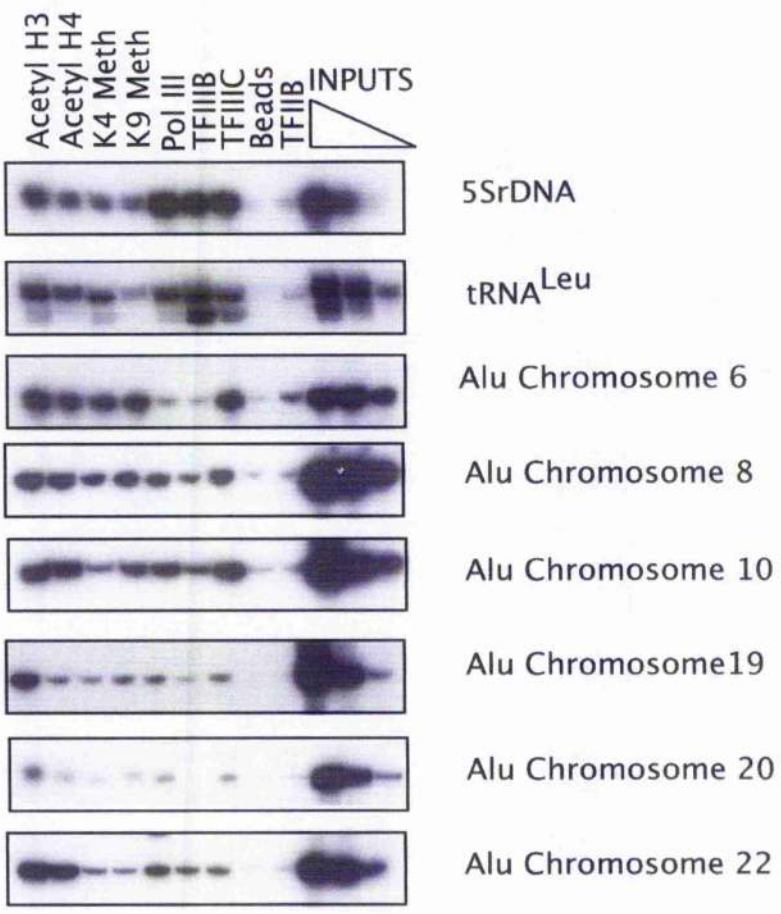


Figure 6.5 Alu genes contain trimethylated H3 Lys^4 and H3 Lys^9.
A ChIP assay was performed using HeLa cells. Antibodies were used against acetylated histone H3, acetylated histone H4, trimethylated H3 Lys^4, trimethylated H3 Lys^9, TFIIIC110, Brf1 (a component of TFIIIB), RPC155 (pol III) and TFIIB (negative control). The samples were then analysed by PCR, alongside genomic DNA (10%, 2% and 0.4% of input) using 5S rRNA, tRNA^{Leu}, Alu chromosome 6, Alu chromosome 8, Alu chromosome 10, Alu chromosome 19, Alu chromosome 20, and Alu chromosome 22 primers (n=2).

6.2.5 The promoter occupancy of the pol III transcriptional complex is correlated with histone methylation

Results from this chapter demonstrate that pol III-transcribed genes have both H3 Lys^4 and H3 Lys^9 methylation along with pol III and its associated factors TFIIIB and TFIIIC. A problem with conventional ChIP assays is that they do not tell you if two proteins are found together or separately. This is because the result gained is from a pool of cells. For instance, when looking at a single gene half the cells might have a high level of H3 Lys^9 methylation and no pol III present, whereas the other half might have no H3 Lys^9 methylation and a high level of pol III. When the two sets of cells are pooled in a ChIP assay it would appear that both methylated H3 Lys^9 and pol III are found on the same gene, it does not discriminate between the two sub populations of cells. This becomes even more of a problem when you are looking at multi-copy genes at the same time. Therefore, a sequential ChIP was performed to determine if pol III, TFIIIB and TFIIIC are found on genes that contain methylated H3 Lys^4 or H3 Lys^9. Formaldehyde cross-linked chromatin was prepared from asynchronously growing HeLa cells. Antibodies against trimethylated H3 Lys^4 and trimethylated H3 Lys^9 were used along with a 4E-BP1 antibody which was used as a negative control. This primary immmunoprecipitation separates two subpopulations of genes that contain trimethylated H3 Lys^4 or H3 Lys^9. The material gained from the first set of immunoprecipitations was then subject to a second immunoprecipitation using antibodies against trimethylated H3 Lys^4, trimethylated H3 Lys^9, RPC155 (a component of pol III), TFIIIC110 and the TFIIIB subunit Brf1. Thus, PCR analysis of the second immunoprecipitation should demonstrate

if components of the pol III transcription machinery are present at the same gene as either trimethylated H3 Lys^4 or H3 Lys^9.

ChIPs using HeLa cells have shown that genes encoding 5S rRNA have an equal level of trimethylated H3 Lys^4 or H3 Lys^9. Therefore, immunoprecipitations using trimethylated H3 Lys^4 or H3 Lys^9 antibodies will contain an equal amount of the 5S rRNA gene. By subjecting this material to a second round of immunoprecipitations using an antibody against pol III you should be able to determine which modification has more pol III associated with it on genes encoding 5S rRNA. Thus, sequential ChIPs in this instance provide a powerful tool for looking at promoter occupancy in relation to histone tail modifications. The sequential ChIP described here was performed (figure 6.6). More pol III was associated with trimethylated H3 Lys^4 compared to trimethylated H3 Lys^9. Further sequential ChIPs were performed using antibodies against Brf1 (a component of TFIIIB) (figure 6.7) and TFIIIC110 (figure 6.8), instead of pol III. A similar pattern is observed in these experiments, with there being more TFIIIB and a small increase in TFIIIC associated with trimethylated H3 Lys^4 compared to trimethylated H3 Lys^9.

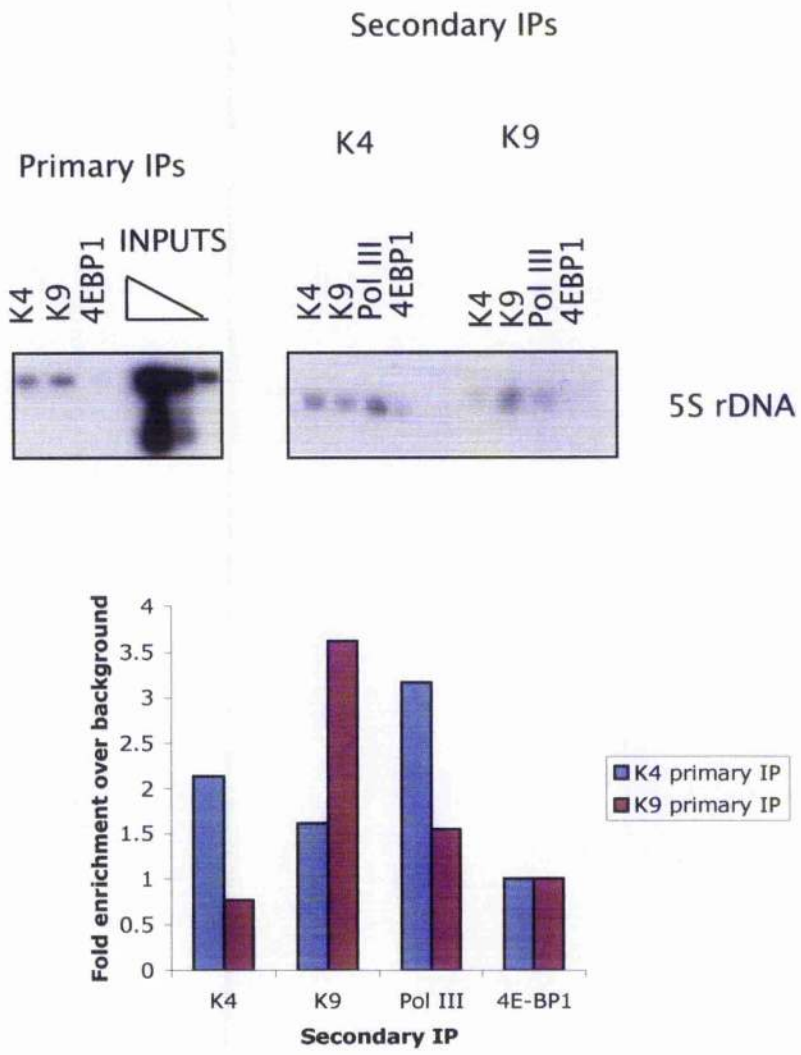


Figure 6.6 Pol III promoter occupancy is associated with the presence of trimethylated H3 Lys^4.

To look at the correlation between histone methylation and the promoter occupancy of TFIIIB a sequential ChIP was performed. **(a)** A primary immunoprecipitation was performed using antibodies against trimethylated H3 Lys^4 and H3 Lys^9. 4EBP-1 was used as a negative control. Material from the primary immunoprecipitation was then subject to a second immunoprecipitation using antibodies against RPC155 (a component of pol III), trimethylated H3 Lys^4 and H3 Lys^9. 4EBP-1 was again used as a negative control. The samples were then analysed by PCR using 5S rRNA primers. **(b)** Results from the second immunoprecipitation were quantified. Fold enrichment was obtained by dividing the intensity of the band by that of the background (4EBP-1) (n=2).

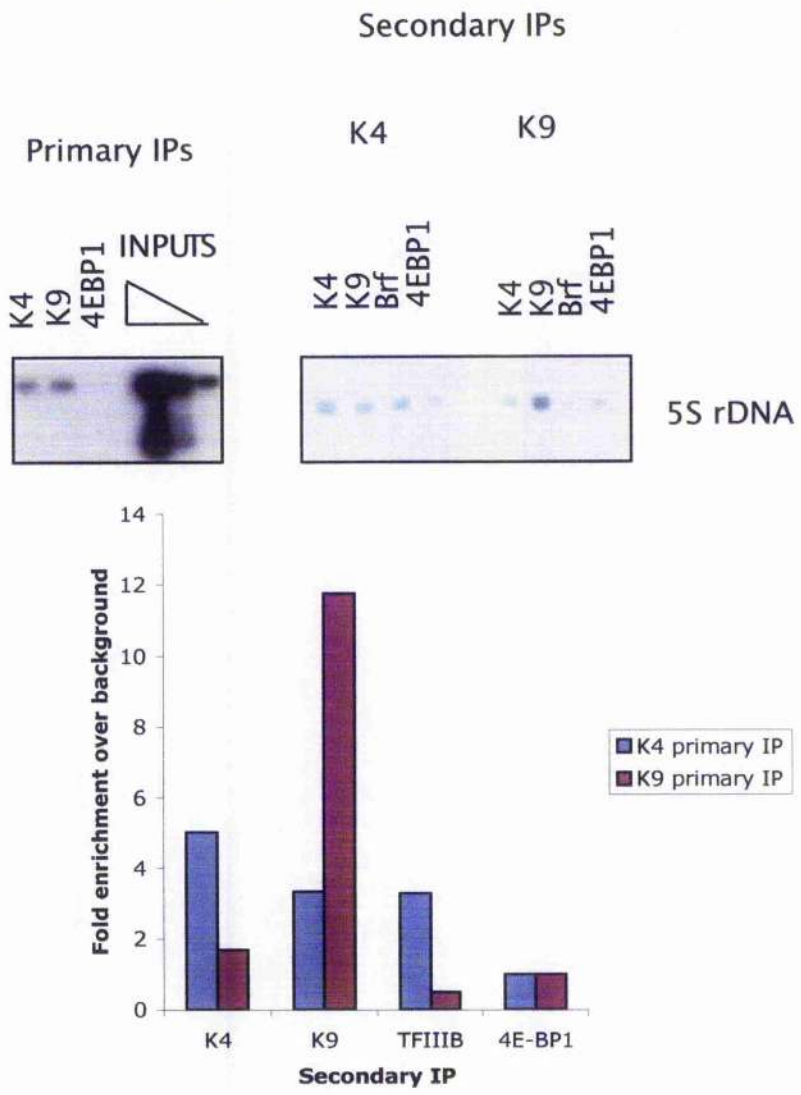


Figure 6.7 TFIIIB promoter occupancy is associated with the presence of trimethylated H3 Lys^4.

