Methods for Decreasing RNA Polymerase III Transcription in Mammalian Cells

by

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The research contained within this Masters thesis was my own work, unless otherwise stated. This document has not been submitted for any other degree.

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Abstract

RNA polymerase III transcription has been found to be abnormally elevated in many types of transformed and tumour cells. These rapidly growing cells demonstrate an increase in pol III transcripts and hence, an accumulation of protein above normal levels. The accretion of protein may lead to uncontrolled cell growth, a hallmark of cancer. Thus, the ability of pol III to increase a cell's biosynthetic capacity links it with malignant growth. Therefore, experiments were conducted to assess whether pol III transcription could be regulated by decreasing transcription in mammalian cells.

Homo sapien and *Mus musculus* cell lines were investigated to consider three different methods for decreasing pol III transcription; targeting a subunit of the transcription machinery, Brf1, by small interfering RNA, targeting the polymerase with the specific drug tagetitoxin, and inducing a negative effector of pol III transcription, Maf1. Levels of pol III transcripts were decreased in response to transfection of Brf1 siRNA, which also had a decreased effect on proliferation rates. When cells were treated with tagetitoxin during electroporation, pol III transcription also decreased. Induction of the pol III suppressor Maf1 decreased pol III transcripts to varying degrees, although faults were found within this system. More significant data was obtained by the application of siRNA against Maf1, as well from the analysis of samples acquired from heterozygous Maf1 cells. These results showed that decreasing Maf1 allows for the deregulation of pol III and an increase in pol III transcription.

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Abbreviations

~	about
α	alpha
β	beta
°C	degrees Celsius
µCi	microCurie
µg	microgram
µM	micromolar
µl	microlitre
-	minus
%	percent
A	adenine
A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
Ago	Argonaute
Amp	ampicillin
APS	ammonium persulphate
Arg	arginine
ARPP P0	acidic ribosomal phosphoprotein P0
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia-telangiectasia and Rad3-related
BCR	breakpoint cluster region
Bdp1	B double prime 1
Bp	base pairs
Brf1	TFIIB-related factor 1
Brf2	TFIIB-related factor 2
BSA	bovine serum albumin
C	cytosine
C-	carboxy-
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
$CHCl_3$	chloroform

CK2	casein kinase 2
Cm	centimetre
CO_2	carbon dioxide
CPZ	chlorpromazine
Ct	C-terminal
Da	Dalton
dATP	2' deoxyadenosine triphosphate
DCR	Dicer
dCTP	2' deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2' deoxyguanosine triphosphate
dH_2O	distilled H_2O
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	2' deoxy (nucleotide) triphosphate
DSE	distal sequence element
dsRBD	dsRNA-binding domain
dsRNA	double stranded RNA
DTT	dithiothreitol
EBV	Epstein-Barr virus
EDTA	ethylenediamine tetra acetic acid
ERK	extracellular signal-regulated kinase
ES	embryonic stem
EtBr	ethidium bromide
FB	final bleed
FBS	foetel bovine serum
g	gram
G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTP	guanosine triphosphate
rGTP	ribosomal guanosine triphosphate
HA	haemagglutinin
HCl	hydrogen chloride

HeLa	Henrietta Lacks
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HPV	human papillomavirus
hr	hour
hsp	heat shock protein
IAA	Isoamyl alcohol
ICR	internal control region
IE	intermediate element
Ig	immunoglobulin
kb	kilobases
KCl	potassium chloride
kDa	kiloDaltons
LB	lysogeny broth
Leu	leucine
M	molar
mA	milliampere
MEK	MAPK/ERK kinase
Met	methionine
mg	milligram
$MgCl_2$	magnesium cloride
$MgSO_4$	magnesium sulfate
min	minutes
miRNA	micro RNA
ml	millilitre
mM	millimolar
mmol	millimole
MMS	methylmethane sulfonate
MOPS	3-(N-morpholino)propane-sulphonic acid
mRNA	messenger RNA
MW	molecular weight
N-	amino-
NaCl	sodium chloride
NaFl	sodium flouride

NaOAc	sodium acetate
NaOH	sodium hydroxide
NIH	National Institute of Health
ng	nanograms
NLS	nuclear localization sequence
nm	nanometer
nM	nanomolar
Nt-	N-terminal
P	phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
PhOH	phenol
PKA	protein kinase A
pmol	picomole
PMSF	phenylmethanesulphonylfluoride
pols	polymerases
pol I	RNA polymerase I
pol II	RNA polymerase II
pol III	RNA polymerase III
PP2A	protein phosphatase 2A
PSE	proximal sequence element
P-Tefb	positive transcription elongation factor b
PVDF	polyvinylidene difluoride
R	restriction point
rasiRNA	repeat-associated short interfering RNAs
rATP	ribosomal adenosine triphosphate
RB	retinoblastoma protein
rCTP	ribosomal cytosine triphosphate
rDNA	ribosomal DNA
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference

RNAP	RNA polymerase
RNase	ribonuclease
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcriptase-PCR
s	second
Sec	Selenocysteine
SDS	sodium dodecyl sulphate
SINE	short interspersed repeat
siRNA	small interfering RNA
$SNAP_c$	snRNA activator protein complex
snRNA	small nuclear RNA
spRNAP-IV	single-polypeptide nuclear RNA polymerase IV
SRP	signal recognition particle
SDS	sodium dodecyl sulphate
T	thymine
™	trademark
TBE	tris-borate EDTA
TBP	TATA-box binding protein
TBS	Tris buffered saline
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TFIIIA	transcription factor IIIA
TFIIIB	transcription factor IIIB
TFIIIC	transcription factor IIIC
Tn	termination site
TOR	target of rapamycin
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	transfer RNA
tTA	tetracycline-controlled transactivator
tyr	tyrosine
U	unit

U	uracil
UBF	upstream binding factor
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
V	volt
v/v	volume per volume
vRNA	vault RNA
W	watt
w/v	weight per volume

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Chapter 1

Introduction

1.1 Transcription

Gene expression is determined by which genes are transcribed. Transcription serves as the first step in gene expression, decoding a genome. During this essential process, DNA is copied into complementary RNA sequence by RNA polymerase. Because transcription is the first step in gene expression, it serves as the most common and important level of regulatory control. Transcriptional regulation is involved in biochemical processes such as a cell's growth and development, serving to assist the cell in adapting to its changing environment. Deficient transcription can result in abundant defects in biological processes resulting in a multitude of disease states such as diabetes, cardiovascular hypertension, developmental abnormalities, and cancer.

1.2 Four nuclear eukaryotic RNA polymerases

Eukaryotes have four nuclear RNA polymerases (pols) which transcribe the nuclear genes, namely RNA polymerases I, II, III and a recently found single-polypeptide nuclear RNA polymerase of mitochondrial origin, spRNAP-IV (pol IV) (Kravchenko *et al.*, 2005; Sentenac, 1985). The identities of pol I, pol II and pol III were found by their different elution from DEAE- Sephadex columns, as well as differing sensitivities to the toxin α-amanitin (Kedinger *et al.*, 1970; Roeder and Rutter, 1969). Recently, it was revealed that pol IV expression originates from an alternative transcript of the mitochondrial RNA polymerase gene *(POLMT)* (Kravchenko *et al.*, 2005). RNA polymerase I (pol I) synthesizes most of the ribosomal (r)RNA, comprising 28S, 18S and 5.8S ribosomal RNAs which are processed from the large 45S RNA precursor molecule (pre-RNA). Pol I may be responsible for 70 to 80% of nuclear

transcription in proliferating cells (White, 2001a). RNA polymerase II (pol II) produces the protein-encoding messenger (m)RNAs and most of the small nuclear (sn)RNAs which are involved in mRNA processing. RNA polymerase III (pol III) is responsible for 10 to 20% of all nuclear transcription and produces small, stable, untranslated RNAs. These genes are less than 400 nucleotides in length and encode structural or catalytic RNAs (Schramm and Hernandez, 2002; White, 2001a) with important roles in cellular metabolism, including; transfer (t)RNAs, 5S rRNA, and VA RNAs which are encoded by adenovirus and used to direct translational machinery of an infected cell towards increased viral load (White, 2001a). 7SL RNA is also synthesized by pol III and is involved in intracellular protein transport as part of the signal recognition particle. Lesser characterised pol III products include U6 snRNA, H1 and MRP RNAs involved in RNA transcript processing, 7SK, and RNAs encoded by the short interspersed repeat (SINE) gene families.

1.3 Class III genes

Genes transcribed by the nuclear eukaryotic RNA polymerases are grouped into classes, determined by which polymerase transcribes that particular set of genes. Therefore, templates transcribed by pols I, II, III are referred to as class I, II and III genes, respectively. A table of class III gene products is located below.

Table 1.1 Summary stating gene products and function of class III genes.*

Product	Function
tRNA	Translational adaptor
5S rRNA	Ribosomal component
U6 snRNA	mRNA splicing
H1 RNA	RNase P component (tRNA processing)
MRP RNA	rRNA splicing and mitochondrial DNA replication
7SL RNA	Signal recognition particle component
7SK RNA	Control of P-Tef-b and mRNA elongation
SINE transcripts	Unknown, might perform a role in cellular stress responses
VA RNA	Translation control (adenovirus)
vault RNAs	Component of cytoplasmic vault nucleoproteins
EBER RNAs	Epstein-Barr virus translational control

*Adapted from White, 2001b.

1.3.1 tRNA

Apart from mitochondrial tRNA, a high level of structural conservation exists between tRNAs from prokaryotes and eukaryotes (Sharp *et al.*, 1984). In *Saccharomyces cerevisiae* and other eukaryotes, early tRNA processing components have been found in the nucleolus, suggesting that transcription of the tRNA genes might also be nucleolar (Thompson *et al.*, 2003). tRNA genes have transcripts that are processed to between 70 and 90 nucleotides, which serve as translational adaptors and enable amino acids to align according to the sequence of nucleotides in the mRNA (Alberts *et al.*, 2002). Accurate

translation of mRNA into a polypeptide chain rests on the ability of a tRNA to read the three residue anti-codon sequence. Eukaryotic cells possess 50 to 100 distinct tRNAs (Sharp *et al.*, 1984), however, the proportions of each tRNA species vary between cell type (Garel, 1976). Because each of the tRNA species has a similar structure, their individual nucleotide sequence creates a specificity that allows one tRNA to recognize only one amino acid. This amino acid is brought to the lengthening peptide chain only if it matches a complementary codon in the mRNA. Errors in this process occur at a surprisingly low rate of one mistake in 40,000 couplings, revealing the importance of this process and its control on protein synthesis (Alberts *et al.*, 2002).

1.3.2 5S rRNA

The 120 nucleotide 5S rRNA is the smallest of the ribosomal RNAs, yet it is essential for all eukaryotic organisms, playing a critical role in translation (Wool, 1979). Although there are exceptions, the 5S rRNA gene is generally present in the common rRNA precursor in bacteria and archaea, but is independently transcribed by pol III in eukaryotes (Lafontaine and Tollervey, 2001). Pre-ribosomal RNA is processed into mature rRNA species which are then transported into the nucleolus, both forms undergoing covalent modifications. After further transport to the cytoplasm, 5S rRNA is incorporated into the large ribosomal subunit which begins the formation of peptide bonds (Wool, 1979). In humans, cells contain 200- 300 5S rRNA genes, while *Saccharomyces cerevisiae* and *Xenopus laevis* contain 140 and over 200,000 copies, respectively (Consortium, 2001; Elion and Warner, 1984; Wolffe and Brown, 1988). In *X. laevis*, most 5S genes are only expressed in

the oocyte to sustain rapid growth during development (Wolffe and Brown, 1988).

1.3.3 U6 snRNA

U6, along with five other small nuclear ribonucleoproteins (snRNPs) and non-small nuclear ribonucleoproteins, functions to remove pre-mRNA introns within the formed spliceosome (Hastings and Krainer, 2001). Through this process, pre-mRNA genes transcribed by pol II are extensively modified and a coding sequence compatible with translation is created. In yeast, U6 snRNA uses a metal ion that is required for the catalytic activity of this process (Yean *et al.*, 2000). U6 is the smallest of the five snRNPs (106 nucleotides) and is the only component of the spliceosome that is not transcribed by pol II (Brow and Guthrie, 1988; Kunkel *et al.*, 1986).

1.3.4 H1 and MRP RNAs

The gene coding for human H1 RNA is only present in one to three copies per cell (Bartkiewicz *et al.*, 1989). This 369 nucleotide long molecule was first isolated from HeLa (human cervical carcinoma) cells (Bartkiewicz *et al.*, 1989), and found to resemble the structure for the RNA subunit of RNase P (Bartkiewicz *et al.*, 1989; Lee and Engelke, 1989). RNase P has endonuclease activity and is involved in processing pre-tRNA (Lee and Engelke, 1989). H1 RNA exhibits some sequence homology to MRP RNA (Gold *et al.*, 1989), both RNA subunits of these two enzymes sharing a highly conserved helical region, P4 (Piccinelli *et al.*, 2005). While both components are involved in RNA processing, RNase P is found in all phylogenetic domains and cleaves a pre-tRNA to produce a mature 5' end of the tRNA, while MRP is only found in

eukaryotes and is important for rRNA processing, cleaving pre-rRNA to 5.8S rRNA.

1.3.5 7SL

7SL is a component of the signal recognition particle (SRP) that mediates co-translational insertion of secretory proteins into the endoplasmic reticulum. Only a few copies of the full length 7SL gene exist within the human nuclear genome, encoding for a highly conserved 300 nucleotide transcript (Ullu and Tschudi, 1984; Ullu and Weiner, 1984). 7SL serves as a scaffold protein in the SRP, which undergoes structural changes during the assembly of the signal recognition complex (Kuglstatter *et al.*, 2002).

1.3.6 7SK

7SK, a 330 nucleotide snRNA, was found to interact with the transcription elongation factor P-TEFb in a reversible manner which is transcription-dependent (Nguyen *et al.*, 2001). The 7SK-P-TEFb interaction contributes to an important feedback loop modulating the activity of pol II in which P-TEFb stimulates transcription of cellular and viral genes by phosphorylating the polymerase (Chen *et al.*, 2004). Phosphorylation of CDK9, a kinase comprising part of P-TEFb, is crucial for the 7SK-P-TEFb interaction.

1.3.7 SINEs

SINEs (short interspersed elements) include the mammalian Alu gene family, as well as the rodent B1 and B2 families, and range from 50 to 500 nucleotides. It is estimated that 5% of the human genome consists of Alu

repeats (~one million copies in the haploid genome), while B1 genes are present at 100,000 copies per haploid mouse genome (Bennett *et al.*, 1984; Consortium, 2001; Rudin and Thompson, 2001). Alu genes are most common in humans, while B1 and B2 families are most prevalent in rodents, B2 being rodent-specific. Alu genes are homologous to B1 genes (~80%) (Bennett *et al.*, 1984), which are thought to be derived from 7SL RNA (Ullu and Tschudi, 1984). B2 genes are believed to have evolved from tRNA (Schmid, 1998).

SINE sequences are retroelements, retroposons that are replicated through an RNA intermediate which is reverse transcribed, amplified and then integrated throughout the genome, accounting for their high prevalence (Williams *et al.*, 2004). SINE functions are largely unknown; however, certain SINE transcripts have been found to be upregulated in a variety of stress conditions such as; heat shock (Allen *et al.*, 2004; Liu *et al.*, 1995), cisplatin treatment, etoposide and gamma radiation (Rudin and Thompson, 2001), cyclohexamide treatment (Liu *et al.*, 1995), and cells infected with various types of virus (Jang and Latchman, 1989; Panning and Smiley, 1993).