To look at the correlation between histone methylation and the promoter occupancy of TFIIIB, a sequential ChIP was performed. **(a)** A primary immunoprecipitation was performed using antibodies against trimethylated H3 Lys^4 and H3 Lys^9. 4EBP-1 was used as a negative control. Material from the primary immunoprecipitation was then subject to a second immunoprecipitation using antibodies against Brf1 (a component of TFIIIB), trimethylated H3 Lys^4 and H3 Lys^9. 4EBP-1 was again used as a negative control. The samples were then analysed by PCR using 5S rRNA primers. **(b)** Results from the second immunoprecipitation were quantified. Fold enrichment was obtained by dividing the intensity of the band by that of the background (4EBP-1) (n=2).

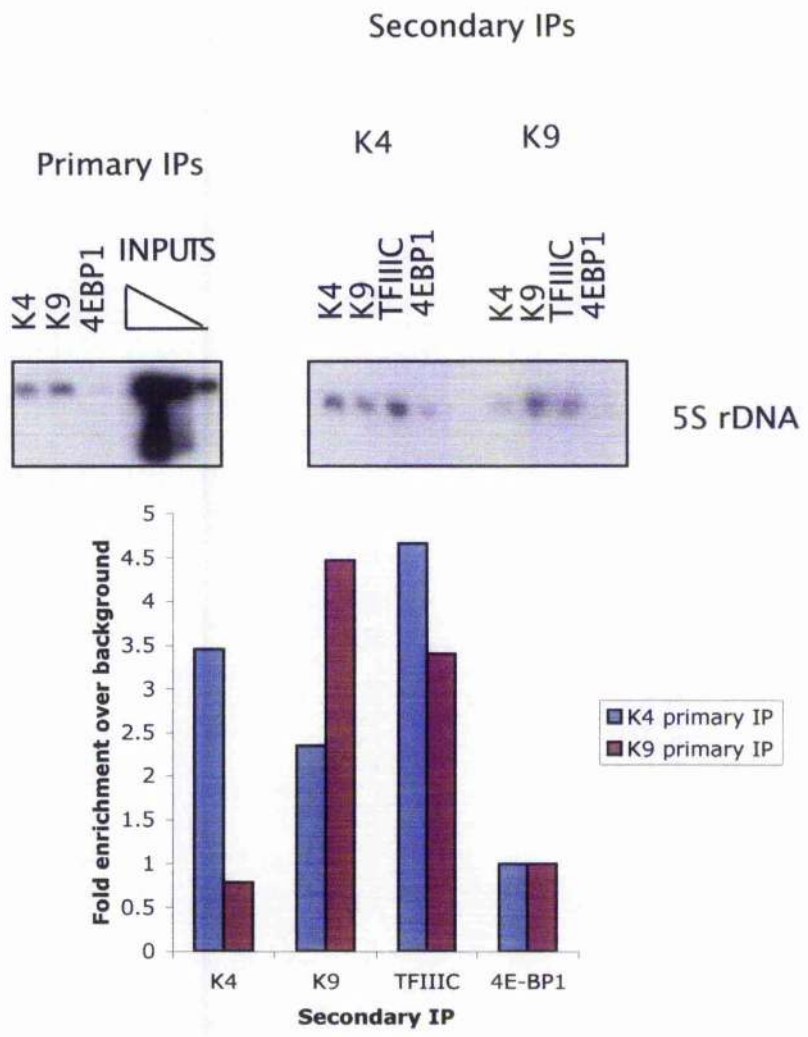


Figure 6.8 TFIIIC promoter occupancy is associated with the presence of trimethylated H3 Lys^4.

To look at the correlation between histone methylation and the promoter occupancy of TFIIIC, a sequential ChIP was performed. **(a)** A primary immunoprecipitation was performed using antibodies against trimethylated H3 Lys^4 and H3 Lys^9. 4EBP-1 was used as a negative control. Material from the primary immunoprecipitation was then subject to a second immunoprecipitation using antibodies against TFIIIC110, trimethylated H3 Lys^4 and H3 Lys^9. 4EBP-1 was again used as a negative control. The samples were then analysed by PCR using 5S rRNA primers. **(b)** Results from the second immunoprecipitation were quantified. Fold enrichment was obtained by dividing the intensity of the band by that of the background (4EBP-1) (n=2).

6.2.6 H3 Lys^4 or H3 Lys^9 methylation on pol III genes are not mutually exclusive

Sequential ChIPs were also performed to see if trimethylated H3 Lys^4 and trimethylated H3 Lys^9 were mutually exclusive or could be found on the same 5S rRNA genes (figures 6.6, 6.7 and 6.8). Again formaldehyde cross-linked chromatin was prepared from asynchronously growing HeLa cells. Antibodies against trimethylated H3 Lys^4 and trimethylated H3 Lys^9 were used. Both immunoprecipitations were then subject to a second immunoprecipitation using the same trimethylated H3 Lys^4 and trimethylated H3 Lys^9 antibodies. PCR analysis revealed that when trimethylated H3 Lys^4 is found on 5S rRNA genes, trimethylated H3 Lys^9 is also present but to a lesser degree. However, there is little trimethylated H3 Lys^4 found on genes that have been immunoprecipitated with trimethylated H3 Lys^9 in the first instance. Conversely, this may reflect the immunoprecipitation efficiency of the two antibodies used. Alternatively the result obtained could be from a group of genes as 5S rRNA genes occur in clusters. Thus, there could be a 5S rRNA gene with H3 Lys^4 methylation and no H3 Lys^9 methylation, and another 5S rRNA gene with H3 Lys^9 methylation and no H3 Lys^4 methylation which are found in close proximity to each other. The resolution of a ChIP may not stringent enough to differentiate between these two 5S rRNA genes, therefore, the result gained is from a combination of a number of genes.

6.3 Discussion:

Results from this chapter have shown that both H3 Lys^4 and H3 Lys^9 methylation are present on pol III-transcribed genes. Different genes show different patterns of H3 Lys^4 and H3 Lys^9 methylation and the presence of these marks is correlated with the promoter occupancy of TFIIIB, TFIIIC and pol III.

Methylated H3 Lys^4 is predominantly associated with the promoters of transcriptionally active genes (Liang et al., 2004), whereas methylated H3 Lys^9 is associated with inactive heterochromatin (Nakayama et al., 2001). Sequential ChIP data from this present study is consistent with these observations, as there is a greater promoter occupancy of pol III, TFIIIB and TFIIIC associated with H3 Lys^4 methylation compared to H3 Lys^9 methylation on genes encoding 5S rRNA. Thus, a correlation is observed between methylated histone marks and the promoter occupancy of the pol III transcriptional machinery. Sequential ChIPs also highlighted the possibility that H3 Lys^4 and H3 Lys^9 methylation are not mutually exclusive on the same 5S rRNA gene. A past study using the sequential ChIP technique has also shown that both H3 Lys^4 and H3 Lys^9 methylation can be found on the same gene (Vakoc et al., 2005). They found that H3 Lys^9 methylation actually increased during activation of transcription and was associated with elongation by pol II on the β-major globin gene (Vakoc et al., 2005). Furthermore, a recent study has shown that H3 Lys^4 methylation is required for the repression of a pol II transcribed rRNA gene (Briggs et al., 2006). This was shown to be dependent on the activity of the H3 Lys^4 methylatransferase, SET1 (Briggs et al., 2006). Therefore, the

black and white image of active H3 Lys^4 methylation and inactive H3 Lys^9 methylation appears to be changing.

Comparison of WI38 and C33A cells have shown that histone H3 Lys^4 methylation is elevated in a transformed cell line when compared to an untransformed cell line. Indeed, previous studies have shown that global changes in histone methylation are associated with cancer and that these changes are predictive of the clinical outcome (Seligson et al., 2005). More specifically, H3 Lys^4 methylation has been shown to change on specific genes during ovarian carcinogenesis (Caslini et al., 2006). However, the cell lines used in this study are from different tissues and may have different patterns of methylation independent of their transformed state. Therefore, a more meaningful result would be obtained by comparing a single cell line before and after transformation.

Kondo and Issa found that Alus are enriched for methylated H3 Lys^9, highlighting a potential mechanism that may repress the expression of these repetitive elements (Kondo and Issa, 2003). Another study has shown that methylated H3 Lys^4 was also present on a number of Alus (Hakimi et al., 2002). This present study is in agreement with these results as both methylated H3 Lys^4 and H3 Lys^9 were found to be present on Alu genes. However, pol III was present on the majority of these genes, even when they displayed a high level of methylated H3 Lys^9 compared to methylated H3 Lys^4. Alus constitute a major part of the human genome, numbering 1,090,000. The vast majority of these genes were not expressed, as ~99% of potentially active Alu repeats are thought to be silenced

by chromatin (Russanova et al., 1995). It seems unlikely that by chance all the Alus looked at in this study are being transcribed if work by Russanova et al. is correct. Therefore, the presence of pol III on these genes represents a surprising result.

Histone methylation has been shown to be associated with the binding of a number of different proteins that can alter chromatin structure (Cruz et al., 2005; Pray-Grant et al., 2005), making genes more or less permissive to transcription. Therefore, methylation of histones found on pol III-transcribed genes may facilitate the recruitment of chromatin modifying enzymes that help control expression. Indeed, Hakimi *et al.* has shown that the present of methylated H3 Lys^4 on Alu genes is associated with the binding of the chromatin remodelling complex SNF2h-NuRD (Hakimi et al., 2002). This may therefore provide a mechanism by which the expression of pol III-transcribed genes is controlled through histone methylation. However, work by Hakimi *et al.* is purely correlative and further work is needed to see if the SNF2h-NuRD complex is required for the direct activation of Alu gene transcription.

The different pattern of histone methylation observed on pol III-transcribed genes may reflect their copy number within the genome. Results from this chapter demonstrate that genes eAlus and B2s have a high level of H3 Lys^9 methylation compared to H3 Lys^4 methylation. Both genes are repetitive elements; Alus number 1,090,000 in the human genome, whereas B2 genes number 328,000 in the mouse genome. However, not all these genes are active, as the majority of Alu and B2 genes are known to be repressed by

chromatin (Russanova et al., 1995). Thus, the high level of H3 Lys9 methylation observed on these genes may in part mediate their transcriptional repression. Genes encoding 5S rRNA are also regulated by chromatin (Russanova et al., 1995), but have a much lower copy number, ranging between 200-300 within the human genome. Results in this chapter have shown that genes encoding 5S rRNA in mouse fibroblast and HeLa cells, have a similar level of H3 Lys^4 and H3 Lys^9 methylation. The higher level of H3 Lys^4 methylation on 5S rRNA genes compared to genes encoding Alus could indicate that the former could proportionately have more active genes compared to the latter. Indeed, a past study has shown that chromatin has a much greater repressive effect on Alu genes compared to 5S rRNA genes (Russanova et al., 1995). Finally tRNA genes are relatively unaffected by chromatin (Kundu et al., 1999; Morse et al., 1992; Russanova et al., 1995) and have a low copy number compared to 5S rRNA, Alu and B2 genes, averaging 10 genes per amino acid tRNA adaptor. Experiments in this chapter have shown that tRNA genes have a high level of H3 Lys^4 methylation and almost no H3 Lys^9 methylation. Thus, correlative data would suggest that the higher the copy number of pol III-transcribed genes the lower the H3 Lys^4 to H3 Lys^9 methylation ratio. Conversely, a low copy number gene has a higher ration of H3 Lys^4 to H3 Lys^9 methylation. It may also suggest a reason why genes with a high copy number transcribed by pol III are highly repressed by chromatin whereas genes with a low copy number are not.