1.3.8 Viral RNAs

There are many viruses that contain short segments of coding region that signal pol III transcription of viral genomes. Pol III viral product VA RNA, which are encoded by adenovirus, divert the translational machinery of a virus infected cell towards efficient production of viral transcripts (White, 2001b). Pol III is essential in its role in adenovirus transcription as this allows the virus to rapidly multiply, producing high levels of transcripts at late stages of infection (Soderlund *et al.*, 1976). The Epstein-Barr virus (EBV) genome

contains EBER-1 and EBER-2 genes that are also transcribed by the pol III transcription machinery. These small nuclear RNAs have regions of homology to VA genes and are 166 and 172 nucleotides long, respectively (Clarke *et al.*, 1992; Rosa *et al.*, 1981). The EBV genome contains these genes, and EBERs have been found to play an oncogenic role in Burkitt's lymphoma cells, showing an ability to repress the cell's antiviral response as well as inducing growth in soft agar and the formation of tumours in mice (Komano *et al.*, 1999).

1.3.9 Vault RNAs

Vault (v)RNAs are components of large cytoplasmic vault nucleoproteins with largely unknown functions and transcribed by pol III (Rome *et al.*, 1991). In humans there are four separate genes (*hvg1-4*) that encode similar vRNAs and vary in length from 86 to 142 nucleotides long (Kickhoefer *et al.*, 1998; Van Zon *et al.*, 2001). Recently, evidence of involvement in drug resistance has been discussed, but is under controversy (Mossink *et al.*, 2003). It was hypothesized that vaults could possibly contribute to drug resistance by transporting or sequestering drugs away from their intracellular targets.

1.4 Class III gene promoters

There are three types of promoter arrangements that are utilized by pol III (White, 2001b). The unusual feature of most class III promoters is the location of the sequence elements downstream of the transcription start site, lying within the transcribed region. These types of promoters are deemed type 1 and 2. Class I and class II genes, transcribed by pols I and II, respectively, mostly

consist of sequence elements upstream of the start site. Pol III's type III promoter structure is more like that of class I and II genes, the sequence elements found upstream of the start site. A schematic of the three promoter types is depicted in Figure 1.1 and described in detail below.

1.4.1 Type I promoters

5S rRNA genes are the only pol III genes to utilize type I promoters. This promoter requires three internal elements: a highly conserved A-block (located between +50 and +64) an intermediate element (IE) (between +67 and +72) and a C-block (between +80 to +97) (Figure 1.1). Studies in *X. laevis* have shown that spacing of the internal elements is important for efficient transcription, as alternations in the spacing of these elements has been found to reduce transcription activity and prevent formation of a stable initiation complex (Pieler *et al.*, 1987).

The region between the A-block and transcriptional start site is an important determinant of 5S rRNA expression, particularly when conditions are unfavourable for transcription (Keller *et al.*, 1990). Mutations within the A- and C- blocks abolish transcription, while the flanking sequences are more resilient to mutations (Keller *et al.*, 1990; White, 2001a).

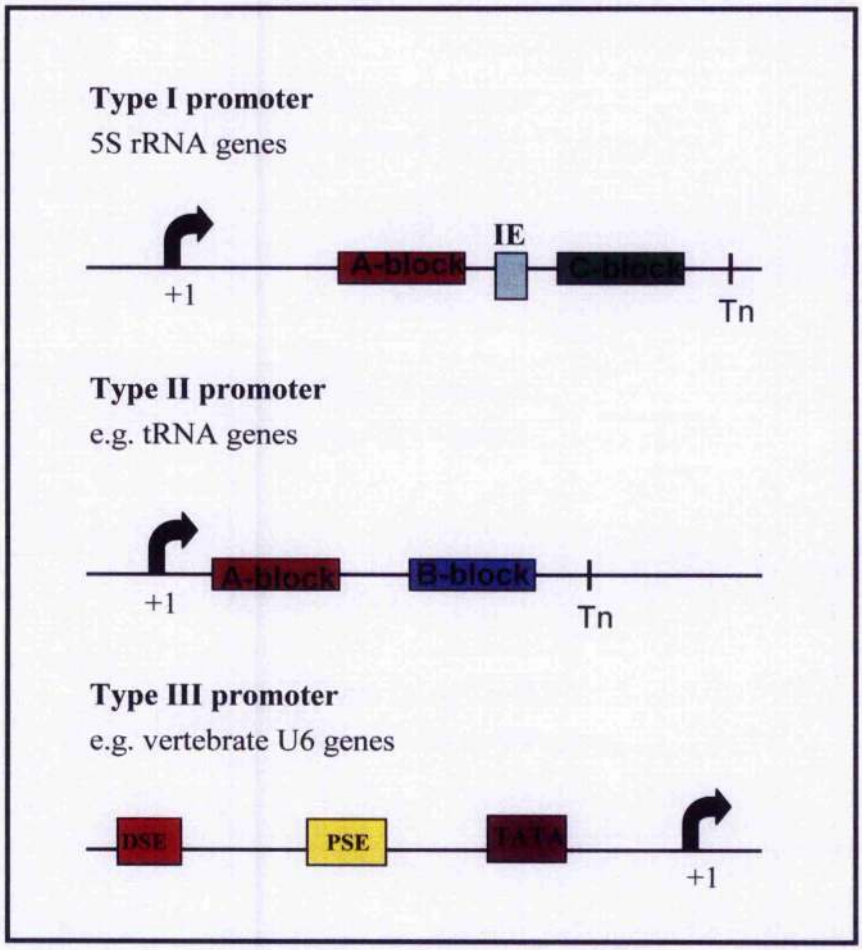


Figure 1.1 Three types of promoter structure that are utilized by pol III.* The coloured boxes represent the promoter elements.

Abbreviations: IE, intermediate element; Tn, termination site; DSE, distal sequence element; PSE, proximal sequence element; TATA, TATA box. *Adapted from White, 2001b.

The region between the A-block and transcriptional start site is an important determinant of 5S rRNA expression, particularly when conditions are unfavourable for transcription (Keller *et al.*, 1990). Mutations within the A- and C- blocks abolish transcription, while the flanking sequences are more resilient to mutations (Keller *et al.*, 1990; White, 2001a).

1.4.2 Type II promoters

The most common promoter employed by pol III is the type II promoter, utilized by tRNA genes, the adenovirus VA genes, and many SINE gene families. Type II promoters are constructed of two 10bp sequence blocks A- and B-, separated by 30 to 40bp (Galli *et al.*, 1981). The A- block is homologous to the A- block sequence of type I promoters, functionally interchangeable in some species (Ciliberto *et al.*, 1983). The location of A-blocks in type I and type II is different, however; in type I promoters the A-block lies ~50bp from the start site, while the type II promoter A- block lies at +10 to +20, much closer to the start site (Galli *et al.*, 1981). The location of the B- block can be extremely variable, a possible reason for this lying in the presence of small introns within the coding regions of some tRNA genes. Optimal binding of the A- and B- blocks occurs with genes that have an A-block and B- block at a distance of 30 to 60 base pairs, however a span of 365 base pairs remains transcriptionally functional (Baker *et al.*, 1987; Fabrizio *et al.*, 1987).

1.4.3 Type III promoters

Vertebrate type III promoters utilized by U6 snRNA, 7SK and MRP genes lack any requirement for intragenic promoter elements (Das *et al.*, 1988; Kunkel

and Pederson, 1989; Murphy *et al.*, 1987; Paule and White, 2000; Yuan and Reddy, 1991). This is unusual, as this type of promoter structure is more similar to that found in genes transcribed by pol I and II. The best characterised type III promoter is that of the U6 gene. Transcription of this gene requires a TATA box (located between -30 and -25), a proximal sequence element (PSE) (between -66 and -47) and a distal sequence element (DSE) (between -244 and -214) (Carbon *et al.*, 1987; Das *et al.*, 1988; Kunkel and Pederson, 1988; Kunkel and Pederson, 1989; Lobo and Hernandez, 1989). The pol II-transcribed U2 snRNA gene and the U6 snRNA genes share commonality in that the U6 PSE and DSE are homologous and interchangeable (Kunkel and Pederson, 1988). An unusual anomaly lies in the ability of the U2 gene (usually lacking a TATA box) to be transcribed by pol III when a TATA box is inserted and, inversely, crippling the U6 TATA box allows U6 to be transcribed by pol II (Lobo and Hernandez, 1989).

1.5 Transcription of class III genes

Before transcription can be initiated, the polymerase and many accessory factors, namely transcription factors, must be assembled onto the promoter. The process of transcription begins when a pre-initiation complex containing all the necessary factors is complete, proceeds with an elongation step, and terminates with the polymerase being recycled for further rounds of transcription.

1.5.1 Assembly of transcription machinery on type II promoters

Due to the three different types of promoters utilized by pol III, there is a different requirement of transcription factors and the order in which they are brought to the promoter. Pol III has little specificity for the above promoter elements and therefore must employ specific transcription factor complexes to create a functional transcription initiation apparatus. Assembly on type II promoters will be discussed first (Figure 1.2), as it is the most commonly utilized promoter type, followed by the remaining promoters.

1.5.1.1 Transcription Factor IIIC (TFIIIC)

TFIIIC is one of the largest and most complicated transcription factors being studied (Geiduschek and Kassavetis, 2001; Paule and White, 2000). TFIIIC, a multi-subunit complex, recognizes the A- and B- blocks of both type I and type III promoters, binding simultaneously. In *Saccharomyces*, TFIIIC has been found to consist of two globular domains, each ~300kDa and composed of six subunits (Schultz *et al.*, 1989). In human cells, two major subunits have been separated chromatographically into two sub-complexes, TFIIIC1 and TFIIIC2 (Yoshinaga *et al.*, 1987). Little is known concerning TFIIIC1, although sedimentation analysis suggests a possible mass of 200kDa and is required for transcription activity (Kovelman and Roeder, 1992). TFIIIC2 is the best characterized of the TFIIIC subunits, with a total of five subunits, binding to the B- block promoter through its 220 kDa subunit (Geiduschek and Kassavetis, 2001). TFIIIC2 initially recognizes the type II promoter, and then recruits TFIIIC1 and transcription factor IIIB (TFIIIB) (Figure 1.2).

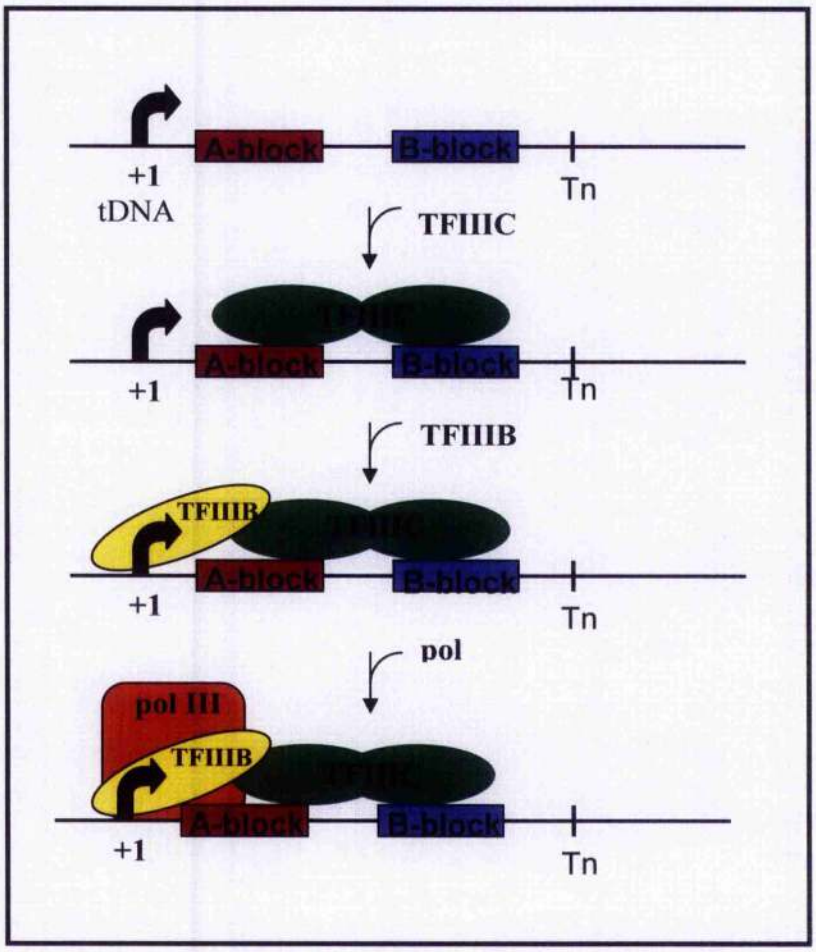


Figure 1.2 Step-by-step assembly of pol III basal transcription machinery on a type 2 promoter.

TFIIIC is recruited first and binds to the A- and B- blocks simultaneously. TFIIIB is then bound to TFIIIC through protein-protein interactions, followed by the binding of the polymerase. The transcription start site is noted as +1 and the termination site as Tn.

TFIIIC subunits 63, 90 and 102 bind to Brf1 (a subunit of TFIIIB), while TFIIIC63 and 102 binds to TATA-binding protein (TBP) (another TFIIIB subunit) (Hsieh *et al.*, 1999b; Hsieh *et al.*, 1999a). Binding of TFIIIC1 and TFIIIB enhances and extends the protein footprint produced by TFIIIC2 to include the A- block (Wang and Roeder, 1998; Yoshinaga *et al.*, 1987). Photocrosslinking experiments have shown that the various subunits of TFIIIC span across the length of the entire gene, creating the appearance of a dumbbell when bound (Bartholomew *et al.*, 1990). Both TFIIIC1 and TFIIIC2 are required for expression of 5S rRNA, VA and tRNA genes, while U6 and 7SK genes require TFIIIC1 but not TFIIIC2 for transcription.

1.5.1.2 Transcription factor IIIB (TFIIIB)

TFIIIC serves to recruit TFIIIB to the promoter, TFIIIB being regarded as an extremely important factor in the pol III transcription system. TFIIIB is composed of at least three proteins in both *Saccharomyces cerevisaie* and humans: TBP, and two TBP-associated factors, currently known as TFIIB-related factor (Brf1) and B double prime (Bdp1). TFIIIB has been shown to be an important target for cell cycle regulation in actively proliferating cells, functioning as a point of control by both tumour suppressors and transforming proteins, helping to determine a cell's biosynthetic capacity (White, 1998). In yeast, TFIIIB alone assembled upstream of the transcription start site can direct multiple rounds of transcription on the tRNA and U6 snRNA genes (Dieci and Sentenac, 2004).

TBP is a 34kDa general transcription factor utilized by the transcription machineries of pol I, II and III (Cormack and Struhl, 1992; White and Jackson,

1992). In the case of pol III, the involvement of TBP was unexpected because most class III genes lack TATA boxes. The first indication that TBP functioned with TFIIIB in an active transcription complex was uncovered when a population of TBP molecules copurified with TFIIIB activity in yeast (Margottin *et al.*, 1991), later found to be bound to Brf, which can be bound in the absence of DNA through a conserved region of TBP (Colbert *et al.*, 1998; Shen *et al.*, 1998).

The 160kDa subunit Bdp1 was first found to copurify with Brf1 (Kassavetis *et al.*, 1991b). Bpd1 is required for transcription from all pol III promoter types and in contrast to Brf1, Bdp1 forms a weak association with TBP when there is no DNA template present (Schramm *et al.*, 2000). As with Brf1, Bdp1 makes direct contacts with TFIIIC, allowing TFIIIC to bring TFIIIB to the promoter (Geiduschek and Kassavetis, 2001). Recently, HsBdp1 activity has been shown to be an essential component in TFIIIC1 and TFIIIC1-like activity when the addition of this protein alone was able to reconstitute transcription from a VA1 gene (Weser *et al.*, 2004).

Brf1 shares N-terminal homology to the pol II transcription factor IIB (TFIIB) (Schramm and Hernandez, 2002). Brf1 is a 90kDa protein that is initially contacted by DNA-bound TFIIIC, which then serves to recruit the pol III subunit C34 (RPC39 in human) (Brun *et al.*, 1997). Brf1 is the only TFIIIB subunit to make direct contacts with pol III itself, localizing pol III to the transcription start site and facilitating transcription initiation. Dividing Brf1 in half and using these two components in an *in vitro* transcription system still

reconstitutes active transcription to the same degree as non-divided Brf1 (Kassavetis *et al.*, 1998).

1.5.2 Assembly of transcription machinery on type I promoters

1.5.2.1 Transcription factor IIIA (TFIIIA)

Transcription initiation at a type I promoter requires the gene-specific transcription factor III-A (TFIIIA), a 40kDa single polypeptide. TFIIIA acts as a platform, allowing TFIIIC to be recruited onto type I promoters, although the manner in which TFIIIA promotes TFIIIC binding is uncertain (Schramm and Hernandez, 2002). This protein consists of nine tandem zinc fingers, the N-terminal three fingers recognizing the C- block, the middle fingers the intermediate element, and the C-terminal three fingers contacting the A- block (Nolte *et al.*, 1998; White, 2001b). C- block binding contributes to 95% of the total binding affinity of full length TFIIIA (Clemens *et al.*, 1992). After TFIIIC is bound to TFIIIA, the basal transcription machinery proceeds in the same order as in a type II promoter, TFIIIC bringing TFIIIB, and hence pol III, to the site of transcription initiation.

1.5.3 Assembly of transcription machinery on type III promoters

The TFIIIB that is recruited does not contain Brf1 as in type I and II promoters, but instead Bdp1, TBP and Brf2, a Brf1-related factor. TFIIIB does not require TFIIIA or TFIIIIC for recruitment, but binds to the TATA box and PSE by the TBP subunit of TFIIIB and SNAPc (snRNA activator protein complex) (Lagna

et al., 1994; Murphy *et al.*, 1992). Recognition of the special promoter structure type III is complex. SNAPc and Oct-1 are recruited to the upstream sequence elements and bind cooperatively to their spatially separated PSE and DSE (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). The affinity of SNAPc to its PSE is dependent on the DNA- bound Oct-1 and protein-protein interactions between SNAPc and Oct-1 increase TFIIIB-SNAPc recruitment (Murphy *et al.*, 1992). Once stabilized, PSE occupancy is stimulated, pol III is recruited and transcription is initiated.

1.5.4 Elongation and termination

Once pol III has been recruited to a promoter it separates the DNA helix around the initiation site, forming a transcription bubble (Geiduschek and Kassavetis, 2001). This process may require the active participation of TFIIIB, possibly through promoter opening, but the exact mechanism remains to be determined (Constanzo *et al.*, 2001; Kassavetis *et al.*, 1998). As pol III transcribes the gene, the DNA bubble moves with it, elongating RNA at an average of ~20 nucleotides per second. Transcription by pol III is not uniform, however, as pol III moves along certain nucleotides more quickly than others (White, 2001a). Studies have shown that pol III severs its connection with TFIIIB at an early stage, after several ribonucleotides have been synthesized (Geiduschek and Kassavetis, 2001). Pol III does not require elongation factors, unlike pol I and pol II. The small size of class III genes may not make this necessary, or it is possible that one or more pol III subunits may carry out this function (White, 2001b).

Transcription termination begins when pol III recognizes short runs of U (Geiduschek and Kassavetis, 2001). The efficiency of termination seems to be influenced by the flanking sequence and increases with greater lengths of U-run. In human VA1 and tRNA genes (as well as yeast class III genes), pol III is recycled without being released from the template and, hence, the slow initiation step is avoided. This mechanism may occur by specific handing-off mechanisms or purely stoichiometric recycling. In yeast, pol III has been shown to be directly transferred from the termination site to the promoter. A preferential termination pathway allows RNA release and transcription reinitiation, without the release of pol III (Dieci and Sentenac, 1996). The precise mechanism exploited by human pol III still remains to be determined, but it has been shown to be retained in the original transcription complex on VA1 and tRNA genes without dissociating after each transcriptional round and may be assisted by TFIIIB and TFIIIC (Ferrari *et al.*, 2004; White, 2001b).

1.6 Proteins influencing pol III transcription

Various proteins have been found to influence pol III transcription, mainly through targeting transcription factor IIIB, including c-Myc, retinoblastoma protein (RB), p53, and protein kinases CK2 and ERK.

1.6.1 Influencing pol III transcription during proliferation

1.6.1.1 c-Myc

Overexpression of the cellular oncogene product c-Myc has been studied in a variety of human cancers including lymphomas and leukaemias (Spencer and Groudine, 1991). c-Myc is able to coordinate cell division and growth through pol II and pol III and enhances pol I transcription of rRNA genes leading to

increased protein biosynthesis (Grandori *et al.*, 2005). c-Myc directly binds to TFIIIB through a protein-protein interaction between TFIIIB and the N-terminal transactivation domain of c-Myc. The interaction between c-Myc and TFIIIB is significant, considering TFIIIB's ability to regulate pol III transcription. Activation of c-Myc has been found to increase translation and growth, which precedes DNA replication and cell division (Rosenwald, 1996; Schmidt, 1999). Loss of endogenous c-Myc depleted from HeLa cells using small interfering RNA caused a decrease in pol III products, namely tRNA and 5S rRNA (Felton-Edkins *et al.*, 2003b). Overabundance of exogenous c-Myc rapidly increases transcription of the latter two gene types (Gomez-Roman *et al.*, 2003). These experiments in combination provide evidence that c-Myc directly regulates pol III transcription via TFIIIB.

1.6.1.2 Retinoblastoma protein (RB)

The discovery of RB was pivotal to the further understanding of the mechanisms that influence a cell's transformation. It has been suggested that the pathway involving RB may be disrupted in all human malignancies (Weinberg, 1995). RB contains regions of homology to both TBP and Brf1, which may assist in its ability to mimic these molecules and therefore disrupt TFIIIB (Larminie *et al.*, 1997). Using extracts prepared from RB-knockout mice, Larminie *et al.*, demonstrated a specific increase in TFIIIB activity when the *Rb* gene was disrupted, as well as a higher rate of pol III transcription (1997). RB is thought to decrease the rate of transcription by disrupting the interaction between TFIIIB and TFIIIC and also disturbing the interaction between TFIIIB and pol III (Sutcliffe *et al.*, 2000). This, in turn, inhibits the synthesis of tRNA and 5S rRNA. In non-proliferating cells, RB is primarily

found in its underphosphorylated form, interacting with many proteins, including TFIIIB (Scott *et al.*, 2001). TFIIIB only associates with this underphosphorylated form of RB. The TFIIIB-RB complex inhibits further binding of TFIIIB to TFIIIC, which decreases class III gene expression (Sutcliffe *et al.*, 2000). Loss of class III gene expression (namely rRNA and tRNA) leads to decreased protein biosynthesis and may constitute an important role in tumour suppression (White, 1998). RB and TFIIIB together play an important role in cell cycle progression, which will be discussed below.

Two closely RB-related proteins, p107 and p130, also appear to play a role in repression of TFIIIB. Sutcliffe *et al.*, demonstrated that a subunit of TFIIIB interacts physically with p107 and p130. This TFIIIB subunit was Brf1, demonstrated by binding of both endogenous and recombinant p107 and p130 to Brf1 during immunoprecipitation assays (Sutcliffe *et al.*, 1999). When p107 and p130 are not repressing TFIIIB, pol III transcription is deregulated and transcription increases, with a consequent increase in the cell's biosynthetic capacity (reviewed in White, 1997). In contrast, pol I transcripts only appear to increase in fibroblasts lacking RB and p130, with no increase in fibroblasts lacking p130 and p107 (Ciarmatori *et al.*, 2001).

1.6.1.3 ERK

Protein kinases ERK and CK2 have been shown to stimulate pol III transcription in mammalian cells, via TFIIIB (Felton-Edkins *et al.*, 2003b; Johnston *et al.*, 2002). ERK is associated with the promotion of growth through activation of translational capacity and ribosome biogenesis (Brandenburger *et al.*, 2001; Whitmarsh and Davis, 2000). Felton-Edkins *et al.*

revealed that activation of ERK is required for TFIIIB binding to pol III and TFIIIC, and that only the activated form of ERK binds TFIIIB (2003). More specifically, the interaction between TFIIIB and ERK was found to be between ERK and the subunit of TFIIIB, Brf1, which is phosphorylated by ERK *in vitro* and *in vivo*. Co-immunoprecipitation assays using cells incubated with serum or MEK inhibitor suggests that ERK activity may promote the interaction of TFIIIB, TFIIIC2, and pol III in proliferating cells, perhaps stimulating initiation complex assembly. However, because phosphorylation of Brf1 is only partially hindered by blocking ERK activity *in vivo*, another protein kinase, such as CK2, may be required for maximal transcription (Felton-Edkins *et al.*, 2003b; Johnston *et al.*, 2002).

1.6.2 Pol III transcription during cellular stress

1.6.2.1 CK2

CK2 has many roles within a cell. One role includes stimulation of pol III transcription in yeast and mammalian cells, via TFIIIB, directly linking it with cellular growth and proliferation (Brown *et al.*, 2000; Carroll and Marsjak, 1989; Felton-Edkins *et al.*, 2003b; Johnston *et al.*, 2002; Munstermann *et al.*, 1990). In another role involving cellular stress in yeast, CK2 relays DNA damage signals to the pol III machinery, releasing its catalytic subunits while the remaining subunits stay bound to TFIIIB. In turn, TFIIIB cannot be phosphorylated, and hence, activated, which results in the repression of pol III transcription (Ghavidel and Schultz, 2001). Johnston *et al.*, demonstrated the association between CK2 and TFIIIB, and CK2's ability to stimulate pol III transcription by binding and phosphorylating TFIIIB and assisting its recruitment by TFIIIC2. More specifically, Brf1 was found to require

phosphorylation by CK2 in order to interact efficiently with TFIIIC2. Transcription by pol III requires phosphorylation by CK2, however, CK2 phosphorylation of Bdp1, Brf2 and TBP together is inhibitory (Hu *et al.*, 2003).

1.6.2.2 p53

p53 is an important tumour suppressor that is inactivated in many human cancers (Vousden, 2000). p53 may become activated in response to cellular stress such as; hypoxia, radiation and oncogenic stimuli, inducing cell death. In most of these cancers the mutation lies in the central core domain, a region fundamental in its ability to regulate TFIIIB (Stein *et al.*, 2002). The tumour suppressor protein p53 targets TFIIIB, blocking its function and therefore serving as a general repressor of pol III transcription (Cairns and White, 1998). p53's N-terminal domain binds TFIIIB, through its direct interaction with TBP, and thereby prevents promoter occupancy by TFIIIB (Chesnokov *et al.*, 1996; Crighton *et al.*, 2003). When this occurs, TFIIIB disassociates from both TFIIIC2 and pol III (Crighton *et al.*, 2003). TFIIIB appears to be less susceptible to p53 once it has assembled into a preinitiation complex. Immunoprecipitation assays have shown that Brf1 can assemble into specific complexes with cellular p53 (Cairns and White, 1998). Since TFIIIB has an important role in a cell's biosynthetic capacity, p53's ability to release TFIIIB from repression may add to the loss of growth control in many types of tumours.

1.6.2.3 Maf1

Maf1, a novel and conserved protein, has recently been found to negatively regulate pol III transcription in *Saccharomyces cerevisiae* (Pluta *et al.*, 2001). Pol III repression by Maf1 occurs in response to a variety of conditions, suggesting that this protein may be a convergence point in major nutritional and stress-signalling pathways. This protein will be discussed further in the results section.

1.7 Regulation of pol III during the cell cycle

TFIIIB has been shown to be an important target for cell cycle regulation of pol III transcription. Through this factor, pol III transcription may be deregulated, providing striking evidence that deregulation through TFIIIB plays an essential role in driving mammalian cells towards tumour development.

There are four phases of the somatic cell cycle; S phase (for DNA Synthesis), where chromosomal replication occurs, M phase (for **M**itosis), and G1 and G2, or **G**ap phases. Two thirds of the way through G1 the restriction point (R) occurs, where the cell is committed to replicate and divide irrespective of the presence of external growth factors. The cell cycle has a profound effect upon gene expression, especially during mitosis, when transcription is generally repressed. TFIIIB subunits TBP and Brf1 have been shown to be hyperphosphorylated during this period, which inactivates TFIIIB and represses pol III transcription (Fairley *et al.*, 2003; White *et al.*, 1995). As TFIIIB leaves mitosis, hyperphosphorylation of TFIIIB is rapidly reversed.

TFIIIB activity remains low in early G1 phase and increases gradually as cells move into S phase (White *et al.*, 1995). Because of this increase, pol III transcription is 2 to 3- fold higher in S and G2 than in early G1. During early G1, active TFIIIB is a limiting factor for pol III transcription, but by S and G2 phases it increases to levels over that of TFIIIC. This increases the rate of pol III transcription at this time point.

This was further demonstrated by Scott *et al.* using serum-starved cells and add-back experiments, where fractions containing TFIIIB stimulated transcription in serum-starved and growing cells (2001). No response was observed when a fraction containing TFIIIC and pol III was added. This suggests that TFIIIB is indeed limiting, while TFIIIC and pol III are in excess. The subunits Brf1 and TBP did not decrease during serum deprivation, suggesting that the amount of TFIIIB is not responsible for its own loss of activity.

RB has proved to be an important factor in cell cycle regulation in its interaction with TFIIIB. RB and RB-related protein p130 bind and repress TFIIIB during G0 and early G1 phase, then dissociate from TFIIIB shortly before S phase, causing an increase in transcription (Brown *et al.*, 2000). RB and p130 were shown to bind to the Brf1 subunit of TFIIIB (Scott *et al.*, 2001). This dissociation is caused by the phosphorylation of RB in mid- to late G1 phase. Because TFIIIB is known to bind only the underphosphorylated form of RB, it suggests that TFIIIB is released from repression by RB at the G1/S transition due to hyperphosphorylation of RB by the cyclin-dependent kinases D and E.

TFIIIB and its ability to regulate pol III transcription in growth-arrested cells has been the source of conflicting experiments. Tower and Sollner-Webb concluded that a specific reduction in TFIIIB activity was responsible for down -regulating pol III transcription in growth-arrested cells; however, it was not determined by what mechanism (1988). Another laboratory demonstrated that HeLa cells down-regulate pol III transcription when grown in low serum, due to a decrease in the activity of TFIIIC2 (Hoeffler *et al.*, 1988). However, HeLa cells grow actively in these studies, despite low serum conditions, so these experiments may not provide a clear picture of the actions of pol III as it exits the cell cycle.