Under the conditions looked at, histone methylation on pol III-transcribed genes does not change. A recent report has shown that Myc influences global chromatin structure through alterations in histone methylation and acetylation (Knoepfler et al., 2006). Myc

has previously been shown to regulate pol III activity (Gomez-Roman et al., 2003). Therefore, histone methylation might provide a potential mechanism for Myc's regulation of pol III-transcribed genes. However, histone methylation was not found to change when comparing wild-type cells to Myc knockouts. Furthermore, addition of TSA, which has been shown to elevate the expression of pol III-transcribed genes (chapter 5) had no effect on the pattern of histone methylation. Thus, alteration in the expression of pol III-transcribed genes either through histone acetylation or through the absence or presence of Myc has no effect on the histone methylation pattern observed on these genes. This may be consistent with the idea that in some cases histone methylation is a permanent mark and may contribute to memory where inheritance of this epigenetic mark takes place following replication (Bannister et al., 2002). This permanent mark may then make the histone more or less susceptible to an additional, reversible modification such as histone acetylation (Bannister et al., 2002).

Data presented in this chapter has shown that histone methylation varies between the different pol III-transcribed templates. Sequential ChIPs highlighted a correlation between histone methylation and the presence of the pol III machinery. Methylation was not shown to change in the conditions looked at, suggesting a more permanent mark that is associated with epigenetic inheritance.

Chapter 7-Final discussion

Work from chapters 3 and 4 has shown that the mTOR pathway plays an important role in the regulation of pol III activity. Furthermore, components of the mTOR pathway, PKB, TSC2 and Rheb, were all shown to play a role in the regulation of pol III. Histone acetylation was also shown to change in response to the activity of mTOR, highlighting a potential mechanism for the regulation of pol III. Work was then carried out on the general effect of histone acetylation and methylation, and what affect this had on the expression of pol III-transcribed genes.

7.1 mTOR regulates pol III

Addition of the mTOR inhibitor rapamycin decreased the expression of pol III-transcribed genes. This is only seen on type 1 and type 2 promoters, as previous work by Emma Graham has shown that rapamycin had no effect on U6 transcription. ChIP analysis revealed that the promoter occupancy of TFIIIB and pol III decreased in response to rapamycin treatment. TFIIIC's promoter occupancy remained unchanged. Further ChIPs also demonstrated that mTOR is present on Pol III-transcribed genes. This would suggest that mTOR is controlling the expression of genes transcribed by pol III through a direct mechanism. Previous work by Emma Graham (data unpublished) has shown that mTOR controls the phosphorylation of TFIIIC110. This was demonstrated *in vivo*, where addition of rapamycin led to a decrease in the phosphorylation of TFIIIC110. Preliminary work in this present study has shown that raptor, an essential component of the mTOR complex, was found to co-immunoprecipitate with TFIIIC110. This led to the model where mTOR directly binds and phosphorylates the 110kDa subunit of TFIIIC,

promoting its interaction with TFIIIB, which in turn elevates the expression of pol III-transcribed genes.

A very recent study has speculated a similar mechanism for the control of pol I transcription in *Saccharomyces cerevisiae* (Li et al., 2006). ChIP analysis showed that Tor1 (a yeast homologue to mTOR) was found on the 35S rDNA promoter (Li et al., 2006). This led to the authors speculating that binding may be important for the phosphorylation of components of the pol I transcriptional machinery by Tor1 (Li et al., 2006). Experiments by Li et al. also characterized the presence of Tor1 in the region between the enhancer and promoter elements of the 35S rDNA gene. In *S. cerevisiae*, this region carries a gene encoding pol III-transcribed 5S rRNA. In agreement with work carried out in chapter 4, ChIP analysis showed that Tor1 was bound to 5S rRNA genes. Furthermore, the association of Tor1 with 5S rRNA genes was diminished by the addition of rapamycin. This was thought to be controlled through cellular localization, as the addition of rapamycin led to the cytoplasmic retention of Tor1.

Future experiments are needed to characterize the response of the pol III-transcriptional machinery to rapamycin. ChIP experiments will be required to look at the promoter occupancy of mTOR on pol III-transcribed genes during rapamycin treatment. Following on from this, the cellular localization of mTOR will need to be determined following rapamycin treatment, to see if there is conservation between the response observed in yeast and in mammalian systems. A previous study in mammalian cells has shown that mTOR has a predominant nuclear localization in a number of normal and malignant cell lines (Zhang et al., 2002). Furthermore, a separate study has shown that mTOR shuttles between the nucleus and the cytoplasm (Kim and Chen, 2000). This was demonstrated

using leptomycin B, which is an inhibitor of the nuclear export receptor Crm1 (Kim and Chen, 2000). Therefore, further work is needed to investigate these potential mechanisms and how they regulate the expression of pol III-transcribed genes. (See figure 7.1 for the proposed mechanism of mTORs regulation of pol III).

7.2 PKB/Akt

Results from chapter 3 suggest that PKB may regulate the expression of pol III-transcribed genes direcetly. This was suggested by an *in vitro* transcription assay using a competitive substrate inhibitor for PKB. Addition of this competitive inhibitor reduced the expression of $tRNA^{Leu}$ in a dose-dependent manner. PKB is a proto-oncogene (Bellacosa et al., 1991) and has been implicated in cell survival and cell cycle progression. This is achieved through its phosphorylation of a number of key targets such as p53, forkhead transcription factors, BAD, caspase 9, p21 and p27 (Downward, 2004). Its overexpression or over-activation is thought to play an important role in cancer (Besson et al., 1999; Mirza et al., 2000; Tang et al., 2006). PKB is activated by phosphatidylinositol 3 phosphates, the product of phosphatidylinositol 3 phosphate kinase (PI3K) (Wullschleger et al., 2006). PKB is commonly overactive in a number of different tumours because of the frequent inactivation of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (Besson et al., 1999; Tang et al., 2006), which negatively regulates levels of phosphatidylinositol 3 phosphate levels (Wullschleger et al., 2006). Furthermore, overexpression of PKB transforms mammalian

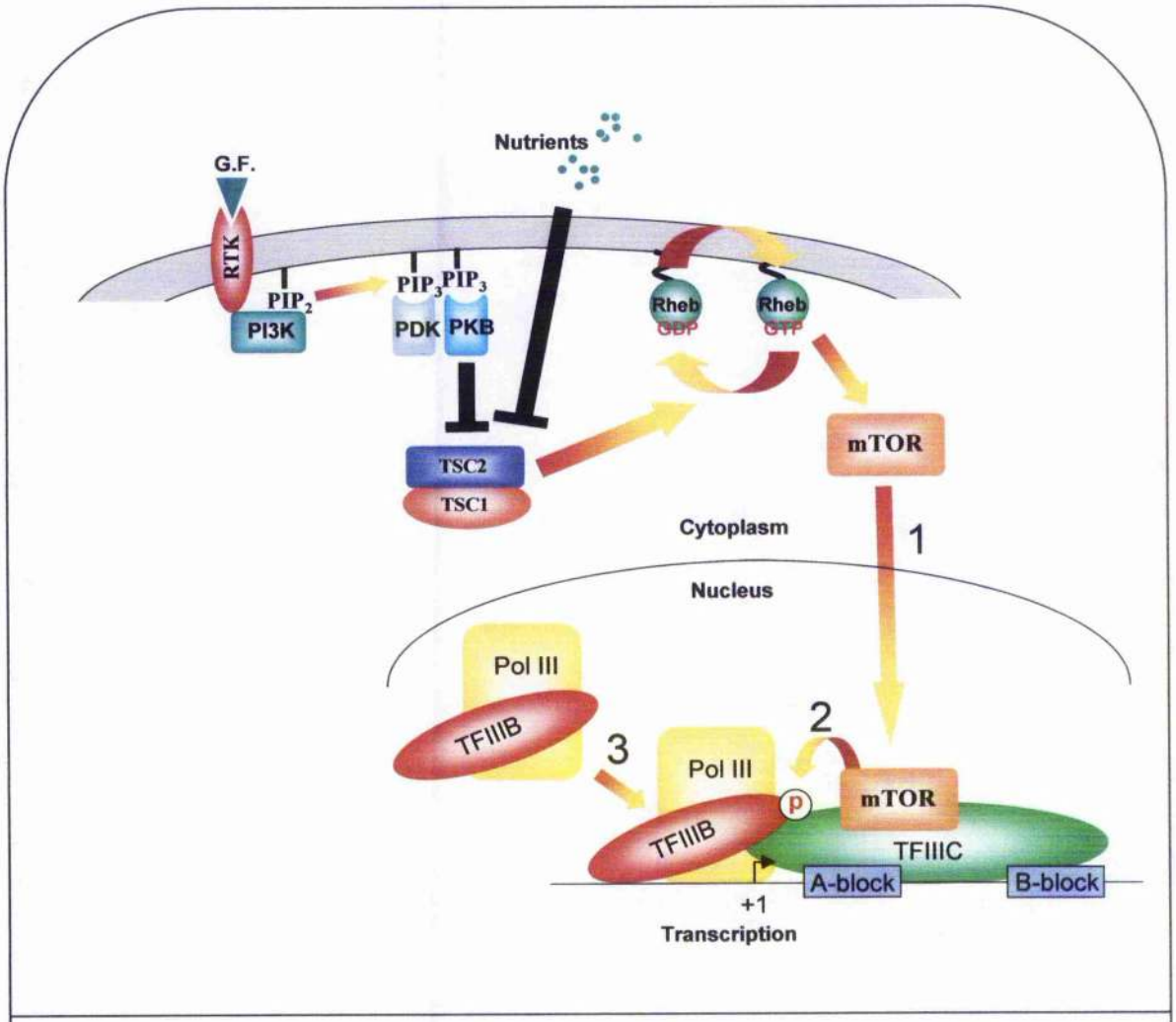


Figure 7.1 Proposed model of how mTOR regulates pol III transcription. mTOR activity is controlled by growth factor and nutrient availability. Active mTOR translocates from the cytoplasm to the nucleus where it directly binds to TFIIIC on the promoter of pol III-transcribed genes (1). Once bound, mTOR phosphorylates TFIIIC (2), which promotes the recruitment of TFIIIB and pol III (3), allowing transcription to take place.

cells in culture (Mirza et al., 2000). Thus, PKB is an important regulator of cellular carcinogenesis.

PKB's ability to regulate the expression of pol III-transcribed genes may reflect its involvement in the mTOR pathway, as it has been shown to phosphorylate and inactivate TSC2 (Cai et al., 2006). PKB has also been shown to regulate the activity of a number of transcription factors. It is known to directly phosphorylate forkhead transcription factors (Brunet et al., 1999). Furthermore, it also regulates the activity of the transcription factors p53 and NF-κB (Downward, 2004). Several papers have shown PKB becomes active and undergoes nuclear translocation upon growth factor stimulation (Borgatti et al., 2000; Wang and Brattain, 2006). Therefore, PKB could be regulating the expression of pol III-transcribed genes through a direct phosphorylation event, which could be controlled by its cellular localization. Future experiments will be needed to determine the mechanism by which PKB regulates the expression of pol III-transcribed genes. An initial experiment will again use the substrate competitive inhibitor in an *in vitro* transcription assay using extract from cells that have previously been treated with rapamycin. This should help determine if PKB is working through mTOR to regulate the expression of pol III-transcribed genes *in vitro*.

7.3 TSC2

Experiments carried out in chapters 3 and 4 demonstrated that TSC2 regulates the expression of genes transcribed by pol III. Knockout of TSC2 results in an elevation in pol III transcription along with an increase in the promoter occupancy of TFIIIC, TFIIIB and pol III. This is in contrast to rapamycin-treated cells, where the promoter occupancy of TFIIIC was unaltered. Western analysis revealed that the 110kDa subunit of TFIIIC was elevated in the TSC2 knockouts, whereas the other subunits remain constant. This is consistent with previous reports that have suggested that this subunit is the rate-limiting component of pol III transcription (Hoeffler et al., 1988; Kovelman and Roeder, 1992; Sinn et al., 1995). Thus, TSC2 might regulate the expression of pol III-transcribed genes through the expression of TFIIIC110.