1.8 Cell growth and cell cycle progression

Although cell growth is necessary for sustaining eukaryotic organisms, the mechanisms in which cell growth and cell cycle progression are coupled together remains fairly elusive. Cell growth is necessary for cell cycle progression, while cell cycle progression is not required for cell growth (Johnston *et al.*, 1977). Further insight was achieved when studies showed that cells must reach a critical mass before DNA replication and cell division occur (Fingar *et al.*, 2002; Johnston *et al.*, 1977; Zetterberg and Killander, 1965). This critical mass is the accumulation of protein that makes up 80 to 90% of a cell's dry mass (Zetterberg and Killander, 1965). The amount of critical mass becomes important when linked to a cell's biosynthetic capacity and how it is controlled. In transformed and tumour cells, the pathways that control the accumulation of mass and, hence, progression into the cell cycle is altered, causing abnormal and uncontrolled cell division. Investigation into the

common proteins and pathways that link cell cycle progression and cell growth may allow further insight into the events that must take place for cell transformation.

1.9 Aims of Masters Thesis

TFIIIB has proved to be an essential molecule involved in pol III basal machinery and regulation and is an important target for cell cycle regulation in actively proliferating cells (White, 1998). TFIIIB is an important determinant of 5S rRNA and tRNA production, affecting a cell's biosynthetic capacity. The importance of this transcription factor was further proven when many key proteins such as tumour suppressors p53 and RB, oncogene c-Myc, kinases CK2 and ERK, and negative pol III regulator Maf1 were found to target this molecule. The mechanisms in which these proteins affect TFIIIB have been researched, but the exact molecular mechanisms of regulation are not yet understood. This is vital when considering that the deregulation of TFIIIB may be a significant step towards tumour development.

My overall project aims were to use three different methods to decrease pol III transcription, and observe transcript expression, proteins levels, and proliferation rates. Brf1 was found to be an important TFIIIB subunit affected by all of the key proteins listed above, therefore, this subunit seemed most likely to have an effect on transcription, and hence, proliferation rates, when targeted by small interfering RNA (siRNA) in mammalian cells (Crighton and Woiwode; Desai *et al.*, 2005; Felton-Edkins *et al.*, 2003b; Gomez-Roman *et al.*, 2003; Johnston *et al.*, 2002; Larminie *et al.*, 1997).

Although siRNA technology can be extremely effective, it can be time-consuming and expensive, so other methods were sought to decrease pol III transcription. *In vitro*, tagetitoxin has been found to specifically inhibit pol III transcription, probably by inhibiting elongation and delaying the accumulation

of full-length transcript (Steinberg and Burgess, 1992; Steinberg *et al.*, 1990). Tagetitoxin seemed an excellent candidate for decreasing pol III transcription in mammalian cells, and may prove to be a more straightforward method, bypassing complications such as siRNA design and transfection efficiencies.

Yet another way to decrease pol III transcription is to use a negative effector of RNA polymerase III, such as Maf1. In *Saccharomyces cerevisiae*, Maf1 was found to be a nuclear protein that interacts with pol III and serves to help regulate the level of cellular tRNA in response to external signals (Pluta *et al.*, 2001). The last aim of this project was to create a Tet-On mammalian cell line that overexpresses Maf1 when induced, to observe the levels of pol III transcripts and proliferation rates. Maf1 siRNA was also used to examine opposing effects.

Thus, the main objective of this Masters is to gain insight into the effects of decreasing pol III transcription by various methods in mammalian cell lines, targeting either the polymerase itself, or proteins that interact with it.

Chapter 2

Materials and Methods

2.0 Materials and Methods

2.1 Cell culture

2.1.1 Culture of HeLa, NIH 3T3, and MCF-7 cells

HeLa and NIH 3T3 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium), supplemented with 10% (v/v) foetal bovine serum (FBS), 2 nM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. MCF-7 cells were grown in RPMI 1640 medium, supplemented with 10% FBS, 2 nM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cell types were grown in a humidified atmosphere which contained 5% CO_2 at 37°C. A class II hood was used for aseptic techniques, along with sterile equipment and reagents. Cells were passaged when subconfluent; approximately every 2 to 3 days. After media was aspirated from the flask, 2 ml of buffered trypsin-EDTA (0.05% (w/v) trypsin, 0.02% (w/v) EDTA) was added to the cells, and then aspirated immediately. A further 2 ml was added and left at room temperature (~22°C) until cells appeared to begin to dislodge from the flask wall, approximately 1-4 minutes, depending on the cell type. The remaining trypsin was then aspirated. The flask was gently tapped and fresh media was immediately added to the dissociated cells in order to neutralise the trypsin.

Cryo-freezing was used for storage of all cell lines. Cells were trypsinized as described above and, following pelleting by centrifugation, cells were resuspended in a solution of 70% FBS, 20% DMEM and 10% dimethylsulphoxide (DMSO) with no other supplements. Cells were aliquotted into cryo-tubes at density of $2x10^6$ cells/ml/tube and frozen in stages by initially being placed at –80°C overnight and subsequently being transferred to liquid nitrogen storage the next day. Thawing of cells was performed rapidly

by placing cryo-tubes in a 37°C water bath until just thawed. Cells were then mixed with fresh media, centrifuged, and the supernatant aspirated off to ensure removal of DMSO prior to resuspension in normal culture media, containing 10% (v/v) FBS DMEM.

2.1.2 Culture of pTRE2.HA.HsMaf1 (Tet-on) HeLa cells

HeLa cells that express the tetracycline-controlled transactivator (tTA) were purchased from Clontech, BD Biosciences and routinely cultured in DMEM supplemented with 10% FBS (Tet-System Approved, Clonetech), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml G418 and 100 μg/ml hygromycin B. All other culturing techniques were performed as in 2.1.1, above.

2.2 Preparation of total cellular RNA

Total cellular RNA was isolated from cells when approximately 80% confluent using TRI reagent (Sigma), a solution of guanidine thiocyanate and phenol, in accordance with the manufacturer's instructions. Media was aspirated off cells grown in 10 cm tissue culture dishes and residual media removed with two washes using 5 ml of ice-cold PBS. Cells from each dish were harvested by scraping in 1 ml of TRI reagent per dish and transferred to a sterile eppendorf tube. Cells were left to stand for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was then added to each tube and the samples vortexed for 15 seconds. The samples were then allowed to stand for a further 15 minutes at room temperature prior to being centrifuged at 13,000g for 15 minutes at 4°C. This resulted in separation of the samples into three phases: a lower red organic phase

containing protein, a middle white interphase containing precipitated DNA and an upper colourless aqueous phase which contains the RNA. The upper phase was carefully removed by pipetting, ensuring no contamination from the remaining phases, and transferred to fresh eppendorf tubes. Isopropanol (500 µl) was added to each of these tubes containing the aqueous RNA and thoroughly mixed by repeated inverting. Following 5–10 minutes incubation at room temperature to allow maximal precipitation of RNA, samples were centrifuged at 13,000g for 10 minutes at 4°C. The supernatant was then removed and the remaining RNA pellet was washed with 1 ml of 75% (v/v) ethanol made up with diethypyrocarbonate (DEPC)-treated dH_2O. DEPC is 0.1% (v/v) diethypyrocarbonate/sterile water which is mixed into solution, left overnight at room temperature and then autoclaved to inactivate the remaining DEPC. The samples were then vortexed briefly, subsequently microcentrifuged at 7,500g for 5 minutes at 4°C and the supernatant aspirated off. Residual supernatant was removed with a P20 pipette following pulse microcentrifugation. Appropriate volumes of DEPC-dH_2O, in the range of 10-30 µl (depending on the size of the RNA pellet), were added to the RNA pellets and the samples were heated in a 65°C water bath for 10 – 15 minutes to facilitate resuspension of the RNA. The samples were stored at –80°C.

2.3 Quantification of nucleic acids

Concentrations of nucleic acids were determined by spectrophotometry, where an OD of 1 at 260 nm correlates to 50 µg/ml of double-stranded DNA and 40 µg/ml of single-stranded DNA and RNA. Readings were zeroed in the same solution in which the sample was diluted. RNA concentration was determined by A_{260} using the calculation:

RNA concentration (μg/ml) = absorbance at 260 nm x 40 x dilution factor.

DNA concentration was determined by the equation:

DNA concentration (μg/ml) = absorbance at 260 nm x 50 x dilution factor.

A ratio of absorbance at 260 nm to 280 nm in the range of 1.8 to 2 indicated the RNA samples were relatively free from contamination with protein.

2.4 Reverse transcriptase-polymerase chain reaction (RT-PCR)

2.4.1 cDNA preparation

cDNAs were prepared from 3 μg of RNA (diluted to 1 μg/μl). 2 μl of random primers (hexanucleotide mix, (Roche)) diluted 1:10 with DEPC- dH_20) was mixed with 19 μl of DEPC-dH_20. Primer annealing was carried out at 80°C in a final volume of 24 μl and allowed to proceed for 10 minutes before transferral to ice, then microfuged briefly. 8 μl of 5 x First Strand Buffer (Invitrogen), 4 μl of 0.1 M dithiothreitol (DTT), 2 μl of 10 mM dNTP mix prepared in DEPC-dH_20) and 1 μl (200U) of Superscript II Reverse Transcriptase (Invitrogen) was added to initiate reverse transcription, which was performed for 1 hour at 37°C before the reaction was stopped by heating at 70°C for 15 minutes. Resulting cDNA was stored at -20°C.

2.4.2 Polymerase chain reaction

PCRs were carried out using a Techne TC-312 PCR thermocycler. 2 μl of cDNA was amplified with 20 pml of the relevant primers under gene-specific denature, cycling and final extension cycling parameters (Table 2.1, below). In addition to the primers, reaction mixtures also contained 0.5 U of *Taq*

polymerase (Promega), 1 X Taq DNA polymerase buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 1 μCi of [α^{32}P] dCTP (Amersham) made up with nuclease-free water to 20 μl final volume.

Table 2.1 Primers employed in RT-PCR reactions

Transcript	**Primers**	**Product Size**	**Cycle Number**	**Program Parameters**
ARPP P0	5'-GCACTGGAAGTCCAACT ACTTC-3' 5'-TGAGGTCCTCCTTGGT GAACAC-3'	265bp	18-22	95°C for 2 min 95°C for 1 min 58°C for 30 s 72°C for 1 min 72°C for 3 min
B2	5'-GGGGCTGGAGAGATGG CT-3' 5'-CCATGTGGTTGCTGGG AT-3'	120bp	15-18	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
Brf1	5'-AAATTCTGTGAGCCTCT TCCGTAGTG-3' 5'-AGACCCATGCTTGTACA TTCCACG-3'	260bp	21-24	95°C for 2 min 95°C for 1 min 60°C for 1 min 72°C for 1 min 72°C for 5 min
Lamin A/C	5'-GCTGAAAGCGCGCA ATAC 5'-CAGTCGGGTCTCATG ACG-3'	333bp	30-35	94°C for 3 min 94°C for 30 s 56° C for 30 s 72°C for 30 s 72° for 4 min

Maf1 (mouse)	5'-GCAGTTCTGCCAGG AGGGCCA-3' 5'-CTCCATGGTGCTGGTCTC CTC-3'	613bp	25-30	95°C for 2 min 95°C for 30 s 60°C for 30 s 72°C for 1 min 72°C for 5 min
Maf1 (human)	5'-CCATCAACTCACAGCTG AC-3' 5'-GGCTCTGCTGAAGTCAT AG-3'	307bp	25-30	95°C for 2 min 95°C for 30 s 58°C for 30 s 72°C for 1 min 72°C for 5 min
$tRNA^{Arg}$	5'-GGCTCTGTGGCGCAATG GATA-3' 5'-TTCGAACCCACAACCTT TGAATTGCTC-3'	74bp	20-22	95° for 2 min 95° for 30 s 66° for 30 s 72° for 15 s 72° for 5 min
$tRNA^{Leu}$	5'-GTCAGGATGGCCGAG TGGTGTAAGGCGCC-3' 5'CCACGCCTCCATACGGAG ACCAGACCC-3'	88bp	25-30	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
$tRNA^{Sec}$	5'-GGATGATCCTCAGTG GTC-3' 5'-GGTGGAATTGAACCA CTC-3'	74bp	21-24	95°C for 3 min 95°C for 30 s 60°C for 30 s 72°C for 30 s 72°C for 5 min

5S rRNA	5'-GGCATACCACCCTGAA CGC-3' 5'-CAGCACCCGGTATTCCC AGG-3'	107bp	19-22	95°C for 3 min 95° for 30 s 58° for 1 min 72° for 1 min 72° for 10 min
7SK	5'-CGATCTGGTTGCGACAT CTG-3' 5'-CGTTCTCCTACAAATG GAC-3'	247bp	25-30	95°C for 3 min 95°C for 30 s 57°C for 30 s 72°C for 30 s 72°C for 10 min
snRNA U6	5'-GCTCGCTTCGGCAGCA CATATAC-3' 5'-TATCGAACGCTTCACGA ATTTGCG-3'	96bp	18-20	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

Reaction products were resolved on 7% (v/v) polyacrylamide sequencing gels containing 7 M urea and 0.5 x TBE (45 mM Tris-HCl, 45 mM boric acid, 0.625 mM EDTA pH 8.0). Gels were pre-run for 30 minutes at 40 W in 0.5 x TBE and 1.5 µl of each sample was loaded after being boiled at 100°C for 2 minutes. Electrophoresis was carried out for a further 1 hour at 40 W and the gel subsequently vacuum-dried at 80°C for 1 hour before being exposed to autoradiography film in order to detect the radiolabelled products.

2.5 Preparation of whole cell extracts

All whole cell extracts were prepared from cells grown in 6-well culture plates or 10 cm tissue culture dishes to facilitate scraping, and were harvested at approximately 80% confluency. Preparation was performed on ice as rapidly

as possible and all solutions and tubes were kept ice-cold to maintain cell activity. Cells were washed twice with 5 ml of PBS before being scraped with a plastic spatula into 5 ml of ice-cold PBS. Cells were collected in chilled 50 ml Falcon tubes and pelleted by slow centrifugation at 1100g for 8 minutes at 4°C. A small volume of fresh ice-cold PBS was used to resuspend the cell pellets and allow the cells to be transferred to eppendorf tubes. These were then microcentrifuged briefly at 4°C to re-pellet the cells and the PBS removed. The volumes of cell pellets were then measured by comparison with pre-measured volumes of water. Optimal microextraction requires pellets to be between 50-150 µl, giving approximately 0.5–3 x 10^7 cells; larger pellets were subdivided. An equal volume of freshly made pre-cooled microextraction buffer (450 mM NaCl, 50 mM NaF, 20 mM Hepes pH 7.8, 25% glycerol, 1 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, 40 µg/ml bestatin, 1 µg/ml trypsin inhibitor, 0.7 µg/ml pepstatin, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin) was added to the cells and, following resuspension, the cells were immediately snap-frozen on dry ice. Cells were then placed in a 30°C water bath until just thawed before being immediately returned to dry-ice. This freeze-thaw procedure was performed a total of 3 times to ensure optimal cell lysis, with cells then being microcentrifuged at 7,000g for 7 minutes at 4°C after the third thaw. The supernatant was carefully decanted into a fresh tube, leaving behind the cell debris, and then promptly aliquotted and snap frozen. These extracts were then stored at –80°C.

2.6 Measuring protein concentration

The concentration of proteins in whole cell extracts was determined by using Bradford's reagent (Biorad). Quantification of the colour reaction produced

when 1 ml of diluted reagent (1:4 in distilled water) was mixed with a volume of sample containing protein in the range of ~1-12 μg gave a precise indication of protein concentration. A Bradford assay standard curve was made by measuring the absorbance of bovine serum albumin (BSA) standards at 595 nm in a UV spectrophotometer, as increasing amounts of protein in Bradford's reagent creates a linear curve. Once a standard curve was created within Microsoft Excel™, sample measurements of absorbance were plugged into the equation created by the standard curve, in this way obtaining the protein concentration. Three dilutions of each sample were measured and averaged to provide accurate concentrations.

2.7 SDS-polyacrylamide gel electrophoresis (PAGE) analysis

2.7.1 Separation of proteins by SDS-PAGE

Proteins were resolved on denaturing polyacrylamide gels according to molecular weight by electrophoresis. Typically, 7.8% polyacrylamide resolving mini-gels (375 mM Tris pH 8.8, 0.1% SDS) were used with a stacking layer comprised of 4% polyacrylamide gel (125 mM Tris pH 6.8, 0.1% SDS) based on the discontinuous buffer system described by Laemmli (Laemmli, 1970). Samples were boiled for 2 minutes in 1 x protein sample buffer (62.5 mM Tris pH 6.8, 0.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.125% bromophenol blue) prior to loading. Electrophoresis was performed in 1 x SDS running buffer (0.1% SDS, 76.8 mM glycine, 10 mM Tris, pH 8.3) at an initial voltage of 100 V while the bromophenol dye front moved through the stacking gel and a subsequent voltage of 150 V after

reaching the resolving gel. Electrophoresis was allowed to proceed until the dye front had reached the bottom of the gel, approximately 1 – 1.5 hours.

2.7.2 Western blotting

Electrophoretic transfer of proteins resolved by SDS-PAGE to PVDF membrane was achieved using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system. Transfer was carried out in 1 x transfer buffer (76.8 mM glycine, 10 mM Tris, pH 8.3, 16.5% methanol) at 50 V for 1 hour. Transfer sandwich was placed in the transfer gel tank alongside an ice pack, or was performed at 4°C. Following transfer, the membrane was blocked in milk buffer (32.5 mM Tris, 150 mM NaCl, 0.2% Tween-20, 4% skimmed milk powder (Marvel)), for 1 hour at room temperature. Membranes were incubated with primary antibodies (typically a 1:1000 dilution in milk buffer) overnight at 4°C, or for 2 hours at room temperature (~22°C). Excess primary antibody was removed by washing the blot 3 times for 2 minutes in fresh milk buffer before incubating for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000 dilution in milk buffer) (DAKO). To ensure removal of excess secondary antibody, the blot was sequentially washed in batches of fresh milk buffer, 3 times for 2 minutes, followed by 2 washes for 15 minutes. After one further 5 minute wash using 1 x TBS (2.5 mM Tris-HCl pH 7.6, 15 mM NaCl), the blot was developed using the enhanced chemiluminescence method (ECL™, Amersham) as directed by the manufacturer.

2.7.3 Western signal detection

Horse radish peroxidase-conjugated secondary antibodies were used to detect signals on Western blots. Chemiluminescence detection (using ECL™,

above), was performed by adding a ratio of 1 part reagent 1 to 1 part reagent 2 to the filter. The solution was applied to the filter for 1 minute at room temperature. The solution was then gently wiped away with blotting paper, covered in cling film, exposed to ECL film (Amersham Pharmacia) for varying lengths of time, and then developed using the X-OMAT film processor.

2.7.4 Primary antibodies

Table 2.2

Protein	Antibody	Type	Company
Actin	C11	Polyclonal	Santa Cruz Biotechnology
Brf1	SK-2839	Polyclonal	In house
Maf1	1167	Polyclonal	In house
Sp1	420	Monoclonal	Santa Cruz Biotechnology

2.8 Transformations

2.8.1 Storage of competent cells

1 ml of bacterial culture was added to 40% (v/v) glycerol solution (in sterile distilled water), before freezing in liquid nitrogen.

2.8.2 Transformation of competent cells

E.coli XL-1 Blue supercompetent cells (Stratagene) were transformed for plasmid storage and propagation. Cells, stored at –80°C and highly temperature sensitive, were thawed on ice to prevent loss of transformation

ability. 0.4 µl of β-mercaptoethanol, which enhances transformation efficiency, was added to the 50 µl of cells that were required per transformation reaction to give a final concentration of 25 mM. Typically 10 – 20 ng of plasmid DNA was then gently mixed into the chilled cells. The contents were gently tapped occasionally during a 30 minute incubation on ice, before being heat shocked at 42°C for exactly 45 seconds and then transferred to ice for a further 2 minutes. Cells were incubated at 37°C for 1 hour on an orbital shaker (225 – 250 rpm) following the addition of 450 µl of preheated (42°C) SOC medium (LB broth, 0.04% (w/v) glucose, 10 mM $MgSO_4$, 10 mM $MgCl_2$). Typically 100 µl and 250 µl of the transformation mixture was then plated on LB agar (2% (w/v) LB, 2% (w/v) agar) plates containing 50 µg/ml ampicillin (Amp) and the plates were incubated at 37°C overnight to allow growth and colony-formation of the transformed cells.

2.9 Techniques used in the creation of the plasmid pTRE2.hyg.Ha.HsMaf1

2.9.1 Preparation of plasmid DNA

For large scale plasmid DNA preparation, a single isolated bacterial colony was selected from a freshly-streaked plate and used to inoculate 5ml of LB medium containing the selective antibiotic (100 µg/ml ampicillin). This was allowed to incubate with vigorous shaking at 37°C for ~7 hours to form a mini-culture and was subsequently used to inoculate 250 ml of LB medium containing 100 µg/ml ampicillin. Following an overnight incubation at 37°C on an orbital shaker (~300 rpm), cells were harvested by centrifugation at

6,000g for 15 minutes at 4°C and plasmid DNA retrieved using the Qiagen Plasmid Maxi Kit.

The bacterial pellet was resuspended in 10 ml of Buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μg/ml RNase A) and then gently, but thoroughly mixed with 10 ml of Buffer P2 (200 mM NaOH, 1% (w/v) SDS) to initiate an alkaline lysis reaction. This reaction was allowed to proceed for 5 minutes at room temperature before neutralising the lysate by the addition of 10ml of chilled Buffer P3 (3 M potassium acetate, pH 5.5), which subsequently resulted in formation of a precipitate of potassium dodecyl sulphate. The SDS-denatured proteins and chromosomal DNA were co-precipitated with the detergent, whilst the plasmid DNA remained in solution due to a lack of close protein associations. Precipitation was enhanced by a 20 minute incubation on ice and the precipitate pelleted by centrifugation at 20,000g for 30 minutes at 4°C. The supernatant containing plasmid DNA was promptly removed and applied to a Qiagen-tip 500 column, pre-equilibrated with 10 ml of Buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol, 0.15% (v/v) Triton X-100). Gravity flow allowed the supernatant to pass through the anion-exchange resin to which plasmid DNA is able to bind tightly. The resin was then washed twice with 30 ml of Buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol) and the purified plasmid DNA was subsequently eluted with 15 ml of Buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% (v/v) isopropanol) and precipitated with 10.5 ml of room-temperature isopropanol. This was immediately followed with a 15,000g centrifugation at 4°C for 30 minutes. The plasmid DNA pellet was then washed with 70% (v/v) ethanol, dried at room-temperature for 5 – 10 minutes and resuspended in an

appropriate volume of sterile water. The DNA was then quantified using the spectrophotometer, as in section 2.3.

2.9.2 Restriction digests

Restriction digests were performed in 20 µl volume and contained; 1 µg DNA, 1 µl enzyme (or 1 µl each, if using two enzymes), 2 µl enzyme buffer (appropriate to the restriction enzyme being used), and were made up to the final volume with sterile distilled water. All enzymes and buffers were supplied by Promega. DNAs were digested with enzymes for 2 hours at 37°C. Amounts of DNA in a restriction digest varied between 1-3 µg for plasmid DNA and 3 - 5 µg for genomic DNA.

Digests were mixed with 6 x Agarose Gel DNA Loading Buffer (0.25% (w/v) Bromophenol Blue, 0.25% (w/v) Xylene Cyanol, 30% (v/v) glycerol), analysed on 1% (w/v) agarose gels containing ethidium bromide (Sigma) and visualised on an ultraviolet light box. The concentrations of nucleic acid solutions were determined spectrophotometrically using a quartz cuvette.

2.9.3 Agarose gel electrophoresis of DNA

DNAs were separated in 1 % (w/v) agarose in 1x TAE (40 mM Tris-acetate, 1 mM EDTA pH 8.0) containing 0.1 µg/ml ethidium bromide, using 1x TAE as the electrophoresis buffer. Sizes were compared to a 1kb ladder (Gibco-BRL). Prior to loading, 6 x loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in water) was added to the samples to a final 1x concentration of loading dye in the sample.

2.9.4 Purification of DNA from gels

DNA bands were excised from the gel using a sterile scalpel blade under a UV light, and the DNA extracted using the Qiagen Gel Extraction Kit according to the manufacturer's instructions. DNA was typically eluted in 30 µl of pre-warmed buffer EB (Qiagen) or H_2O.

2.9.5 DNA ligations

For cloning inserts into vectors, plasmid DNA containing the vector and the insert were digested with appropriate restriction enzymes, as described in sections 2.9.2 and 2.9.6. For directional cloning of inserts, two different enzymes were used to restrict the vector and the insert, which permits cloning of the insert into the vector in a predicted and directional manner. After the restriction digest, both vector and insert were electrophoresed on a 1% (w/v) agarose gel and the DNA bands excised from the gel and gel-purified (see section 2.9.4).

For the ligation reaction, molecular ratios of 1:1, 3:1, and 6:1 (insert:vector) were used and typically 50-100 ng of vector. Ligation reactions were carried out with 2 µl 10x T4 ligase buffer (Gibco BRL), with 1 µl (1 U) of T4 ligase (Gibco BRL) in a final volume of 20 µl with sterile water. The ligation was carried out overnight at 14°C. For transformation of XL1-Blue, 5 µl of the ligase reaction was used.

2.9.6 Subcloning of Maf1 into pCDNA3.HA

In order to apply an HA- tag to Maf1, Maf1 was first subcloned into the pCDNA3.HA vector, then digested out. The plasmid, pGEM-T.HsMaf1 was a

gift from Olivier Lefebvre (CEA/Saclay, France). This plasmid was digested with *Bam*HI and *Sal*I. PCRs were then performed to insert a *Bam*HI site onto the 5' end of the gene (5'-GTAGCTGGATCCATGAAGCTATTGGA-3' and 5'-GGCGGCTCTAGAACGCGTCGACTCAAATAC-3'). The PCR product was then ran out on an agarose DNA gel, and the correct band excised. The excised product was then purified using the Qiagen Gel Extraction Kit. pCDNA3.HA and the HsMaf1 vector were then digested with *Bam*HI and *Xba*I. The separate bands were cut from an agarose DNA gel and the products purified, as above. pCDNA3.HA and HsMaf1 were then ligated together. After ligation, *E.coli* XL-1 Blue supercompetent cells (Stratagene) were transformed with the ligated pCDNA3.HA.HsMaf1. In order to produce a high plasmid yield, cells were cultured in 5 mls (mini prep), then 250 mls (maxi prep). The presence of the plasmid was checked by digestion in both mini and maxi prep stages. PCR was then performed to add an *Nhe*I site onto the 5' end of the gene (5'-GCACGCGCTAGCATGGAATTCT-3'and 5'-GTAGCTGGATCCATGAAG CTATTGGAGAAC-3'). The PCR product was then run on an agarose DNA gel and the proper band excised and purified. This procedure produced HA.HsMaf1.

2.9.7 Subcloning of HA.HsMaf1 into pTRE2.hyg

HA.HsMaf1 was digested with *Nhe*I and pTRE2.hyg with *Nhe*I and *Sal*I. A PCR cleanup was performed using the QIAquick PCR Purification Kit (Qiagen), as per the manufacturer's instructions. HA.Maf1 was then ligated into pTRE2.hyg, to create pTRE2.hyg.HA.HsMaf1. Using the same competent cells as above, transformations were done with ligated plasmid. As above, mini, and then maxi preps were cultured to increase DNA yields of the

plasmid. At each culture volume the plasmid was checked by digestion with *Nhe*I and *Sal*I enzymes. The final plasmid, pTRE2.hyg.HA.Hs.Maf1 was sent for sequencing and yielded ~6.1 kb plasmid.

2.9.8 Stable transfection

HeLa-derived Tet-On cells for stable transfection were plated out at 2 x 10^5 cells/10 cm dish 24 hours prior to transfection, resulting in a confluency of ~70 – 80% at the time of transfection. Two 10 cm dishes were transfected with 5 µg of pTRE2.HA.Maf1.hyg construct and 5 µg of the empty, pTRE2.hyg vector. Mastermixes for each set of 10 cm dishes were made up comprising the appropriate plasmid DNA and Opti-MEM I reduced serum medium (Gibco BRL) to give a volume of 100 µl per dish. Then, for each transfection, 25 µl of Lipofectamine reagent was diluted into 100 µl of serum-free medium. The two solutions were combined, mixed gently and incubated at room temperature for 45 minutes in the dark to allow DNA-liposome complexes to form. While complexes were forming, cells were washed once with 3 mls of serum-free medium. For each transfection, 0.8 ml of serum-free medium was added to the tube containing the complexes. This was mixed gently and the diluted complex solution overlayed onto the rinsed cells. Cells were incubated with the complexes for 3 hours at 37°C in a CO_2 incubator. Following incubation, 10 mls of normal growth medium, plus G418 100 µg/ml, was added to each dish. Cells were allowed to grow for a further 24 hours before adding the selection agent hygromycin B (Melford Laboratories), at a concentration of 600 µg/ml, to the culture medium. To ensure Tet-On gene expression system remained 'switched off' during the selection process, cells were cultured with Tet-approved FBS (BD Biosciences, Clonetech). Fresh complete selection

medium, containing hygromycin, was replaced every four days. After about four days, cells began to die but were split if they reached confluency before massive cell death began. After two to four weeks, healthy hygromycin-resistant colonies were isolated and transferred to individual plates or wells. Potential clones were then screened using Maf1 specific assays to identify the clones with the lowest background of Maf1 gene expression and highest induction of Maf1 gene expression. This was achieved by western blot using antibodies to both transfected Maf1 (with the HA tag), and total Maf1, as well as RT-PCRs for Maf1.

2.10 Electroporation of HeLa and MCF-7 cells

For all electroporation experiments, an Amaxa Nucleofector (Amaxa GmbH) was used. HeLa cells were electroporated using the Cell Line Nucleofector™ Kit R (program I-20), while MCF-7 cells were electroporated using the Cell Line Nucleofector™ Kit V (program P-20) following the procedures listed in the manufacturer's instructions.

All siRNAs were designed and synthesised by Qiagen.