An inducible HeLa cell line was used to test the theory that TFIIIC110 is the rate-limiting component of pol III transcription. Results from chapter 5 demonstrated that induction of HA-TFIIIC110 had no effect on the expression of pol III-transcribed genes. This disputes the mechanism proposed in previous papers where TFIIIC110 was suggested to be 'the central controlling subunit for transcription by RNA polymerase III' in HeLa cells (Sinn et al., 1995). Instead, Brf1, a component of TFIIIB, has been shown to be the rate-limiting component. Its overexpression has been shown to induce the expression of 5S rRNA and $tRNA^{Leu}$ in HeLa cells (Innes et al., 2006). It should be remembered that TSC2 was knocked out in MEF cells, whereas the overexpression of TFIIIC110 was performed in HeLa cells. However, work in chapter 5 has gone some way to dispel the idea that TFIIIC110 is the rate-limiting component of pol III transcription.

Disruption of TSC2 function leads to the development of tuberous sclerosis, characterized by the formation of benign tumours in the central nervous system, kidney, heart, lung and skin (Mak and Yeung, 2004). Loss of TSC2 function in tumours results in the elevation of mTOR signalling (El-Hashemite et al., 2003). Rodent models have shown that germline mutations within TSC2 result in the formation of tumours that are sensitive to rapamycin treatment (Kenerson et al., 2002). Thus, loss of TSC2 function results in the formation of tumours, which is mediated through an elevation in the activity of mTOR. Work in chapters 3 and 4 have shown that mTOR controls the expression of pol III-transcribed genes. Therefore, TSC2's ability to control the expression of pol III-transcribed highlights a novel role for this tumour suppressor

7.4 Rheb

Rheb acts downstream of TSC1/TSC2 and upstream of mTOR to regulate cellular growth (Inoki et al., 2003a). Work in chapter 3 demonstrated that the knockdown of Rheb resulted in a decrease in the presence of pol III transcripts. This, along with other data from chapters 3 and 4, would suggest that the knockdown of Rheb causes a decrease in the activity of mTOR, which leads to a reduction in the expression of pol III-transcribed genes.

Rheb is ubiquitously expressed in human tissue (Gromov et al., 1995) and plays a role in cellular transformation (Basso et al., 2005; Gromov et al., 1995; Mak and Yeung, 2004).

Its expression has been shown to be upregulated in SV40-transformed cells (Gromov et al., 1995). Furthermore, as previously stated, Rheb is known to be regulated by the tumour suppressor TSC2 (Inoki et al., 2003a). Overexpression of the TSC1/TSC2 complex results in a decrease in the activity of Rheb (Inoki et al., 2003a). Rheb is also known to be a target for an anticancer drug, SCH66336 (Basso et al., 2005). SCH66336 inhibits Rheb's farnesylation, which is thought to be required for its membrane localization (Basso et al., 2005). Addition of this drug reduces Rheb's activity and results in a decrease in mTOR signalling which is correlated with the anti-tumour properties of SCH66336 (Basso et al., 2005). Therefore, Rheb is an important factor in the control of cellular growth and a target for cancer treatment.

7.5 S6K

Previous studies have shown that S6K, a kinase downstream of mTOR, regulates the expression of pol I-transcribed genes (Hannan et al., 2003). As pol I and pol III are regulated by similar mechanisms (White, 2005), it was thought that regulation of pol III by mTOR might also require S6K. However, knockdown of either S6K1 or S6K2 had no effect on the expression of pol III-transcribed genes, whereas the presence of pol I transcripts was diminished, highlighting a difference in the regulation of pol I and pol III-transcribed genes.

7.6 Maf1 and mTOR

mTOR coordinates cell growth with nutrient availability. Previous studies in yeast have shown that the addition of the mTOR inhibitor rapamycin reduces the expression of pol III transcripts (Upadhya et al., 2002b). This effect is lost when Maf1 (a repressor of pol III transcription) is deleted (Upadhya et al., 2002b). Cells that lack Maf1 are also unable to repress the expression of pol III-transcribed when subjected to nutrient deprivation, DNA damage or oxidative stress (Desai et al., 2005; Upadhya et al., 2002b). Thus, Maf1 is a common component of multiple signalling pathways that repress pol III transcription in yeast. Recent studies in yeast have shown that Maf1 binds to pol III-transcribed genes (Oficjalska-Pham et al., 2006; Roberts et al., 2006). Furthermore, its binding is elevated after the addition of rapamycin, which is correlated with a decrease in the promoter occupancy of TFIIIB and pol III (Oficjalska-Pham et al., 2006; Roberts et al., 2006). Results in chapter 3 show a similar response to rapamycin treatment with a decrease in the promoter occupancy of both pol III and TFIIIB. Therefore, Maf1 may be an important mediator of the rapamycin response.

Maf1's activity is controlled through its phosphorylation (Oficjalska-Pham et al., 2006; Roberts et al., 2006). When cells are treated with rapamycin, Maf1 becomes hypophosphorylated through the phosphatase PP2A (Oficjalska-Pham et al., 2006). This allows Maf1 to accumulate in the nucleus where it represses the expression of pol III-transcribed genes (Oficjalska-Pham et al., 2006). However, work in chapter 4 has shown that rapamycin-mediated repression of pol III-transcribed genes is not alleviated with the use of PP2A inhibitors. This preliminary result may reflect differences in response

observed in yeast and mammalian systems, though further work is needed to look at this in greater detail.

Although homologues of Maf1 have been discovered in higher eukaryotes (Pluta et al., 2001) its cellular role thus far has only been reported in yeast. Therefore, future experiments will look at Maf1 to see if it also represses the expression of pol III-transcribed genes in a mammalian system. ChIP analysis will be used to determine the promoter occupancy of Maf1 on pol III-transcribed genes, to see if it has a similar role to that observed in yeast. Current work, subsequent to the completion of this thesis has indicated that Maf1 plays a pivotal role in the regulation of pol III-transcribed genes in response to rapamycin.

7.7 mTOR and histone acetylation

ChIP assays in chapter 3 showed that the promoter occupancy of pol III and TFIIIB is diminished when mTOR signalling is blocked by rapamycin. In parallel, there is a decrease in the level of histone H3 acetylation. Indeed, previous studies in yeast have shown that mTOR regulates the acetylation of histones found on pol I-transcribed genes (Claypool et al., 2003; Tsang et al., 2003). Experiments in chapter 5 demonstrated that acetylation is important for the expression of pol III-transcribed genes. Addition of the HDAC inhibitor TSA led to an increase in the acetylation of histone H3 and H4 and an elevation in the promoter occupancy of TFIIIB and pol III. Therefore, on pol III-transcribed genes, correlative data suggest that the promoter occupancy of pol III and

TFIIIB is elevated when there is an increase in the level of histone acetylation. Consistent with this, histone acetylation is seen as a marker for transcriptional activation in many systems (Eberharter and Becker, 2002). Previous *in vitro* studies have also shown that the acetylation of histone tails by HATs promotes transcriptional activation (An et al., 2004; An et al., 2002). Thus, mTOR might promote the expression of pol III-transcribed genes through the recruitment of HATs.

Future experiments will look at the role of histone acetylation and how this is affected by the inhibition of the mTOR pathway. Work will focus on the recruitment of HATs and HDACs that are associated with histone H3 acetylation and deactylation. This will be carried out using ChIP assays to observe the promoter occupancy of these proteins on pol III-transcribed genes in response to the activity of mTOR.

7.8 Factor acetylation

The role of acetylation is not confined to histones, as a number of other proteins, including transcription factors, are known to be regulated by this covalent modification (Glozak et al., 2005). Work in chapter 5 using TSA demonstrated that acetylation has an effect on pol III activity on naked DNA templates where no histones are. This highlights a potential role for factor acetylation instead of histone acetylation in the regulation of genes transcribed by pol III. Furthermore, use of recombinant HATs in an *in vitro* transcription assay demonstrated that p300 and TIP60 mildly stimulate the expression of pol III-transcribed genes. This would suggest that they are mediating their effects

through factor acetylation instead of histone acetylation. Both p300 and TIP60 have previously been shown to acetylate transcription factors (Halkidou et al., 2004; Perrot and Rechler, 2005). Therefore, future work will focus on the role of these HATs and their histone-independent function. ChIP assays will be used to determine if they are found on pol III-transcribed genes. Co-immunoprecipitation will then be used to identify any potential target within the pol III transcriptional machinery.

7.9 Histone methylation

Work in chapter 6 has shown that pol III-transcribed genes are associated both histone H3 Lys^4 and histone H3 Lys^9 methylation. H3 Lys^4 methylation is associated with transcriptionally active genes (Liang et al., 2004), whereas H3 Lys^9 methylation is associated with transcriptionally repressed genes (Nakayama et al., 2001). Consistent with this, sequential ChIPs demonstrated that the pol III transcriptional machinery had greater promoter occupancy when there was an elevated level of H3 Lys^4 methylation compared to H3 Lys^9 methylation.

Future work is needed to identify the histone methyltransferases and demethylases involved in the regulation of histone H3 Lys^4 and histone H3 Lys^9 methylation on pol III-transcribed genes. Over the last few years, a number of studies have identified histone methyltransferases which target histone H3. Examples include set1 and Suv39h, which methylate H3 Lys^4 and H3 Lys^9, respectively (Lachner et al., 2001; Milne et al., 2002; Rea et al., 2000; Schneider et al., 2005). Following on from this, recent publications

have demonstrated that histone methylation is a reversible process, with the discovery of histone demethylases. These include LSD1 and members of the Jmjc family, which have been shown to demethylate both H3 Lys^4 and H3 Lys^9 (Klose et al., 2006; Shi et al., 2004; Tsukada et al., 2006; Whetstine et al., 2006; Yamane et al., 2006). Thus, histone methyltransferases and demethylases described here may provide potential targets when looking at the regulation of pol III-transcribed genes.

Histone methylation provides binding sites for the recruitment of chromodomain proteins that regulate the expression of genes. Methylation of histone H3 Lys^4 can promote the recruitment of Chd1, which is associated with the transcriptional activation of genes (Pray-Grant et al., 2005). Conversely, methylated histone H3 Lys^9 is associated with the recruitment of HP1, a protein associated with gene silencing (Lachner et al., 2001). Again, these proteins may provide potential targets when looking at the regulation of pol III-transcribed genes through histone methylation.

7.10 Importance of these findings

The rate of cellular growth is dependent on the rate of protein synthesis (Baxter and Stanners, 1978). The process of translation mediates protein synthesis, where ribosomes synthesize proteins from mRNA templates. Therefore, ribosomes assume a central role in the process of growth. This is reinforced by the finding that ribosome content is directly proportional to the rate of cellular growth (Kief and Warner, 1981). During mitogenic stimulation, there is an increase in the synthesis of rRNA and ribosomal

proteins which help facilitate an increase in protein synthesis (Johnson et al., 1974; Kief and Warner, 1981; Mauck and Green, 1974). Mitogenic stimulation also leads to an increase in transcription by pol III (Clarke et al., 1996; Felton-Edkins et al., 2003a; Scott et al., 2001; White et al., 1995). Levels of pol III transcripts, such as tRNA play important role in protein synthesis (Francis and Rajbhandary, 1990). Thus, the regulation of pol III is an important determinant of the cells translational capacity and growth.

mTOR also responds to mitogenic stimuli by regulating cellular growth and proliferation (Inoki et al., 2005; Wullschleger et al., 2006). This is in part mediated through its phosphorylation of S6K and 4E-BP1, both of which are involved in protein translation (Inoki et al., 2005; Wullschleger et al., 2006). However, recent studies have demonstrated that mTOR controls rRNA expression through its regulation of components of the pol I transcriptional complex (Hannan et al., 2003; James and Zomerdijk, 2004; Mayer et al., 2004). Work in this present study demonstrates that mTOR also controls the expression of genes encoding rRNA through the regulation of the pol III transcriptional machinery. This highlights a mechanism by which the expression of pol III-transcribed genes are controlled through mitogenic stimuli. Furthermore, ChIP assays demonstrated that mTOR directly binds to pol III-transcribed genes. This suggests, for the first time, that mTOR regulates transcription through a direct interaction with a genes transcriptional machinery in situ on DNA.