Lamin A/C [sense: r(CUGGACUUCCAGAAGAACA)dTdT; antisense: r(UGUUCUUCUGGAAGUCCAG)dTdT]

Brf1 siRNA-1 [sense: r(GGAUGCAAUUGAGAUUGAA)dTdT; antisense: r(UUCAAUCUCAAUUGCAUCC)dTdG]

Brf1 siRNA-2 [sense: r(GGAGGAGGUUGAAGGUGAA)dTdT; antisense: r(UUCACCUUCAACCUCCUCC)dAdG]

2.10.1 Electroporation of HeLa cells in Brf1 and Maf1 siRNA experiments

HeLa cells were grown to ~80% confluency before being used for electroporation. Cells were removed from flasks via trypinization (as above), and centrifuged at 1,100g to produce a cell pellet. This pellet was resuspended in a specific volume of fresh media and cells were counted with a haemocytometer (Sigma). 1 X 10^6 cells were used per nucleofection cuvette. The manufacturer's instructions for nucleofection were followed, utilizing the HeLa-specific I-13 program on the nucleofector machine. 3 μg of each siRNA (Lamin A/C, HsMaf1 pooled, Brf1 siRNA-1, Brf1siRNA-2 or a pool of both Brf1 siRNAs) was used per reaction cuvette. Each reaction was split evenly into a 6-well plate, with the addition of fresh media. Cells were harvested 48 hours later. siRNA sequences were as follows:

Lamin A/C [sense: r(CUGGACUUCCAGAAGAACA)dTdT; antisense: r(UGUUCUUCUGGAAGUCCAG)dTdT]

Brf1 siRNA-1 [sense: r(GGAUGCAAUUGAGAUUGAA)dTdT; antisense: r(UUCAAUCUCAAUUGCAUCC)dTdG]

Brf1 siRNA-2 [sense: r(GGAGGAGGUUGAAGGUGAA)dTdT; antisense: r(UUCACCUUCAACCUCCUCC)dAdG]

HsMaf1 siRNAs (1 μg of each siRNA was used for a pool of 3 μg total): Target 1 [sense: r(GAUGGCGGGAGAUGAUAAA)dTdT; antisense: r(UUUAUCAUCUCCCGCCAUC)dTdT]; Target 2 [sense: r(GUCAUGAAU UCAGCCGAGA)dTdT]; antisense: r(UCUCGGCUGAAUUCAUGAC)dTdT] Target 3 [sense: r(AUGCAGUCAACUGCAGCCU)dTdT]; antisense: r(AGGCUGCAGUUGACUGCAU)dTdT

All siRNA sequences were designed and synthesised by Qiagen. "Scrambled siRNA" is a non-targeting negative control duplex known to have no limited sequence similarity to known genes. The sequence is not provided when purchased.

2.10.2 HeLa and MCF-7 siRNA proliferation rate assays

Cells were grown in normal growth medium to ~80% confluency before nucelofection. 1 X 10^6 cells and 3 µg siRNA were used per reaction, following the manufacturer's instructions for nucleofection, the correct solutions, and program, above. After electroporation, cells were pooled into 45 mls fresh media, gently pipetted up and down, and aliquotted into four 10 cm culture dishes. This allowed for even distribution of the cells. Time 0 was counted with a haemocytometer by immediately counting the cells placed within a 10 cm plate. The cells were grown for a period of 72 hours, replacing medium after 2 days. Total numbers of dead cells were calculated by trypsinizing cells as discussed previously, and resuspending in DMEM and trypan blue (Sigma), although these counts averaged less than 1 cell per haemocytometer (Sigma), and therefore were dismissed. Counts were made in triplicate with at least two 10 cm plates being used for each condition examined. Viable cell counts were plotted against day number to show proliferation rates of the cells, and the changes from time 0 to 72 hours plotted as a percentage change.

2.10.3 pTRE2.hyg.HA.HsMaf1 proliferation rate analysis

Cells were grown to ~80% confluency before plating. 2.0 X 10^5 cells were plated per 10 cm plate. Cells counted at time point 0 were counted, via a haemocytometer, immediately after application to the 10 cm plate. 24 hours

after plating, half the cells were induced by the addition of 1 mg/ml doxycyclin, and fresh media was applied to all cells. Cells were counted at 96 and 120 hours after induction. Counts were made in triplicate, with at least two 10 cm plates being counted for each condition. Viable cell counts were plotted against day number to visualise proliferation rates graphically.

2.11 Tagetitoxin experiments

2.11.1 Direct application of tagetitoxin to NIH 3T3 cells

Commercially made tagetitoxin, Tagetin™, was purchased from Epicentre®. NIH 3T3 cells were seeded at 1 X 10^5 per well of a 6-well culture plate, and were left at normal culture conditions for 24 hours. 45 μM Tagetin™ was then applied to half of the wells, along with 500 μl of normal culture media. After 2 hours incubation cells were harvested for RNA.

2.11.2 RNA pol III *in vitro* transcription assay

In vitro transcription of class III genes was reconstituted using 20 μg of HeLa nuclear extract to provide the basal pol III transcription components. This was supplemented with the addition of 250 ng of plasmid DNA to supply a specific pol III template and reactions were carried out in a 25 μl volume with a final concentration of 12 mM HEPES pH 7.9, 60 mM KCl, 7.2 mM $MgCl_2$, 0.28 mM EDTA, 1.2 mM DTT, 10% (v/v) glycerol, 1 mM creatine phosphate, 0.5 mM each of rATP, rCTP and rGTP and 10 μCi [α-^{32}P] UTP (400 mCi/mmol) (Amersham). In one half of the reaction tubes, HeLa nuclear extract was incubated for 10 minutes at 30°C with 2.5 μl of 100 μM Tagetin™ before the addition of the nucleotides, radioisotopes, etc, listed above. The other half of the reaction tubes (the control tubes) were supplemented with 2.5 μl sterile

distilled water and incubated for 10 minutes at 30°C. After this 10 minute incubation, 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 the synthesised RNA. Phenol-chloroform extraction of the samples was performed to remove protein and DNA by adding 250 µl of a 25:24:1 ratio solution of 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 eppendorf tube containing 750 µl of 96% ethanol in order to precipitate the RNA. The samples were thoroughly mixed by repeated inversion, and left at –20°C overnight before being microcentrifuged at 13,000g for 20 minutes to pellet the precipitated RNA. The supernatant was carefully removed and 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 37°C for 5 – 10 minutes to dry. 4 µl of formamide loading buffer (98% formamide, 10 mM EDTA pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol FF) was added to each sample, which was then vortexed for 20 minutes to ensure the RNA was fully redissolved. 2 µl of each sample was loaded on a pre-run 7% polyacrylamide sequencing gel containing 7 M urea and 0.5 x TBE (45 mM Tris, 45 mM boric acid, 0.625 mM EDTA pH 8.0) after being boiled at 100°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.

2.11.3 Plasmid templates used in the *in vitro* transcription assay

The plasmid templates used for the *in vitro* transcription assay were as follows: pVA_I is a 221 bp *Sal*I-*Bal*I fragment of adenovirus 2 DNA containing the VA_I gene subcloned into pUC18 (Dean and Berk, 1988); pLeu is a 240 bp *Eco*RI-*Hind*III fragment of human genomic DNA carrying a $tRNA^{Leu}$ gene, subcloned into pAT153 (McLaren and Goddard, 1986); PHu5S3.1 is a 638 bp *Bam*HI-*Sac*I fragment of human genomic DNA containing a 5S rRNA gene, subcloned into pBluescript SK+; and the pol I template was pHrP2 (Lescure *et al.*, 1994), which has an 800 bp *Sau*3A fragment of the human rRNA promoter, from -411 to +378, subcloned into *Bam*H1-linearised pUC9.

2.11.4 Application of tagetitoxin to NIH 3T3 cells by heat shock

Cells were seeded at 1 X 10^5 per well of a 6-well culture plate and left at normal culture conditions for 24 hours. Medium was aspirated off and 500 µl of fresh media was applied to cells. 45 µM Tagetin™ was then applied to half of the cells, and incubated for 60 minutes at 37°C. Cells were then heat-shocked for 25 minutes by placing them in an incubator at 45°C. Cells were then allowed to recover for 40 minutes at 37°C before RNA extracts were made.

2.11.5 Nucleofection of HeLa cells with tagetitoxin

Electroporation of cells with Tagetin™ was performed in the same manner as the siRNA experiments, per the manufacturer's instructions, using Cell Line Nucleofector™ Kit R and program I-13. 1 X 10^6 cells were used per reaction cuvette, along with distilled sterile water as a control or 30 µl (22 µM) Tagetin™. The nucleofected cells were added drop wise to 2 mls media that

had been pre-incubated in 6-well culture plates. Cells were harvested 48 hours later for RNA extracts.

Chapter 3

Decreasing Brf1 levels by small interfering RNA decreases RNA polymerase III transcription

3.1 Introduction

3.1.1 *Saccharomyces cerevisiae* and Brf1 discovery

Early research on Brf1-containing transcription factor –IIIB was mostly done in *S. cerevisiae*. Klekamp and Weil and Kassavetis *et al.* purified TFIIIB and found that a fraction was capable of reconstituting tRNA transcription when incubated with TFIIIC and pol III (Kassavetis *et al.*, 1991a; Klekamp and Weil, 1986). Further chromatography split this fractionation into two constituents, designated B' and B," and both components proved essential for tRNA transcription when added to TFIIIC and pol III (Kassavetis *et al.*, 1991a). To examine these fractions further, Bartholomew *et al.*, probed the polypeptide composition of TFIIIB by photocrosslinking and detected two polypeptides of 70 and 90 kDa (Bartholomew *et al.*, 1991). The 70 kDa component was found in the B' fraction of TFIIIB, while the 90 kDa component was found in the B" fraction (Kassavetis *et al.*, 1991a).

These discoveries heralded the cloning of the Brf1 gene by three groups, encoding a protein of 596 amino acids with a predicted molecular mass of 67kDa (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-De-Leon, 1992). Extracts deficient in Brf1 are defective in pol III transcription but can be reconstituted by the addition of recombinant Brf1 (Colbert and Hahn, 1992). Mutation of the Brf1 gene showed a decrease in class III gene expression (namely tRNA and 5S rRNA), but had no effect on *in vivo* expression of class I or class II genes (Lopez-De-Leon, 1992). This discovery implied that Brf1 may be needed for transcription complex assembly on class III genes, but is not necessary on class I or II genes.

As stated in the introduction, Brf1 was named to reflect its homology with TFIIB. Yeast Brf1 displays 23% identity and 44% similarity to TFIIB in its 320 N-terminus residues and two imperfect repeats of 76 amino acids in the amino region (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-De-Leon, 1992). The C-terminal half is dissimilar, although three regions of conservation have been noted and designated as homology regions I, II and III (HI, HII, HIII) (Khoo *et al.*, 1994). These regions might later prove to be significant sequences, possibly determining the function of Brf1 through mechanisms such as phosphorylation.

3.1.2 Brf1 interactions within the pol III initiation complex

3.1.2.1 TBP

Because TFIIB and TBP were known to interact, it was postulated that Brf1 might interact with TBP in a similar manner (Ha *et al.*, 1993). Indeed, further analysis by mutagenesis showed that Brf1 contains two separate TBP-binding domains that interact with opposite faces of complexed TBP-DNA (Colbert *et al.*, 1998; Kassavetis *et al.*, 1998). While Brf1 binds to the TFIIIC-tRNA gene complex weakly, the interaction is made stable by two binding sites on TBP, the TBP- Brf1 complex being extremely stable (Kassavetis *et al.*, 1992). One binding site is assigned to the N-terminal half, in the conserved direct repeat region, while the C-terminal half of Brf1 (namely the HII domain) arbitrates another interaction with TBP (Andrau *et al.*, 1999; Colbert *et al.*, 1998; Khoo *et al.*, 1994). This was proved further when *in vitro* experiments demonstrated that recombinant TBP and Brf1 adequately reconstitute all known properties of the B' fraction (Kassavetis *et al.*, 1992), mimicking the properties of the

proteins isolated in the B' complex from the same laboratory one year earlier (Kassavetis *et al.*, 1991a).

3.1.2.2 TFIIIC

Brf1 contacts DNA-bound TFIIIC by protein-to-protein interactions, therefore recruiting TFIIIB to genes containing type I or II promoters. In mammalian cells, TFIIIC binds to Brf1 through its 63, 90, and 102 kDa subunits (Hsieh *et al.*, 1999b; Hsieh *et al.*, 1999a), while in yeast TFIIIC subunit Tfc4 binds to Brf1 (Kassavetis *et al.*, 1992). Once recruited to a promoter through TFIIIC, TFIIIB locks to the promoter in an incredibly stable complex (Kassavetis *et al.*, 1990). This binding is important as it allows TFIIIC to act as a "positioner," dictating the optimal location for TFIIIB on the promoter (Joazeiro *et al.*, 1996).

3.1.2.3 Pol III

Werner *et al.*, used a two-hybrid system with expression monitored by the GAL1-*lacZ* reporter to investigate protein-protein interactions between various pol III subunits and Brf1 (1993). Pol III subunit C34 was found to interact with Brf1, but not subunits C82, C34, or C31. This discovery places even more importance on the TFIIIB subunit Brf1, as it shows that this interaction directs pol III to its associated genes, influencing transcription.

3.1.2.4 Bdp1

Brf1 plays a critical role in holding TFIIIB together through its interactions with not only TBP, but also Bdp1. Binding of Brf1 and Bdp1 to the TBP-TFIIIC-DNA complex is extremely important, cementing the protein to protein

interactions, helping to determine the physical properties of the TFIIIB-DNA complex, and actually bending the DNA (Colbert *et al.*, 1998; Kumar *et al.*, 1997; Shah *et al.*, 1999). Interactions of Brf1, held together by Bdp1, help to protect the DNA from DNase I (Shah *et al.*, 1999). Brf1 and Bdp1 have overlapping binding sites on Tfc4 (TFIIIC), competing for binding in TFIIIC-directed TFIIIB complex assembly (Liao *et al.*, 2003).

By using Brf1 fragments and photochemical protein-DNA cross-linking, Kassavetis *et al.*, established the sites of Brf1 attachment to Bdp1 (Kassavetis *et al.*, 1998). The TBP- and Bdp1- interacting domains of the C-terminal half of Brf1 both reside within the amino acid 435- 45 segment, which contains Brf homology region 2. This region of Brf1 is necessary and sufficient for Bdp1 recruitment to a stable DNA complex (Kassavetis *et al.*, 1998). Although it was found to be a weaker interaction, the N- proximal half of Brf1 also binds to Bdp1 (Kassavetis *et al.*, 1998; Khoo *et al.*, 1994). Both halves of Brf1 are able to recruit Bdp1 to the TFIIIB-DNA complex (Kassavetis *et al.*, 1998). Further recent analysis from Kassavetis *et al.* showed that the principal attachment site of Brf1 for Bdp1 is a 66-amino acid segment of Brf1, serving as a two-sided adhesive surface, the side chains projecting away from its interface with TBP anchoring Bdp1 binding (2006).

Brf1 and Bdp1 also have connected roles during mitosis through phosphorylation. Fairley *et al.* observed that hyperphosphorylation of Brf1 during mitosis may reduce its affinity for Bdp1, allowing the release of Brf1 and thereby inhibiting transcription (2003). Therefore, even though the

structures of Bdp1 and Brf1 are very different, their roles within the transcription apparatus seem to be closely connected.

3.1.3 Brf1 is an influential binding sight for tumour suppressors, oncogenes, and kinases

(Summarized in Figure 3.1).

3.1.3.1 Tumour suppressors RB and p53

Brf1 emanates further importance in its role as a influential binding protein by directly binding with known tumour suppressors RB and p53. Recombinant and endogenous RB was shown to bind to Brf1 in pull-down and immunoprecipitation experiments (Chu *et al.*, 1997; Larminie *et al.*, 1997). This binding disrupts the interaction between TFIIIB and TFIIIC, dissociating TFIIIB from its functional association with TFIIIC (Sutcliffe *et al.*, 2000). By RB binding to TFIIIB, RB is able to function and instil its tumour suppressor properties on pol III transcription, keeping levels of pol III transcription under check. This is vital, when considering that mutations in the *Rb* gene are found in many human cancers, while other proportions contain the wild type *Rb* but its function is disrupted (Grana *et al.*, 1998; Herwig and Strauss, 1997; Mulligan and Jacks, 1998; Weinberg, 1995).

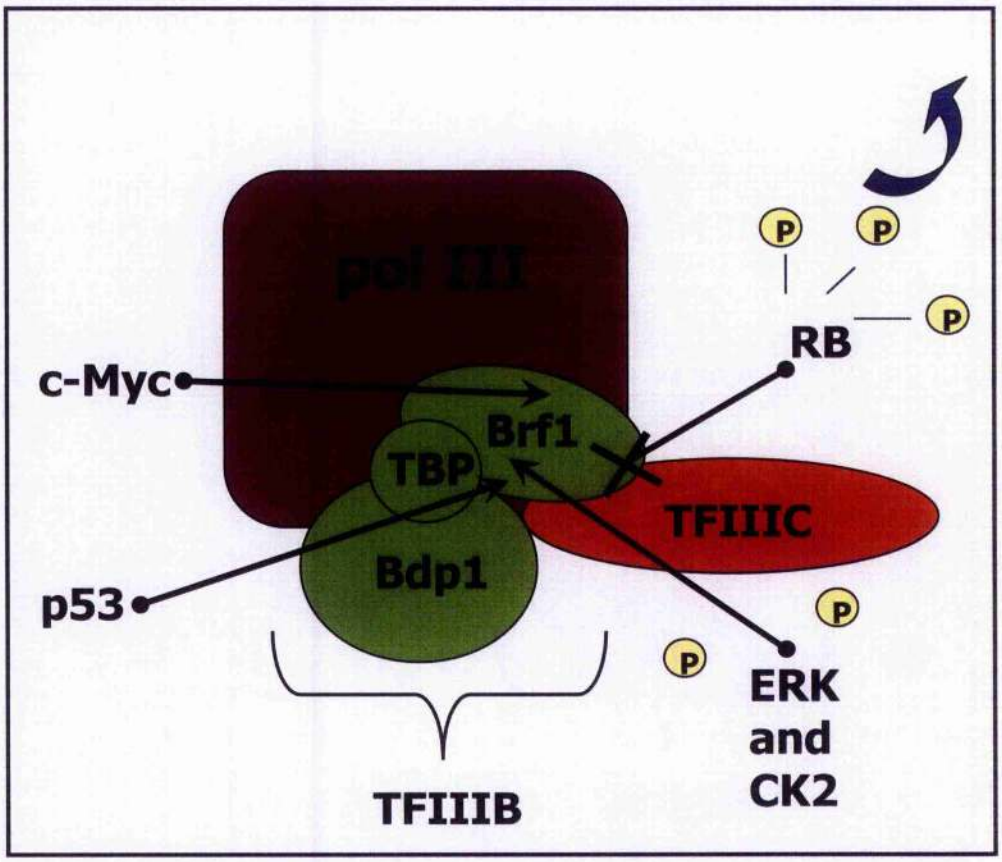


Figure 3.1. Brf1 is an influential binding site for tumour suppressors, oncogenes and kinases.

Brf1 binds to pol III subunit C34, while TFIIIC binds to Brf1 through its 63, 90, and 102 kDa subunits. TBP binds with both the C- and N-terminal halves of Brf1. Bdp1 and Brf1 compete for binding sites on TFIIIC. Residues 1-262 of c-Myc, encompassing its transactivation domain, were found to bind to Brf1. When p53 is bound to TFIIIB, TFIIIB is unable to interact with TFIIIC or be recruited to pol III templates. Hyperphosphorylaton of RB causes dissociation from TFIIIB, therefore derepressing transcription. ERK and CK2 both phosphorylate Brf1.

As stated previously, when p53 is bound to TFIIIB, TFIIIB is unable to interact with TFIIIC or be recruited to pol III templates (Crighton *et al.*, 2003). p53 was found to specifically bind Brf1 during immunoprecipitations in both endogenous and *in vitro* experiments in amounts comparable to p53- TBP. Interestingly, many missense substitution mutations (75%) found to be expressed in human malignancies with mutant forms of p53 are located in the core domain- the very region that is required for p53 regulation of TFIIIB (Bullock *et al.*, 2000; Hollstein *et al.*, 1994; Stein *et al.*, 2002a; Stein *et al.*, 2002b).

This further substantiates Brf1 as an important subunit in the transcription factor- IIIB complex, as it would argue that loss or mutation of p53 in the domain that binds Brf1 will contribute to the derepression of TFIIIB, and hence the deregulation of pol III transcription in a substantial portion of human malignancies.

3.1.3.2 Proto-oncogene c-Myc

Investigations by Gomez-Roman *et al.*, showed that c-Myc is able to directly activate pol III transcription by binding to Brf1 (2003). Residues 1-262 of c-Myc, encompassing its transactivation domain, were found to bind to Brf1 through protein-protein interactions *in vitro* using gluthathione *S*- transferase (GST) fusion proteins. Endogenous experiments demonstrated that c-Myc also fractionates with TFIIIB activity on several columns, binding stably and specifically (Gomez-Roman *et al.*, 2003).

The binding of c-Myc to Brf1 gains considerable importance when the implications of this binding were discovered. c-Myc-TFIIIB binding potently stimulates transcription of pol III transcripts $tRNA^{Leu}$, $tRNA^{Tyr}$ and 5S rRNA within cells (Gomez-Roman *et al.*, 2003; Hirst and Grandori, 2000). This increase in biosynthesis would be expected to impact strongly on cell growth, as c-Myc also targets genes transcribed by pol II that are involved in metabolism and translation (Boon *et al.*, 2001; Rosenwald, 1996). Indeed, deregulation of c-Myc was found to be a commonality in some Burkitt's lymphomas, neuroblastomas and colon carcinomas (Dang, 1999; Nesbit *et al.*, 1999). In summary, Brf1-c-Myc binding contributes to the deregulation of pol III transcription, which in turn may influence c-Myc's role in growth control.

3.1.3.3 Kinases ERK and CK2

ERK is connected to pol III transcription not only through its binding with TFIIIB, but also through c-Myc activation, causing a rapid increase in translation and growth (Rosenwald, 1996; Schmidt, 1999). ERK is able to co-regulate both pol I and pol III, where the phosphorylation of UBF in the pol I system can regulate DNA-binding properties, while the phosphorylation of TFIIIB enhances its ability to bind to TFIIIC and pol III. This coordinates the production of pol I products 5.8S, 18S and 28S rRNA and pol III products 5S rRNA and tRNA, which are required in equimolar quantities within the cell (Felton-Edkins *et al.*, 2003a; Schmidt, 1999; Stenfanovsky *et al.*, 2001).

The phosphorylation of TFIIIB by ERK is applied to the Brf1 subunit and ERK must be activated in order for it to bind (Felton-Edkins *et al.*, 2003a). Once activated in the cytoplasm, ERK must translocate to the nucleus to interact

with TFIIIB. Further analysis by Felton-Edkins *et al.* by mutations of Brf1s ERK phosphorylation site or putative docking domain showed a significant reduction of pol III activation in fibroblasts (2003a). However, when ERK activity was blocked by a MEK inhibitor, Brf1 phosphorylation was only partially reduced, making it likely that another kinase, such as CK2, is required for the uppermost levels of pol III transcription (Felton-Edkins *et al.*, 2003a; Ghavidel *et al.*, 1999; Ghavidel and Schultz, 1997; Johnston *et al.*, 2002).

The ubiquitous protein kinase CK2 was found to cofractionate and coimmunoprecipitate with TFIIIB, namely the Brf1 subunit, in yeast and mammalian cells (Ghavidel and Schultz, 1997; Johnston *et al.*, 2002). As with ERK, CK2 promotes interactions between TFIIIB and TFIIIC and phosphorylation by CK2 may be required for Brf1 to interact with TFIIIC (Johnston *et al.*, 2002). CK2 was also found to phosphorylate the HII region of Brf1 (L.E. Mitchell and R.J. White, unpublished observations).

As abnormal CK2 activity is linked to a variety of human cancers (Faust *et al.*, 1996; Munstermann *et al.*, 1990; Notterman *et al.*, 2001) and CK2 activation of pol III transcription through phosphorylation of Brf1 stimulates the synthesis of pol III products (Johnston *et al.*, 2002), the relationship between Brf1 and CK2 is clearly important.

3.1.4 Brf1 and human papillomavirus 16 (HPV16)

The most prevalent cases of cervical cancers were found to correlate with infection by HPV 16 (zur Housen, 2000). The pathway a cervical cell follows to transformation includes the expression of oncogenes E6 and E7, which serve

to inactivate tumour suppressors p53 and Rb (Dyson *et al.*, 1989; Munger *et al.*, 1989). Futhermore, infection by HPV 16 was linked to elevated Brf1 mRNA in cervical biopsies tested by Daly *et al.* which does not result from inactivation of RB or p53 (2004). In cervical cells Brf1 levels are limiting for production of tRNA and 5S rRNA, making it a major player in a cell's biosynthetic capacity. These results provided the first evidence that a virus may induce Brf1 expression, although the mechanism still remains elusive.

3.1.5 Small interfering RNA (siRNA)

3.1.5.1 siRNA and evolution

It is believed that RNA-triggered silencing arose from an organism's need to survive without unwanted gene expression and to keep transposons and viruses at bay (reviewed in Fire, 2001). Global "policing" mechanisms include not only RNA interference (RNAi), but also encompass diverse pathways such as: preferential methylation of transposon sequences, repeat-dependent silencing, and nonsense-mediated mRNA decay. These pathways all share a commonality in that in each case a nucleic acid conformation that is not associated with normal gene expression is used by the organism to detect potentially detrimental situations. One response to this situation may be to stop the production or expression of potentially detrimental RNAs.

Both plants and animals were found to express genes that encode short forms of fold-back dsRNA (Bartel, 2004). These dsRNAs are processed into microRNAs (miRNAs, discussed below), which are evolutionarily conserved. In plants these miRNAs function to guide the cleavage of sequence-complementary mRNAs, but in animals these miRNAs take on a different role,

inhibiting translation by targeting partially complementary sequences located within the 3' untranslated region (UTR) of mRNAs (reviewed in Meister and Tuschl, 2004).

Observations of the underlying mechanisms involved in gene silencing in plants, fungi, and animals, reveal remarkable similarities that emphasize a common ancestor (Mello and Conte, 2004). Gene silencing was first discovered in plants, where it was known as post-transcriptional gene silencing, and many of the techniques used to reveal the components within this system have been used with similarity within the mammalian system (Baulcombe, 2004; Meister and Tuschl, 2004).

3.1.5.2 The discovery of RNAi in mammals

The importance of RNAi and its implications in research applications is emphasized by the reward of the 2006 Nobel Prize in Physiology or Medicine given to Andrew Z. Fire and Craig C. Mello for their work with the nematode *Caenorhabditis elegans*. The Mello research group found that double-stranded RNA (dsRNA) was a potent trigger for RNAi in *C. elegans*, and this interference could spread from tissue to tissue as well as be passed through the sperm or egg for up to several generations (Fire *et al.*, 1998). This discovery created a new type of research tool that could be used to decode gene function. RNAi is now used to manipulate gene expression experimentally as well as probing gene function on a genome-wide scale. After Fire *et al.* discovered the RNAi trigger, Tuschl and colleagues showed that transfection of mammalian cells with host RNAs could induce the sequence-specific RNAi pathway,

which surmounted the obstacle of using RNAi as a genetic tool in mammals (Elbashir *et al.*, 2001a).

3.1.5.3 The mechanism of gene silencing by siRNA

There are three types of naturally occurring small RNA that differ according to their origins: siRNAs, miRNAs and repeat-associated short interfering RNAs (rasiRNAs) (reviewed in Meister and Tuschl, 2004). dsRNA can be produced naturally from viruses by RNA-templated RNA polymerization, or by hybridization of overlapping transcripts which give rise to siRNAs or rasiRNAs. dsRNA hairpins that are formed from endogenous transcripts that contain inverted repeats are later processed into miRNAs. Both dsRNAs and siRNAs have been used as a tool for gene silencing *in vivo*.

The mechanism of silencing by siRNA was first found in plants, where short RNAs from 20-25 nucleotides long were produced from a RNAi-related process (Hamilton and Baulcombe, 1999). Deciphering this mechanism further, in the same year an RNAi reaction was reconstituted *in vitro* using fruitfly extracts. Elbashir *et al.* discovered that the long dsRNA were chopped up into short RNAs with two 21-nucleotide strands of RNA in a staggered duplex, with 19 nucleotides of dsRNA and two unpaired nucleotides at the ends (2001b). These experiments led to a wave of research uncovering the mechanism of RNAi, although there is still much to be revealed, especially within the mammalian system.

The first step in the RNA-interference pathway (designated the initiation phase) involves the ribonuclease-III enzyme Dicer (DCR), which processes

long dsRNAs into 21-23-nt siRNAs that have 5' phosphates and 2-nucleotide 3' overhangs during the initiation step of RNAi (Bernstein *et al.*, 2001) (see Figure 3.2).

In *C. elegans* and mammals a Dicer-interacting dsRNA-binding domain (dsRBD) protein is thought to allow Dicer to recognize different sources of dsRNAs, however, this protein (or proteins) has yet to be identified. Many organisms contain more than one Dicer gene, each Dicer preferentially processing dsRNAs that come from a specific source, although in mammalian systems there is only one Dicer gene (Meister and Tuschl, 2004). Micro RNAs do not use Dicer, but instead use another endonuclease named Drosha.

DCR has been linked to the Argonaute (Ago) proteins, a family of proteins that are characterized by the presence of two homology domains, PAZ and PIWI. DCR binds directly to Ago proteins AGO2 and PIWI through an RNase-III domain of DCR and the PIWI domain of the Ago proteins (Tahbaz *et al.*, 2004). The 21 to 23nt duplexes are then incorporated into a nuclease-containing multi-protein complex named RNA-induced silencing complex (RISC) (Hammond *et al.*, 2000). The exact components of this complex remain elusive, however it is hypothesized that AGO1 and AGO2 are involved in the 160 kDa minimal RISC mammalian complex, functioning to target-RNA binding and cleavage (Martinez and Tuschl, 2004; Meister *et al.*, 2004).