PKB, TSC2 and Rheb have all been shown to regulate the expression of pol III-transcribed genes and are components of the mTOR pathway. Each has been shown to be a target for oncogenic activation (Basso et al., 2005; Besson et al., 1999; Gromov et al., 1995; Mak and Yeung, 2004; Tang et al., 2006). The pol III transcriptional machinery has been shown to be the target of a number of other oncogenes and tumour suppressors (White, 2005). High levels of pol III activity activity are required to sustain rapid growth (White, 2003). Therefore, mTOR's ability to control the expression of pol III-transcribed genes and its deregulation in cancer may provide a mechanism by which cellular growth is increased in transformed cells.

Chapter 8-**References**

Abraham, R. T., and Wiederrecht, G. J. (1996). Immunopharmacology of rapamycin. Annu Rev Immunol *14*, 483-510.

Allen, T. A., Von Kaenel, S., Goodrich, J. A., and Kugel, J. F. (2004). The SINE-encoded mouse B2 RNA represses mRNA transcription in response to heat shock. Nature Structural and Mol Biol *11*, 816-821.

An, W., Kim, J., and Roeder, R. G. (2004). Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53. Cell *117*, 735-748.

An, W., Palhan, V. B., Karymov, M. A., Leuba, S. H., and Roeder, R. G. (2002). Selective requirements for histone H3 and H4 N termini in p300-dependent transcriptional activation from chromatin. Mol cell *9*, 811-821.

Baker, R. E., Camier, S., Sentenac, A., and Hall, B. D. (1987). Gene size differentially affects the binding of yeast transcription factor t to two intragenic regions. Proc Natl Acad Sci USA *84*, 8768-8772.

Baker, R. E., Gabrielsen, O., and Hall, B. D. (1986). Effects of tRNATyr point mutations on the binding of yeast RNA polymerase III transcription factor C. J Biol Chem *261*, 5275-5282.

Bannister, A. J., Schneider, R., and Kouzarides, T. (2002). Histone methylation: dynamic or static? Cell *109*, 801-806.

Basso, A. D., Miraz, A., Liu, G., Long, B. J., Bishop, W. R., and Kirschmeier, P. (2005). The farnesyl transferase inhibitor (FTI) SCH66336 (lonafarni) inhibits rheb farnesylation and mTOR signaling. J Biol Chem *280*, 31101-31108.

Baxter, G. C., and Stanners, C. P. (1978). The effect of protein degradation on cellular growth characteristics. J Cell Physiol *96*, 139-146.

Beck, T., and Hall, M. N. (1999). The TOR signalling pathway controls the nuclear localization of nutrient regulated transcription factors. Nature *402*, 689-692.

Bellacosa, A., Testa, J. R., Staal, S. P., and Tsichlis, P. N. (1991). A retroviral oncogene, akt, encoding a serine-theronine kinase containing a SH2-like region. Science *254*, 274-277.

Besson, A., Robbins, S. M., and Yong, V. W. (1999). PTEN/MMAC1/TEP1 in signal transduction and tumourigenesis. Eur J Biochem *263*, 605-611.

Bhat, R. A., and Thimmappaya, B. (1985). Construction and analysis of additional adenovirus substitution mutants confirm the complementation of VAI RNA function by two small RNAs encoded by Epstein-Barr virus. J Virol *56*, 750-756.

Bladon, T. S., Fregeau, C. J., and McBurney, M. W. (1990). Synthesis and processing of small B2 transcripts in mouse embryonal carcinoma cells. Mol Cell Biol *10*, 4058-4067.

Borgatti, P., Martelli, A. M., Bellacosa, A., Casto, R., Massari, L., Capitani, S., and Neri, L. M. (2000). Translocation to the nucleus of osteoblast-like MC3T3-E1 cells exposed to proliferative growth factors. FEBS Lett *477*, 27-32.

Boudeau, J., Sapkota, G., and Alessi, D. R. (2003). LKB1, a protein kinase regulating cell proliferation and polarity. FEBS Lett *546*, 159-165.

Bouvet, P., Dimitrov, S., and Wolffe, A. P. (1994). Specific regulation of Xenopus chromosomal 5S rRNA gene transcription in vivo by histone H1. Genes Dev *8*, 1147-1159.

Briggs, S. D., Bryk, M., Strahl, B. D., Cheung, W. L., Davie, J. K., Dent, S. Y. R., Winston, F., and Allis, C. D. (2006). Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in Saccharomyces cerevisiae. Genes Dev *15*, 3286-3295.

Brown, D. D., Wensink, P. C., and Jordan, E. (1971). Purification and some characteristics of 5S DNA from Xenopus laevis. Proc Natl Acad Sci USA *68*, 3175-3179.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell *96*, 857-868.

Burnol, A.-F., Margottin, F., Huet, J., Almouzni, G., Prioleau, M.-N., Mechali, M., and Sentenac, A. (1993a). TFIIIC relieves repression of U6 snRNA transcription by chromatin. Nature *362*, 475-477.

Burnol, A.-F., Margottin, F., Schultz, P., Marsolier, M.-C., Oudet, P., and Sentenac, A. (1993b). Basal promoter and enhancer element of yeast U6 snRNA gene. J Mol Biol *233*, 644-658.

Byvoet, P. (1972). In vivo turnover and distribution of radio-N-methyl in arginine-rich histones from rat tissue. Arch Biochem Biophys *152*, 887-888.

Cai, S. L., Tee, A. R., Short, J. D., Bergeron, J. M., Kim, J., Shen, J., Guo, R., Johnson, C. L., Kiguchi, K., and Walker, C. L. (2006). Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. J Biol Chem *173*, 279-289.

Caslini, C., Capo-Chichi, C. D., Roland, I. H., Nicolas, E., Yeung, A. T., and Xu, X. X. (2006). Histone modifications silence the GATA transcription factor genes in ovarian cancer. Oncogene.

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

Chiu, M. I., Katz, H., and Berlin, V. (1994). RAPT1, a mammalian homolog of yeast Tor, interacts with FKBP12/rapamycin complex. Proc Natl Acad Sci USA *91*, 12574-12578.

Chu, W. M., Ballard, R., Carpick, B. W., Williams, B. R., and Schmid, C. W. (1998). Potential Alu function: regulation of the activity of double-stranded RNA-activated kinase PKR. Mol Cell Biol *18*, 58-68.

Ciliberto, G., Raugei, S., Constanzo, F., Dente, L., and Cortese, R. (1983). Common and interchangeable elements in the promoters of genes transcribed by RNA polymerase III. Cell *32*, 725-733.

Clarke, E. M., Peterson, C. L., Brainard, A. V., and Riggs, D. L. (1996). Regulation of the RNA polymerase I and III transcription systems in response to growth conditions. J Biol Chem *271*, 22189-22195.

Claypool, J. A., French, S. L., Johzuka, K., Eliason, K., Vu, L., Dodd, J. A., Beyer, A. L., and Nomura, M. (2003). Tor pathway regulates Rrn3p-dependent recruitment of yeast RNA polymerase I to the promoter but does not participate in alteration of the number of active genes. Mol Cell Biol *15*, 946-956.

Clemens, K. R., Liao, X., Wolf, V., Wright, P. E., and Gottesfeld, J. M. (1992). Definition of the binding sites of individual zinc fingers in the transcription factor IIIA-5S RNA gene complex. Proc Natl Acad Sci USA *89*, 10822-10826.

Cohen, P., Holmes, C. F. B., and Tsukitani, Y. (1990). Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem Sci *15*, 98-102.

Consortium, I. H. G. S. (2001). Initial sequencing and analysis of the human genome. Nature *409*, 860-921.

Cormack, B. P., and Struhl, K. (1992). The TATA-binding protein is required for transcription by all three nuclear RNA polymerases in yeast cells. *69*, 685-696.

Crighton, D., Woiwode, A., Zhang, C., Mandavia, N., Morton, J. P., Warnock, L. J., Milner, J., White, R. J., and Johnson, D. L. (2003). p53 represses RNA polymerase III transcription by targeting TBP and inhibiting promoter occupancy by TFIIIB. EMBO J *22*, 2810-2820.

Cruz, X., Lois, S., Sanchez-Molina, S., and Martinez-Balbas, M. A. (2005). Do protein motifs read the histone code? Bioessays *27*, 164-175.

Daly, N. L., Arvanitis, D. A., Fairley, J. A., Gomez-Roman, N., Morton, J. P., Spandidos, D. A., and White, R. J. (2005). Derfegulation of RNA polymerase III transcription in cervical epithelium in response to high-risk human papillomavirus. Oncogene *24*, 880-888.

DeRuijter, A. J. M., Van Gennip, A. H., Caron, H. N., Kemp, S., and Van Kuilenburg, B. P. (2003). Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J *370*, 737-749.

Desai, N., Lee, J. H., Upadhya, R., Chu, Y., Moir, R. D., and Willis, I. M. (2005). Two steps in Maf1-dependent repression of transcription by RNA polymerase III. J Biol Chem *280*, 6455-6462.

Dieci, G., and Sentenac, A. (1996). Facilitated recycling pathway for RNA polymerase III. Cell *84*, 245-252.

Downward, J. (2002). Targeting Ras signalling pathways in cancer therapy. Nature Rev Cancer *3*, 11-22.

Downward, J. (2004). PI 3-kinase, Akt and cell survival. Semin Cell Dev Biol *15*, 177-182.

Duerre, J. A., and Lee, C. T. (1974). In vivo methylation and turnover of rat brain histones. J Neurochem *23*, 541-547.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. Genes Dev *12*, 2245-2262.

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

Eberharter, A., and Becker, P. B. (2002). Histone acetylation: a switch between repressive and permissive chromatin. EMBO Rep *3*, 224-229.

Edmondson, D. G., and Roth, S. Y. (1996). Chromatin and transcription. FASEB *10*, 1173-1182.

Eisenman, R. N. (2001). Deconstructing Myc. Genes Dev *15*, 2023-2030.

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

El-Hashemite, N., Zhang, H., Henske, E. P., and Kwiatkowski, D. J. (2003). Mutation in TSC2 and activation of mammalian target of rapamycin signalling pathway in renal angiomyolipoma. Lancet *361*, 1348-1349.

Elion, E. A., and Warner, J. R. (1984). The major promoter element of rRNA transcription in yeast lies 2 kb upstream. Cell *39*, 663-673.

Englander, E. W., and Howard, B. H. (1995). Nucleosome positioning by human Alu elements in chromatin. J Biol Chem *270*, 10091-10096.

Englander, E. W., Wolffe, A. P., and Howard, B. H. (1993). Nucleosome interactions with a human Alu element. J Biol Chem *268*, 19565-19573.

Espinoza, C. A., Allen, T. A., Hieb, A. R., Kugel, J. F., and Goodrich, J. A. (2004). B2 RNA binds directly to RNA polymerase II to repress transcript synthesis. Nature Structural and Mol Biol *11*, 822-829.

Fabrizio, P., Coppo, A., Fruscoloni, P., Benedetti, P., Di Segni, G., and Tocchini-Valentini, G. P. (1987). Comparative mutational analysis of wild-type and stretched tRNALeu3 gene promoters. Proc Natl Acad Sci USA *84*, 8763-8767.

Fairley, J. A., Scott, P. H., and White, R. J. (2003). TFIIIB is phosphorylated, disrupted and selectively released from tRNA promoters during mitosis in vivo. EMBO J *22*, 5841-5850.

Faust, R. A., Gapany, M., Tristani, P., Davis, A., Adams, G. L., and Ahmed, K. (1996a). Elevated protein kinase CK2 activity in chromatin of head and neck tumours: associated with malignant transformations. Cancer Lett *101*, 31-35.

Faust, R. A., Gapany, M., Tristani, P., Davis, A., Adams, G. L., and Ahmed, K. (1996b). Elevated protein kinase CK2 activity in chromatin of head and neck tumours: association with malignant transformation. Cancer Lett *101*, 31-35.

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

Felton-Edkins, Z. A., Kenneth, N. S., Brown, T. R. P., Daly, N. L., Gomez-Roman, N., Grandori, C., Eisenman, R. N., and White, R. J. (2003b). Direct regulation of RNA polymerase III transcription by RB, p53 and c-Myc. Cell Cycle *3*, 181-184.

Felton-Edkins, Z. A., and White, R. J. (2002). Multiple mechanisms contribute to the activation of RNA polymerase III transcription in cells transformed by papovaviruses. J Biol Chem *277*, 48182-48191.

Ferrari, R., Rivetti, C., Acker, J., and Dieci, G. (2004). Distinct roles of transcription factors TFIIIB and TFIIIC in RNA polymerase III transcription reinitiation. Proc Natl Acad Sci USA *101*, 13442-13447.

Francis, M. A., and Rajbhandary, U. L. (1990). Expression and function of a human initiator tRNA gene in the yeast Saccharomyces cerevisiae. Mol Cell Biol *10*, 4486-4494.

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

Gao, X., Zhang, Y., Arrazola, P., Hino, O., Kobayashi, T., Yeung, R. S., Ru§, B., and Pan, D. (2003). Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nature Cell Biol *4*, 699-704.

Garaza, J., Aude, Y. W., and Saucedo, J. F. (2002). Can we prevent in-stent restenosis. Curr Opin Cardiol *17*, 518-525.

Geiduschek, E. P., and Kassavetis, G. A. (2001). The RNA polymerase III transcription apparatus. J Mol Biol *310*, 1-26.

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

Gingras, A. C., Kennedy, S. C., O'Leary, M. A., Sonenberg, N., and Hay, N. (1998). 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt (PKB) signalling pathway. Genes Dev *12*, 502-513.

Glozak, M. A., Sengupta, N., Zhang, X., and Seto, E. (2005). Acetylation and deacetylation of non-histone proteins. Gene *363*, 15-23.

Gold, H. A., Topper, J. N., Clayton, D. A., and Craft, J. (1989). The RNA processing enzyme RNase MRP is identical to the RNP and related to RNase P. Science *245*, 1377-1380.

Gomez, M. R. (1991). Phenotypes of the tuberous sclerosis complex with revision of diagnostic criteria. Ann N Y Acad Sci *615*, 1-7.

Gomez-Roman, N., Grandori, C., Eisenman, R. N., and White, R. J. (2003). Direct activation of RNA polymerase III transcription by c-Myc. Nature *421*, 290-294.

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

Graham, E. L. (Data not published). The regulation of pol III transcription by mTOR.

Gromov, P. S., Madsen, P., Tomerup, N., and Celis, J. E. (1995). A novel approach for expression cloning of small GTPases: identification, tissue distribution and chromosome mapping of the human homolog rheb. FEBS Lett *377*, 221-226.

Gu, M., Lynch, J., and Brecher, P. (2000). Nitric oxide increases p21(Waf1/Cip1) expression by a cGMP-dependent pathway that includes activation of extracellular signal-regulated kinase p70 (S6K). J Biol Chem *275*, 11389-11396.

Hakimi, M.-A., Bochar, D. A., Schmiesing, J. A., Dong, Y., Barak, O. G., Speicher, D. W., Yokomori, K., and Shiekhattar, R. (2002). A chromatin remodelling complex that loads cohesin onto human chromosomes. Nature *418*, 994-998.

Halkidou, K., Logan, I. R., Cook, S., Neal, D. E., and Robson, C. N. (2004). Putative involvement of the histone acetyltransferase Tip60 in ribosomal gene transcription. Nucleic acids re *32*, 1654-1665.

Han, M., and Grunstein, M. (1988). Nucleosome loss activates yeast downstream promoters in vivo. Cell *55*, 1137-1145.

Hannan, K. M., Brandenburger, Y., Jenkins, A., Sharkey, K., Cavanaugh, A., Rothblum, L., Moss, T., Poortinga, G., McArthur, G. A., Pearson, R. B., and Hannan, R. D. (2003). mTOR-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF. Mol Cell Biol *23*, 8862-8877.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Tokunaga, C., Avruch, J., and Yonezawa, K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell *110*, 177-189.

Hara, K., Yonezawa, K., Weng, Q.-P., Kozlowski, M. T., Belham, C., and Avruch, J. (1998). Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common mechanism. J Biol Chem *273*, 14484-14494.

Harrington, L. S., Findlay, G. M., Gray, A., Tolkacheva, T., Wigfield, S., Rebholz, H., Barnett, J., Leslie, N. R., Cheng, S., Shepard, P. R., *et al.* (2006). The TSC1-2 tumour suppressor controls insulin-PI3K signalling via regulation of IRS proteins. J Biol Chem *166*, 213-223.

Hartl, P., Gottesfeld, J., and Forbes, D. J. (1993). Mitotic repression of transcription in vitro. J Cell Biol *120*, 613-624.

Hartley, D., and Cooper, G. M. (20002). Role of mTOR in the degradation of IRS-1:regulation of PP2A activity. J Cell Biochem *85*, 304-314.

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

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

Hsieh, Y.-J., Kundu, T. K., Wang, Z., Kovelman, R., and Roeder, R. G. (1999a). The TFIIIC90 subunit of TFIIIC interacts with multiple components of the RNA polymerase

III machinery and contains a histone-specific acetyltransferase activity. Mol Cell Biol *19*, 7697-7704.

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

Hu, P., Wu, S., and Hernandez, N. (2003). A minimal RNA polymerase III transcription system from human cells reveals positive and negative roles for CK2. Mol Cell *12*, 699-709.

Huang, Y., Fang, J., Bedford, M. T., Zhang, Y., and Xu, R. M. (2006). Recognition of histone H3 lysine-4 methylation by the double tudor domain of JMJD2A. Science *312*, 748-751.

Innes, F., Ramsbottom, B., and White, R. J. (2006). A test of the model that RNA polymerase III transcription is regulated by selective induction of the 110 kDa subunit of TFIIIC. Nucleic Acids Res *34*, 3399-3407.

Inoki, K., Li, Y., Xu, X., and Guan, K.-L. (2003a). Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev *17*, 1829-1834.

Inoki, K., Ouyang, H., Li, Y., and Guan, K. L. (2005). Signaling by target of rapamycin proteins in cell growth control. Microbiol Mol Biol Rev *69*, 79-100.

Inoki, K., Zhu, T., and Guan, K. L. (2003b). TSC2 mediates cellular energy response to control cell growth and survival. Cell *115*, 577-590.

James, M. J., and Zomerdijk, J. C. B. M. (2004). Phosphatidylinositol 3-kinase and mTOR signaling pathways regulate RNA polymerase I transcription in response to IGF-1 and nutrients. J Biol Chem *279*, 8911-8918.

Johnson, L. F., Abelson, H. T., Green, H., and Penman, S. (1974). Changes in RNA in relation to growth of the fibroblast. Amounts of mRNA, rRNA, and tRNA in resting and growing cells. Cell *1*, 95-100.

Johnston, I. M., Allison, S. J., Morton, J. P., Schramm, L., Scott, P. H., and White, R. J. (2002). CK2 forms a stable complex with TFIIIB and activates RNA polymerase III transcription in human cells. Mol Cell Biol *22*, 3757-3768.

Jung, M. (2001). Inhibitors of histone deacetylase as new anticancer agents. Curr Med Chem *8*, 1505-1511.

Kandolf, H. (1994). The H1A histone variant is an in vivo repressor of oocyte-type 5S gene transcription in Xenopus laevis embryos. Proc Natl Acad Sci USA *91*, 7257-7261.

Kassavetis, G. A., Blanco, J. A., Johnson, T. E., and Geiduschek, E. P. (1992). Formation of open and elongating transcription complexes by RNA polymerase III. J Mol Biol *226*, 47-58.

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

Kassavetis, G. A., Kumar, A., Letts, G. A., and Geiduschek, E. P. (1998). A post-recruitment function for the RNA polymerase III transcription-initiation factor IIIB. Proc Natl Acad Sci USA *95*, 9196-9201.

Kassavetis, G. A., Nguyen, S. T., Kobayashi, R., Kumar, A., Geiduschek, E. P., and Pisano, M. (1995). Cloning, expression, and function of TFC5, the gene encoding the B" component of the Saccharomyces cerevisiae RNA polymerase III transcription factor TFIIIB. Proc Natl Acad Sci USA *92*, 9786-9790.

Keller, H. J., You, Q. M., Romaniuk, P. J., and Gottesfeld, J. M. (1990). Additional intragenic promoter elements of the Xenopus 5S RNA genes upstream from the TFIIIA-binding site. Mol Cell Biol *10*, 5166-5176.

Kenerson, H. L., Aicher, L. D., True, L. D., and Yeung, R. S. (2002). Activated mammalian target of rapamycin pathway in the pathogenesis of tuberous sclerosis complex renal tumours. Cancer Res *62*, 5645-5650.

Kief, D. R., and Warner, J. R. (1981). Coordinate control of syntheses of ribosomal ribonucleic acid and ribosomal proteins during nutritional shift-up in Saccharomyces cerevisiae. Mol Cell Biol *1*, 1007-1015.

Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Lateck, R. R., Erjument-Bromage, H., Tempest, P., and Sabatini, D. M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*, 163-175.

Kim, D. H., Sarbassov, D. D., Ali, S. M., Lateck, R. R., Guntur, K. V., Erdjument-Bromage, H., Tempest, P., and Sabatini, D. M. (2003). GbetaL, a positive regulator of rapamycin-sensitive pathway required for nutrient-sensitive interaction between raptor and mTOR. Mol cell *11*, 895-904.

Kim, J. E., and Chen, J. (2000). Cytoplasmic-nuclear shuttling of FKBP12-rapamycin associated protein is involved in rapamycin-sensitive signalling and translation initiation. Proc Natl Acad Sci USA *97*, 14340-14345.

Kim, T. K., and Roeder, R. G. (1994). Involvement of the basic repeat domain of TATA-binding protein (TBP) in transcription by RNA polymerases I, II, and III. J Biol Chem *269*, 4891-4894.

Klose, R. J., Yamane, K., Bae, Y., Zhang, D., Erdjument-Bromage, H., Tempest, P., Wong, J., and Zhang, Y. (2006). The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36. Nature *442*, 312-316.

Knoepfler, P. S., Zhang, X., Cheng, P. F., Gafken, P. R., McMahon, S. B., and Eisenman, R. N. (2006). Myc influences global chromatin structure. EMBO J *25*, 2723-2734.

Kobayashi, T., Minowa, O., Sugitani, Y., Takai, S., Mitani, H., Kobayashi, E., Noda, T., and Hino, O. (2001). A germ-line Tsc1 mutation causes tumour development and

embryonic lethality that are similar, but not identical to, those caused by Tsc2 mutations in mice. Proc Natl Acad Sci USA *98*, 8762-8767.

Kondo, Y., and Issa, J.-P. J. (2003). Enrichment for histone H3 lysine 9 methylation at Alu repeats in human cells. J Biol Chem *278*, 27658-27662.

Kouzarides, T. (2002). Histone methylation in transcriptional control. Curr Opin Genet Dev *12*, 198-209.

Kovelman, R., and Roeder, R. G. (1992). Purification and characterization of two forms of human transcription factor IIIC. J Biol Chem *267*, 24446-24456.

Kravchenko, J. E., Rogozin, I. B., Koonin, E. V., and Chumakov, P. M. (2005). Transcription of mammalian messenger RNAs by nuclear RNA polymerase of mitochondrial origin. Nature *436*, 735-739.

Kristof, A. S., Marks-Konczalik, J., Billings, E., and Moss, J. (2003). Stimulation of signal transducer and activator of transcription-1 (STAT1)-dependent gene transcription by lipopolysaccharide and interferon-gamma is regulated by mammalian target of rapamycin. J Biol Chem *278*, 33637-33644.

Kundu, T. K., Wang, Z., and Roeder, R. G. (1999). Human TFIIIC relieves chromatin-mediated repression of RNA polymerase III transcription and contains an intrinsic histone acetyltransferase activity. Mol Cell Biol *19*, 1605-1615.

Kunz, J., Henriquez, R., Schneider, U., Deuter Reinhard, M., Movva, N. R., and Hall, M. N. (1993). Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell *73*, 585-596.

Kwon, H. S., Huang, B., Jeoung N, H., Wu, P., Steussy, C. N., and Harris, R. A. (2006). Retinoic acids and trichostatin A (TSA), a histone deacetylase inhibitor, induce human pyruvate dehydrogenase kinase 4 (PDK4) gene expression. Biochem Biophys Acta *1759*, 141-151.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116-120.

Lachner, M., O'Sullivan, R. J., and Jenuwein, T. (2003). An epigenetic road map for histone lysine methylation. J Cell Sci *116*, 2117-2124.

Lagna, G., Kovelman, R., Sukegawa, J., and Roeder, R. G. (1994). Cloning and characterization of an evolutionarily divergent DNA-binding subunit of mammalian TFIIIC. Mol Cell Biol *14*, 3053-3064.

Larminie, C. G. C., Alzuherri, H. M., Cairns, C. A., McLees, A., and White, R. J. (1998). Transcription by RNA polymerases I and III: a potential link between cell growth, protein synthesis and the retinoblastoma protein. J Mol Med *76*, 94-103.

Larminie, C. G. C., Sutcliffe, J. E., Tosh, K., Winter, A. G., Felton-Edkins, Z. A., and White, R. J. (1999). Activation of RNA polymerase III transcription in cells transformed by simian virus 40. Mol Cell Biol *19*, 4927-4934.

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

Lee, J.-Y., and Engelke, D. R. (1989). Partial characterization of an RNA component that copurifies with Saccharomyces cerevisiae RNase P. Mol Cell Biol *9*, 2536-2543.

Leresche, A., Wolf, V. J., and Gottesfeld, J. M. (1996). Repression of RNA polymerase II and III transcription during M phase of the cell cycle. Exp Cell Res *229*, 282-288.

Li, H., Tsang, C. K., Watkins, M., Bertram, P. G., and Zheng, X. F. S. (2006). Nutrient regulates Tor1 nuclear localization and association with rDNA promoter. Nature *[Epub ahead of print]*.

Li, Y., Inoki, K., and Guan, K. L. (2004). Biochemical and functional characterization of mall GTPase Rheb and TSC2 GAP activity. Mol Cell Biol *24*, 7965-7975.

Liang, G., Lin, J. C. J., Wei, V., Yoo, C., Cheng, J. C., Nguyen, C. T., Weisenberger, D. J., Egger, G., Gonzales, F. A., and Jones, P. A. (2004). Distinct localization of histone H3 acetylation and H3-K4 methylation to the transcription start sites in the human genome. Proc Natl Acad Sci USA *101*, 7357-7362.

Liu, W.-M., Chu, W.-M., Choudary, P. V., and Schmid, C. W. (1995). Cell stress and translational inhibitors transiently increase the abundance of mammalian SINE transcripts. Nucleic Acids Res *23*, 1758-1765.

Liu, W.-M., Maraia, R. J., Rubin, C. M., and Schmid, C. W. (1994). Alu transcripts: cytoplasmic localisation and regulation by DNA methylation. Nucleic Acids Res *22*, 1087-1095.

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., and Avruch, J. (2005a). Rheb binds and regulates the mTOR kinase. Current Biology *15*, 702-713.

Long, X., Ortiz-Vega, S., Lin, Y., and Avruch, J. (2005b). Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. J Biol Chem *280*, 23433-23436.

Luger, K., Rechsteiner, T. J., Flaus, A. J., Waye, M. Y., and Richmond, T. J. (1997). Characterization of nucleosome core particles containing histone proteins made in bacteria. J Mol Biol *272*, 301-311.

Majahan (1994). Modulation of transcription of rRNA genes by rapamycin. Int J Immunopharmac *16*, 711-721.

Mak, B. C., and Yeung, R. S. (2004). The Tuberous sclerosis complex genes in tumour development. Cancer Invest *22*, 588-603.

Makalowski, W. (2003). Not junk after all. Science *300*, 1246-1247.

Manning, B. D., Logsdon, M. N., Lipovsky, A. I., Abbott, D., Kwiatkowski, D. J., and Cantley, L. C. (2006). Feedback inhibition of Akt signalling limits the growth of tumour lacking Tsc2. Genes Dev *19*, 1773-1778.

Marsolier, M.-C., Tanaka, S., Livingstone-Zatchej, M., Grunstein, M., Thoma, F., and Sentenac, A. (1995). Reciprocal interferences between nucleosomal organization and transcriptional activity of the yeast SNR6 gene. Genes Dev *9*, 410-422.

Mauck, J. C., and Green, H. (1974). Regulation of pre-transfer RNA synthesis during transition from resting to growing state. Cell *3*, 171-177.

Mayer, C., Zhao, J., Yuan, X., and Grummt, I. (2004). mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. Genes Dev *18*, 423-434.

McLaren, A., and Goddard, J. P. (1986). The nucleotide sequence of a human tRNALeu gene. Nucleic Acids Res *14*, 5938.

Milne, T. A., Briggs, S. D., Brock, H. W., Martin, M. E., Gibbs, D., Allis, C. D., and Hess, J. L. (2002). MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol cell *10*, 1107-1117.

Mirza, A. M., Kohn, A. D., Roth, R. A., and McMahon, M. (2000). Oncogenic transformation of cells by a conditionally active form of the protein kinase Akt/PKB. Cell Growth Differ *11*, 279-292.

Mittal, V., Cleary, M. A., Herr, W., and Hernandez, N. (1996). The Oct-1 POU-specific domain can stimulate small nuclear RNA gene transcription by stabilizing the basal transcription complex SNAPc. Mol Cell Biol *16*, 1955-1965.

Mittal, V., and Hernandez, N. (1997). Role for the amino-terminal region of human TBP in U6 snRNA transcription. Science *275*, 1136-1140.

Morrissey, J. P., and Tollervey, D. (1995). Birth of the snRNPs: the evolution of RNase MRP and the eukaryotic pre-rRNA-processing system. Trends Biochem Sci *20*, 78-82.

Morry, M. J., and Harding, J. D. (1986). Modulation of transcriptional activity and stable complex formation by 5'-flanking regions of mouse tRNAHis genes. Mol Cell Biol *6*, 105-115.

Morse, R. H., Roth, S. Y., and Simpson, R. T. (1992). A transcriptionally active tRNA gene interferes with nucleosome positioning in vivo. Mol Cell Biol *12*, 4015-4025.

Moss, T., and Stefanovsky, V. Y. (2002). At the centre of eukaryotic life. Cell *109*, 545-548.

Mulligan, G., and Jacks, T. (1998). The retinoblastoma gene family: cousins with overlapping interests. Trends Genet *14*, 223-229.

Munger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E., and Howley, P. M. (1989). Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumour suppressor gene product. EMBO J *8*, 4099-4105.

Munstermann, U., Fritz, G., Seitz, G., Lu, Y. P., Schneider, H. R., and Issinger, O. G. (1990). Casein kinase II is elevated in solid human tumours and rapidly proliferating non-neoplastic tissue. Eur J Biochem *189*, 251-257.

Muth, V., Nadaud, S., Grummt, I., and Voit, R. (2001). Acetylation of TAFi68, a subunit of TIF-IB/SL1, activates RNA polymerase I transcription. EMBO J *20*, 1353-1362.

Nakayama, J., Rice, J. C., strahl, B. D., Allis, C. D., and Grewal, S. I. S. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science *292*, 110-113.

Nojima, H., Tokunga, C., Eguchi, S., Oshiro, N., Hidaya, S., Tanaka, N., Avruch, J., and Yonezawa, K. (2003). The mammalian target of rapamycin (mTOR) partner, Raptor, binds the mTOR substrate p70 S6 kinase and 4E-BP1 through their TOR signalling (TOS) motif. J Biol Chem *278*, 15461-15464.

Nolte, R. T., Conlin, R. M., Harrison, S. C., and Brown, R. S. (1998). Differing roles for zinc fingers in DNA recognition: structure of six-finger transcription factor IIIA complex. Proc Natl Acad Sci USA *95*, 2938-2943.

Notterman, D. A., Alon, U., Sierk, A. J., and Levine, A. J. (2001). Transcriptional gene expression profiles of colorectal adenoma, adenocarcinoma, and normal tissue examined by oligonucleotide arrays. Cancer Res *61*, 3124-3130.

Oettel, S., Hartel, F., Kober, I., Iben, S., and Seifart, K. H. (1997). Human transcription factors IIIC2, IIIC1 and a novel component IIIC0 fulfil different aspects of DNA binding to various pol III genes. Nucleic Acids Res *25*, 2440-2447.

Oficjalska-Pham, D., Harismendy, O., Smagowicz, W. J., Gonzalez de Peredo, A., Boguta, M., Sentenac, A., and Lefebvre, O. (2006). General repression of RNA

polymerase III transcription is trigger by protein phosphatase type 2A-mediated dephosphorylation of Maf1. Mol cell *22*, 623-632.

Onda, H., Lueck, A., Marks, P. W., Warren, H. B., and Kwiatkowski, D. J. (1999). Tsc2(+/-) mice develop tumours in multiple sites that express gelsolin and are influenced by genetic background. J Clin Investig *104*, 687-695.

Orlandini, M., Semplici, F., Ferruzzi, R., Meggio, F., Pinna, L. A., and Oliviero, S. (1998). Protein kinase CK2a' is induced by serum as a delayed early gene and cooperates with Ha-ras in fibroblast transformation. J Biol Chem *273*, 21291-21297.

Pende, M., Um, S. H., Mieulet, V., Sticker, M., Goss, V. L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S. C., and Thomas, G. (2004). S6K1-/-/S6K2-/- mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. Mol Cell Biol *24*, 3112-3124.

Perrot, V., and Rechler, M. M. (2005). The coactivator p300 directly acetylates the forkhead transcription factor Foxo1 and stimulates Foxo1- induced transcription. Mol Endocrinol *19*, 2283-2298.

Pieler, T., Hamm, J., and Roeder, R. G. (1987). The 5S gene internal control region is composed of three distinct sequence elements, organized as two functional domains with variable spacing. Cell *48*, 91-100.

Pluta, K., Lefebvre, O., Martin, N. C., Smagowicz, W. J., Stanford, D. R., Ellis, S. R., Hopper, A. K., Sentenac, A., and Boguta, M. (2001). Maf1p, a negative effector of RNA polymerase III in Saccharomyces cerevisiae. Mol Cell Biol *21*, 5031-5040.

Potter, C. J., Huang, H., and Xu, T. (2001). Drosophila Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. Cell *105*, 357-368.

Pray-Grant, M. G., Daniel, J. A., Schieltz, D., Yates, J. r., and Grant, P. A. (2005). Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. Nature *433*, 434-438.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z.-W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593-599.

Reddy, R., Henning, D., Das, G., Harless, M., and Wright, D. (1987). The capped U6 small nuclear RNA is transcribed by RNA polymerase III. J Biol Chem *262*, 75-81.

Roberts, D. N., Wilson, B., Huff, J. T., Stewart, A. J., and Cairns, B. R. (2006). Dephosphorylation and genome-wide association of Maf1 with pol III-transcribed genes during repression. Mol cell *22*, 633-644.

Rohde, J. R., and Cardenas, M. E. (2003). The tor pathway regulates gene expression by linking nutrient sensing to histone acetylation. Mol Cell Biol *23*, 629-635.

Rosa, M. D., Gottlieb, E., Lerner, M., and Steitz, J. A. (1981). Striking similarities are exhibited by two small Epstein-Barr virus-encoded ribonucleic acids and the adenovirus-associated ribonucleic acids VAI and VAII. Mol Cell Biol *1*, 785-796.

Rudin, C. M., and Thompson, C. B. (2001). Transcriptional activation of short interspersed elements by DNA-damaging agents. Genes Chromosomes Cancer *30*, 64-71.

Russanova, V. R., Driscoll, C. T., and Howard, B. H. (1995). Adenovirus type 2 preferentially stimulates polymerase III transcription of Alu elements by relieving repression: a potential role for chromatin. Mol Cell Biol *15*, 4282-4290.

Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P., and Meyuhas, O. (2006). Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes Dev *19*, 2199-2211.

Saucedo, L. J., Gao, X., Chiarelli, D. A., Li, L., Pan, D., and Edgar, B. A. (2003). Rheb promotes cell growth as a component of the insulin/TOR signalling network. Nature Cell Biol *5*, 566-680.

Schneider, J., Wood, A., Lee, J.-S., Schuster, R., Dueker, J., Maguire, C., Swanson, S. K., Florens, L., Washburn, M. P., and Shilatifard, A. (2005). Molecular regulation of histone H3 trimethylation by COMPASS and the regulation of gene expression. Mol cell *19*, 849-856.

Schmitt, M. E., and Clayton, D. A. (1993). Nuclear RNase MRP is required for correct processing of pre-5.8S rRNA in Saccharomyces cerevisiae. Mol Cell Biol *13*, 7935-7941.

Schneider, J., Wood, A., Lee, J.-S., Schuster, R., Dueker, J., Maguire, C., Swanson, S. K., Florens, L., Washburn, M. P., and Shilatifard, A. (2005). Molecular regulation of histone H3 trimethylation by COMPASS and the regulation of gene expression. Mol cell *19*, 849-856.

Schramm, L., and Hernandez, N. (2002). Recruitment of RNA polymerase III to its target promoters. Genes Dev *16*, 2593-2620.

Schramm, L., Pendergrast, P. S., Sun, Y., and Hernandez, N. (2000). Different human TFIIIB activities direct RNA polymerase III transcription from TATA-containing and TATA-less promoters. Genes Dev *14*, 2650-2663.

Scott, P. H., Cairns, C. A., Sutcliffe, J. E., Alzuherri, H. M., Meloes, A., Winter, A. G., and White, R. J. (2001). Regulation of RNA polymerase III transcription during cell cycle entry. J Biol Chem *276*, 1005-1014.

Seligson, D. B., Horvath, S., Shi, T., Yze, S., Grunstein, M., and Kurdistani, S. K. (2005). Global histone modification patterns predict risk of prostate cancer recurrence. Nature *435*, 1262-1266.

Shah, O. J., Wang, Z., and Hunter, T. (2004). Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. Curr Biol *14*, 1650-1656.

Sharp, S. J., Schaack, J., Cooley, L., Burke, D. J., and Soll, D. (1984). Structure and transcription of eukaryotic tRNA genes. CRC Crit Rev Biochem *19*, 107-144.

Shaw, R. J., Bardeesy, N., Manning, B. D., Lopez, L., Kosmatka, M., DePinho, R. A., and Cantley, L. C. (2004). The LKB1 tumour suppressor negatively regulates mTOR signalling. Cancer Cell *6*, 91-99.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., Casero, R. A., and Shi, Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941-953.

Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., and Kozma, S. C. (1998). Disruption of the p70s6k/p85s6k gene reveals a small mouse phenotype and a new functional S6 kinase. EMBO J *17*, 6649-6659.

Shivaswamy, S., Kassavetis, G. A., and Bhargava, P. (2004). High-level activation of transcription of the yeast U6 snRNA gene in chromatin of the basal RNA polymerase III transcription factor TFIIIC. Mol Cell Biol *24*, 3596-3606.

Sinn, E., Wang, Z., Kovelman, R., and Roeder, R. G. (1995). Cloning and characterization of a TFIIIC2 subunit (TFIIICb) whose presence correlates with activation of RNA polymerase III-mediated transcription by adenovirus E1A expression and serum factors. Genes Dev *9*, 675-685.

Sinnett, D., Richer, C., Deragon, J. M., and Labuda, D. (1992). Alu RNA transcripts in human embryonal carcinoma cells. Model of post-transcriptional selection of master sequences. J Mol Biol *226*, 689-706.

Smith, E. M., Finnn, S. G., Tee, A. R., Browne, G. J., and Proud, C. G. (2004). The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian

target of rapamycin by amino acids and certain cellular stresses. J Biol Chem *280*, 18717-18727.

Soderlund, H., Pettersson, U., Vennstom, B., Philipson, L., and Mathews, M. B. (1976). A new species of virus-coded low molecular weight RNA from cells infected with Adenovirus type 2. Cell *7*, 585-593.

Song, D. H., Sussman, D. J., and Seldin, D. C. (2000). Endogenous protein kinase CK2 participates in Wnt signaling in mammary epithelial cells. J Biol Chem *275*, 23790-23797.

Stefanovsky, V., Langlois, F., Gagnon-Kugler, T., Rothblum, L. I., and Moss, T. (2006). Growth factor signalling regulates elongation of RNA polymerase I transcription in mammals via UBF phosphorylation and r-chromatin remodelling. Mol cell *21*, 629-639.

Stein, T., Crighton, D., Boyle, J. M., Varley, J. M., and White, R. J. (2002). RNA polymerase III transcription can be depressed by oncogenes or mutations that compromise p53 function in tumours and Li-Fraumeni syndrome. Oncogene *21*, 2961-2970.

Strahl, B. D., and Allis, C. D. (2000). The language of covalent modifications. Nature *403*, 41-45.

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

Tang, J.-M., He, Q.-Y., Guo, R.-X., and Chang, X.-J. (2006). Phosphorylated Akt overexpression and loss of PTEN in non-small cell lung cancer confers poor prognosis. Lung cancer *51*, 181-191.

Thimmappaya, B., Weinberger, C., Schneider, R. J., and Shenk, T. (1982). Adenovirus VAI RNA is required for efficient translation of viral mRNA at late times after infection. Cell *31*, 543-551.

Tsang, C. K., Bertram, P. G., Ai, W., Drenan, R., and Zheng, X. F. S. (2003). Chromatin-mediated regulation of the structure and RNA pol I localization by TOR. EMBO J *22*, 6045-6056.

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M. E., Borchers, C. H., Tempest, P., and Zhang, Y. (2006). Histone demethylation by a family of jmjc domain-containing proteins. Nature *439*, 811-816.

Ullu, E., and Tschudi, C. (1984). Alu sequences are processed 7SL RNA genes. Nature *312*, 171-172.

Ullu, E., and Weiner, A. M. (1985). Upstream sequences modulate the internal promoter of the human 7SL RNA gene. Nature *318*, 371-374.

Upadhya, R., Lee, J., and Willis, I. M. (2002a). Maf1 is an essential mediator of diverse signals that repress RNA polymerase III transcription. Mol Cell *10*, 1489-1494.

Upadhya, R., Lee, J. H., and Willis, I. M. (2002b). Maf1 is an essential mediator of diverse signals that repress RNA polymerase III transcription. Mol cell *10*, 1489-1498.

Vakoc, C. R., Mandat, S. A., Olenchock, B. A., and Blobel, G. A. (2005). Histone H3 lysine 9 methylation and HP1gamma are associated with transcriptional elongation through mammalian chromatin. Mol cell *19*, 381-391.

van Slegtenhorst, M., Nellist, M., Nagelkerken, B., Cheadle, J., Snell, R., van den Ouweland, A., Sampson, J., Halley, D., and van der Sluijs, P. (1998). Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products. Hum Mol Genet *7*, 1053-1057.

Van Zyl, W., Huang, W., Sneddon, A. A., Stark, M., Camier, S., Werner, M., Marck, C., Sentenac, A., and Broach, J. R. (1992). Inactivation of the protein phosphatase 2A regulatory subunit A results in morphological and transcriptional defects in Saccharomyces cerevisiae. Mol Cell Biol *12*, 4946-4959.

Vousden, K. H., and Lu, X. (2002). Live or let die: the cell's response to p53. Nature Rev Cancer *2*, 594-604.

Walter, P., and Blobel, G. (1982). Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. Nature *299*, 691-698.

Wang, R., and Brattain, M. G. (2006). AKT can be activated in the nucleus. Cell Signal *18*, 1722-1731.

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D. R., and Proud, C. G. (2001). Regulation of elongation factor kinase by p90RSK1 and p70 S6 kinase. EMBO J *20*, 4370-4379.

Wang, Z., and Roeder, R. G. (1995). Structure and function of a human transcription factor TFIIIB subunit that is evolutionarily conserved and contains both TFIIB- and high-mobility-group protein 2-related domains. Proc Natl Acad Sci USA *92*, 7026-7030.

Weiner, A. M., Deininger, P. L., and Efstratiadis, A. (1986). Nonviral retroposons: genes, pseudogenes, and transposable elements generated by the reverse flow of genetic information. Annu Rev Biochem *55*, 631-661.

Werness, B. A., Levine, A. J., and Howley, P. M. (1990). Association of human papillomavirus types 16 and 18 E6 protein with p53. Science *248*, 76-79.

Whetstine, J. R., Nottke, A., Lan, F., Huarte, M., Smolikov, S., Chen, Z., Spooner, E., Li, E., Zhang, G., Colaiacova, M., and Shi, Y. (2006). Reversal of histone lysine trimethylation by JMJD2 family of histone demethylases. Cell *125*, 467-481.

White, R. J. (2001a). Gene Transcription. Mechanisms and control. Blackwell Science Ltd.

White, R. J. (2001b). RNA polymerase III transcription. Landes Bioscience *2nd edition*.

White, R. J. (2002). RNA polymerase III transcription, 3rd Edition. Landes Bioscience, Austin *http://www.eurekah.com*.

White, R. J. (2003). rRNA and tRNA production is closely tied to cell growth. In Cell Growth Control *Eds Hall, M., Raff, M. and Thomas, G.*

White, R. J. (2005). RNA polymerases I and III, growth control and cancer. Nat Rev Mol Cell Biol *6*, 69-78.

White, R. J., Gottlieb, T. M., Downes, C. S., and Jackson, S. P. (1995). Cell cycle regulation of RNA polymerase III transcription. Mol Cell Biol *15*, 6653-6662.

White, R. J., Trouche, D., Martin, K., Jackson, S. P., and Kouzarides, T. (1996). Repression of RNA polymerase III transcription by the retinoblastoma protein. Nature *382*, 88-90.

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

Wolffe, A. P., and Brown, D. D. (1988). Developmental regulation of two 5S ribosomal RNA genes. Science *241*, 1626-1632.

Wullschleger, S., Loewith, R., and Hall, M. N. (2006). TOR signalling in growth and metabolism. Cell *124*, 471-484.

Yamane, K., Toumazou, C., Tsukada, Y., Erdjument-Bromage, H., Tempest, P., and Zhang, Y. (2006). JHDMA2A, a Jmjc-containing H3K9 demtheylase, facilitates transcription activation by androgen receptor. Cell *125*, 483-495.

Yoon, J.-B., Murphy, S., Bai, L., Wang, Z., and Roeder, R. G. (1995). Proximal sequence element-binding transcription factor (PTF) is a multisubunit complex required for transcription of both RNA polymerase II- and RNA polymerase III-dependent small nuclear RNA genes. Mol Cell Biol *15*, 2019-2027.

Yoshinaga, S. K., Boulanger, P. A., and Berk, A. J. (1987). Resolution of human transcription factor TFIIIC into two functional components. Proc Natl Acad Sci USA *84*, 3585-3589.

Zang, C., Comai, L., and Johnson, D. L. (2005). PTEN represses RNA polymerase I transcription by disrupting the SL1 complex. Mol Cell Biol *25*, 6899-6911.

Zaragoza, D., Ghavidel, A., Heitman, J., and Schultz, M. C. (1998). Rapamycin induces the G0 program of transcriptional repression in yeast by interfering with the TOR signaling pathway. Mol Cell Biol *18*, 4463-4470.

Zhang, C., Comai, L., and Johnson, D. L. (2005). PTEN represses RNA polymerase I transcription by disrupting the SL1 complex. Mol Cell Biol *25*, 6899-6911.

Zhang, H., Cicchetti, G., Onda, H., Koon, H. B., Asrican, K., Bajraszewski, N., Vazquez, F., Carpenter, C. L., and Kwaitkowski, D. J. (2003a). Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Act signalling through downregulation of PDGFR. J Clin Investig *112*, 1223-1233.

Zhang, X., Shu, L., Hosoi, H., Murti, K. G., and Houghton, P. J. (2002). Predominant nuclear localization of mammalian target of rapamycin in normal and malignant cells in culture. J Biol Chem *277*, 28127-28134.

Zhang, Y., Gao, X., Saucedo, J., Ru, B., Edgar, B. A., and Pan, D. (2003b). Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nature Cell Biol *5*, 578-582.

zur Hausen, H. (2000). Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J Nat Cancer Inst *92*, 690-698.

zur Hausen, H. (2002). Papillomaviruses and cancer: from basic studies to clinical application. Nature Rev Cancer *2*, 342-350.