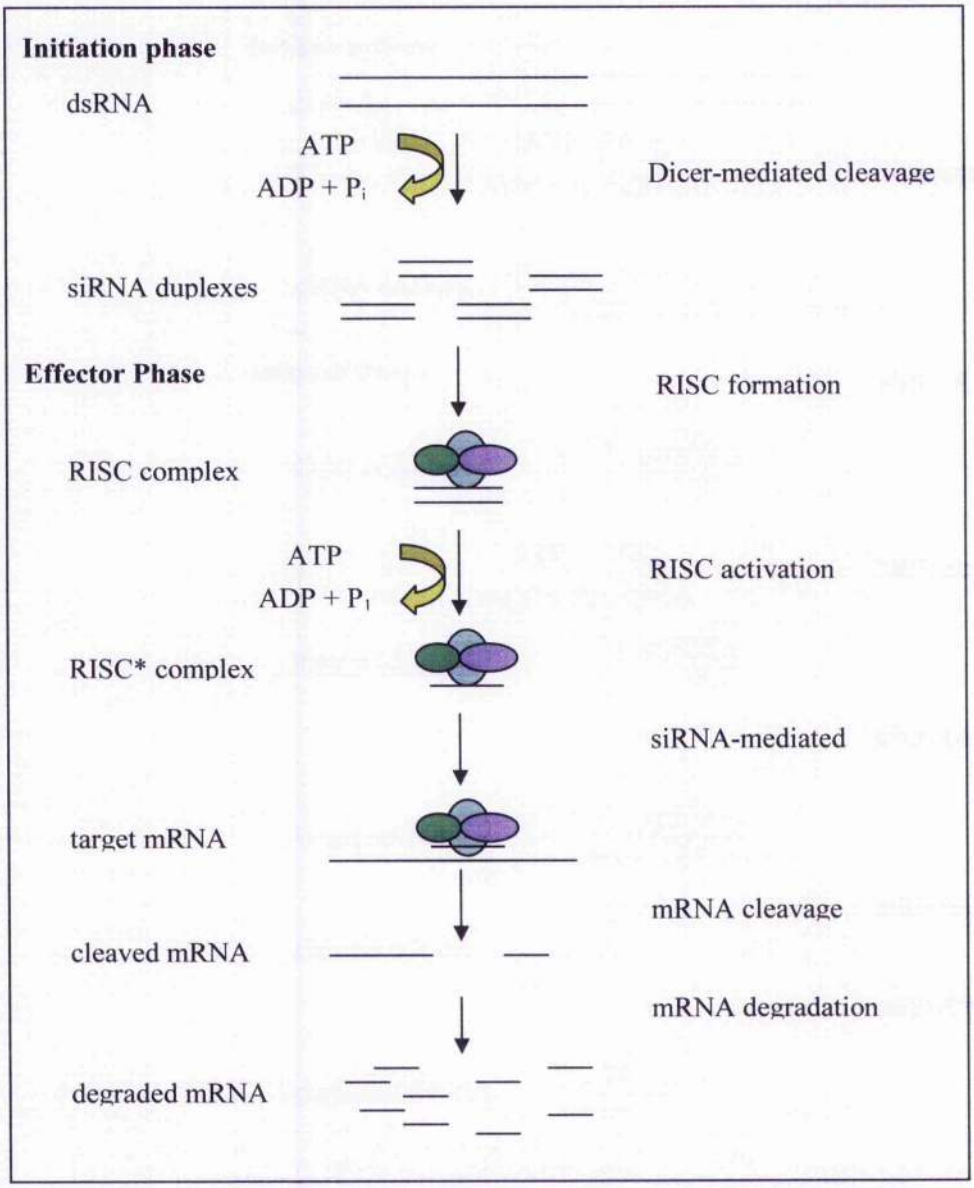


Figure 3.2 Mechanism of RNAi in mammalian systems. (Adapted from Aigner, 2005).

Every RISC complex (in a variety of species) has been found to contain at least one Argonaute protein. The PAZ domain of Ago is implicated in RNA binding, recognizing the terminus of the base-paired helix of the siRNA duplexes, while the PIWI domain provides RISC with effector-nuclease activity (Meister *et al.*, 2004; Song *et al.*, 2004). The PAZ domain also ensures that unrelated RNA is not processed into RISC, by only letting RNA with 2-nucleotide 3' overhangs be incorporated (Lingel *et al.*, 2004). In addition to Ago proteins, fragile X mental retardation protein (FMRP) was found to incorporate with siRNAs into the RISC complex, although the function of this protein is still unknown. RISC assembly is ATP-dependent, which reflects the need for energy for the unwinding of siRNA duplexes and/or spatial changes within the RISC complex (Nykänen *et al.*, 2001).

Synthetic 21-nt long siRNAs are thought to bypass Dicer, as they still function as potent RNAi triggers and do not undergo dsRNA processing (Elbashir *et al.*, 2001a). Relative thermal stability dictates rules for RISC entry for non-processed siRNAs (Khvorova *et al.*, 2003). The RISC incorporated "guide" strand becomes the strand that has its 5' terminus at the end of the siRNA duplex, which is less stably base-paired than the other strand. The non-incorporated strand is simply discarded.

Once RISC has unwound the siRNA, cleavage of the target mRNA in the middle of the complementary region, ten nucleotides upstream of the nucleotide paired with the 5' end of the guide siRNA occurs (Elbashir *et al.*, 2001b). This process was found to be ATP-independent, however multiple rounds of cleavage functions more efficiently in the presence of ATP. This

suggests that a RNA helicase yet unknown might be cleaving the siRNA and working as RISC's endonuclease subunit (Hutvágner and Zamore, 2002). This cleavage is a hydrolysis reaction yielding 5' phosphate and 3' hydroxyl termini which requires magnesium ions and parallels the reaction in which Dicer generates siRNA duplexes from dsRNA precursors (Martinez and Tuschl, 2004). It is hypothesized that Ago proteins may act as nucleases, performing the cleavage reactions of the target mRNA, however in humans only Ago2 but not Ago1 and Ago3 have been found to have nuclease activity (Ma *et al.*, 2004). Although there have been many recent advancements in the research surrounding RNAi, it is clear that there is still much to be uncovered.

3.1.5.4 siRNA: an indispensable tool

siRNA has opened the doors for the testing of individual gene function. Indeed, in *C. elegans* at least 19,000 genes (nearly all) have been analysed, and now similar strategies are being used to ascertain gene function in various other organisms. Traditionally gene function is analysed by using a 'forward genetic' approach, identifying an organism with abnormal physical or behavioural traits, and then isolating the mutant gene(s). This technique is usually limited to organisms that reproduce rapidly. In the mammalian system, 'reverse genetic' approaches allow researchers to knock out a specific gene and later identify its function (i.e. knockout mice), although these methods can be laborious and expensive. In contrast, RNAi allows the option of analyzing many silenced genes at once, can be less costly, and offers a higher-throughput.

Upon initial use within the mammalian system, the drawback of siRNA seemed to be that the effects were only transient, as mammals lack the mechanisms that amplify silencing in worms and plants. This problem was soon solved, when 500-base-pair dsRNA expressed in cell lines was found to stably suppress targeted genes (Paddison *et al.*, 2002). However, a caveat to this approach is that it could only be performed in cell lines that lacked generic responses to dsRNA, such as the RNA-dependent protein kinase (PKR) pathway. Fortunately, another technique in which short hairpin RNAs (shRNAs) can be expressed from pol III promoters to induce a stable suppression of target genes has been found (Brummelkamp *et al.*, 2002). Stable expression allows for the observation of induced phenotypes over longer time spans and stable cell lines can be assayed *in vitro* or *in vivo*. When looking from the therapeutic standpoint, shRNAs can be used to deliver gene silencing effects via gene delivery vehicles to target problem genes, such as an activated oncogene.

3.1.5.5 siRNA: potential pitfalls

Although there are many benefits in using siRNA as a research tool, there are also some potential problems, most of which can be avoided if experiments are performed diligently. RNAi has been found to target genes and proteins that it was not designed to target, and scientists are currently trying to determine how widespread the effects are, how they can be avoided, and why they occur (Couzin, 2004).

An example of this occurrence is found in laboratory experiments performed by Sledz *et al.* and many others working on RNAi (Bridge *et al.*, 2003; Judge

et al., 2005; Sledz *et al.*, 2003). Assays determined that when GAPDH siRNA (a commonly used control siRNA) was transfected into mammalian cells, responding GAPDH mRNA levels were decreased but there was an upregulation of Stat1, a component of the Jak-Stat signalling pathway resulting in interferon mediated activation. The interferon system has been determined as an off-target effect of some siRNAs. The interferon system is an innate immune response that can be activated by dsRNA. This system is commonly the first line of defence against viral infection, where upon infection cytokines are stimulated to start a complex signalling cascade, culminating in the induction of interferon-stimulated genes within the nucleus. Sledz *et al.* found that by transfecting various 21-bp dssiRNAs, interferon-mediated activation of the Jak-Stat signalling pathway occurred (2003). The side effects are not only cause for concern when using RNAi as a basic research tool, but also for use in therapeutics when an immune response by the interferon system would be detrimental to the target.

Although there seems to be many ways in which siRNAs can provoke off-target effects, many of these mechanisms remain elusive. The pertinent question is: how can laboratories avoid or minimize these off-target effects when possible? Currently, this question seems to have at least two possible answers, focusing on the specific design of the siRNA and the concentrations of siRNA introduced into the cell or organism.

Biotechnology companies can chemically synthesize siRNA against a target sequence after picking a sequence that has undergone a stringent algorithm of design rules based on current research. These design rules are also used in

"self-made" siRNAs, in which a target gene is chosen and a target sequence is picked using similar recommendations, usually with the aid of an online design tool. The siRNAS are then made via *in vitro* transcription from oligonucleotide templates (Brown *et al.*, 2002). Below is a table depicting a number of current design criteria, and why each characteristic is important in siRNA design (Table 3.1). However, even though a siRNA may meet all current design criteria, the off-target effects still may occur, making further testing of the siRNA critical.

Researchers have found that the concentration of siRNA is an important determinant of its efficacy. Three independent labs determined that off-target effects are least likely to occur when concentrations between 1 and 30 nM are used (Chi *et al.*, 2003; Jackson *et al.*, 2003; Semizarov *et al.*, 2003). However, even when these concentrations are used, genome-wide studies show that off-target effects can still occur (Jackson and Linsley, 2004; Snøve and Holen, 2004).

Why off-target effects occur continues to remain a matter of debate. One possibility is that mammalian cells may be mistaking foreign siRNAs for microRNAs, because both siRNAs and microRNAs use many of the same enzymes within their pathway (Couzin, 2004). There may be a fine line between triggering one pathways versus another, and the microRNA pathway is considered much less specific, targeting sequences that only partly match their own.

Table 3.1 Common techniques used in siRNA design

siRNA Characteristics	Rationale
The sense strand of the siRNA is the same sequence as the target mRNA sequence, except lacking the 5' AA sequence	Facilitates binding of the target sequence, as the antisense strand binds to the target gene
Sequence of 21nt dsRNA with 3' overhanging dimers of thymidine uridine	May enhance nuclease resistance in the cell culture medium and transfected cells[1]
AA and downstream 19 nucleotides are compared to an appropriate genome database	Decreases risk of off-targets as sequence is checked for homology to other genes
siRNA target sequences should be ~80 nucleotides from the AUG start codon on the target RNA	This criterion is controversial. Elbashir *et al.* suggests that this may facilitate binding of the "Slicer" apparatus[1], while other groups have found that the location of the siRNA target site is not a predictable indicator of siRNA silencing activity[2]
Hign GC content at 5' end Low GC content at 3' end	Sense strand is preferentially loaded into RISC[3]
No long repeats at siRNA ends that are complimentary	May form inactive hairpins

[1]Elbashir *et al.* 2001a, [2]Brown *et al.*2003, [3]Ambion, Inc.

Scientists often use microarrays to look for off-target effects, but this technique could provide ineffective because microarrays only show gene expression levels and not protein levels. If the siRNA happens to be triggering the microRNA pathway, microRNAs may change how RNA is translated into protein.

Peter Linsley's group found that the specific part of the siRNA that was contributing to weak sequence matching between siRNAs and genes is the 5' end (Jackson *et al.*, 2003). If this 5' end matches a sequence in another gene, there may be a risk of off-target binding. Strengthening this research, Schwarz *et al.*, reported that siRNAs with certain sequences and structures may unravel in different manners and the differences in this unwinding may determine how well they target the right gene (Schwarz *et al.*, 2003). Nevertheless, with stringent controls, data from siRNA research may be convincing and certainly influential.

In June of 2003 the editors of *Nature Cell Biology* published an editorial listing possible controls that could be utilized during siRNA experiments. This list is based on a May 2003 Horizon symposium on RNA and provides clear explanations on proper uses of RNAi controls and standards that must be in place for publications. Currently, the rescue control is regarded as the most pertinent control. This involves rescue by expression of the target-gene in a form unaffected by the siRNA. An example of this is a vector-based siRNA system, namely an inducible system. Expression of a target gene could be silenced by inducing production of siRNA, which could then be halted to reveal a return to a normal phenotypic state. This type of control may not

always be possible, however, as some genes (if expressed in leaky induction systems) may cause death of the cell or organism. In the cases where rescue controls are impossible, basic controls including reduction of mRNA and protein levels are necessary and termed 'classical' RNAi. Quantitative controls are also highly desired, whereby titration to the lowest possible level reduces the chance of side effects and provides gradual data on the effect of the siRNA. This is important because the RISC complex has shown to be saturable in some settings (Haley and Zamore, 2004). Other important controls include mismatch or scrambled siRNAs and testing multiple siRNAs designed against different sites on the same target gene.

3.1.5.6 siRNA used in therapeutics

The prospects of using RNAi for therapy are promising. Possible disease targets include conditions that arise from aberrant gene expression including; RNA viruses (such as HIV), cancer, and neurode generative disorders, although this list is not all-encompassing (Boden *et al.*, 2004; Brummelkamp *et al.*, 2002; Kao *et al.*, 2004; Wilda *et al.*, 2002). Boden *et al.* demonstrated the use of a retroviral vector to transfer shRNA expression cassettes genetically into human cells (Boden *et al.*, 2004). Using a specific shRNA to target the viral transactivator protein tat, the group reported HIV-1 replication decreased by greater than 95%. In addition to silencing pathogenic viral genes, siRNAs have been used to effectively knockdown expression of oncogenes such as BCR-ABL (Wilda *et al.*, 2002) and K-RAS (Brummelkamp *et al.*, 2002). Some studies on siRNA silencing of oncogenes include not only decreases in mRNA and protein levels, but also suppression of monolayer and anchorage-independent growth, and even a decrease in tumour size in mouse models

(Calvo *et al.*, 2006; Yoshinouchi *et al.*, 2003). New research techniques include the sensitization of cancerous cell lines to chemotherapeutic drugs with the use of siRNA against a target oncogene. Silencing the BCR-ABL oncogene was reported to increase sensitivity of leukaemia cells to imatinib (Glivec) and radiation (Wohlbold *et al.*, 2003), while RNAi against HPV oncogenes in cervical cancer cells resulted in increased sensitivity to cisplatin (Putral *et al.*, 2005). Clearly the utilization of siRNA as a therapeutic tool has vast implications for the treatment of a wide range of diseases; however, the need for development of efficient delivery systems is essential.

Although using siRNA in mammalian systems is of great use in studying the functions of genes and for understanding the genetic basis of cellular physiology, the molecular make-up of siRNA can make the conveyance difficult *in vivo*. There are two main factors that cause siRNA to be difficult to deliver *in vivo*. First, the physicochemical properties of siRNA, such as the large polar surface area and molecular size, make for poor intrinsic membrane permeability. Pre-clinical methods which have somewhat enhanced delivery involves the hydrodynamic intravenous injection of naked siRNA or shRNA (McCaffrey *et al.*, 2002). This technique involves injecting a large volume of siRNA solution over a short period of time. While this method had been successful in mouse models, delivery is still a problem in clinical applications as hydrodynamic *intra venous* infusion cannot be applied to humans in a safe manner.

The second factor in efficient RNAi delivery is that siRNA is rapidly degraded by both extracellular and intracellular nucleases. Chemical modifications can

often increase stability, which increases the overall cellular uptake of siRNA (Soutschek *et al.*, 2004). Other vehicles, such as chemically unmodified siRNAs bound to polymers or liposomes have shown promise (Urban-Klein *et al.*, 2005; Yano *et al.*, 2004). One effective partnership is the complex formed by the binding of siRNA with polyethylenimines (PEIs), synthetic linear or branched polymers which contain characteristics that allow them to form non-covalent complexes with DNA and siRNA (Boussif *et al.*, 1995; Urban-Klein *et al.*, 2005). The formation of complexes with PEI leads to stable and uniformly sized particles that completely cover the siRNA, protecting the siRNA from the presence of RNAse (*in vitro*) and serum nucleases (*in vivo*) (Grezelinski *et al.*, 2006). Injection of PEI/siRNA into peritoneal or subcutaneous tissue of mice delivered intact radiolabled siRNAs into several organs, but not the blood (Urban-Klein *et al.*, 2005). The complexation of siRNAs with other molecules is a potentially powerful tool to achieve maximal function *in vivo*.

3.1.6 Summary

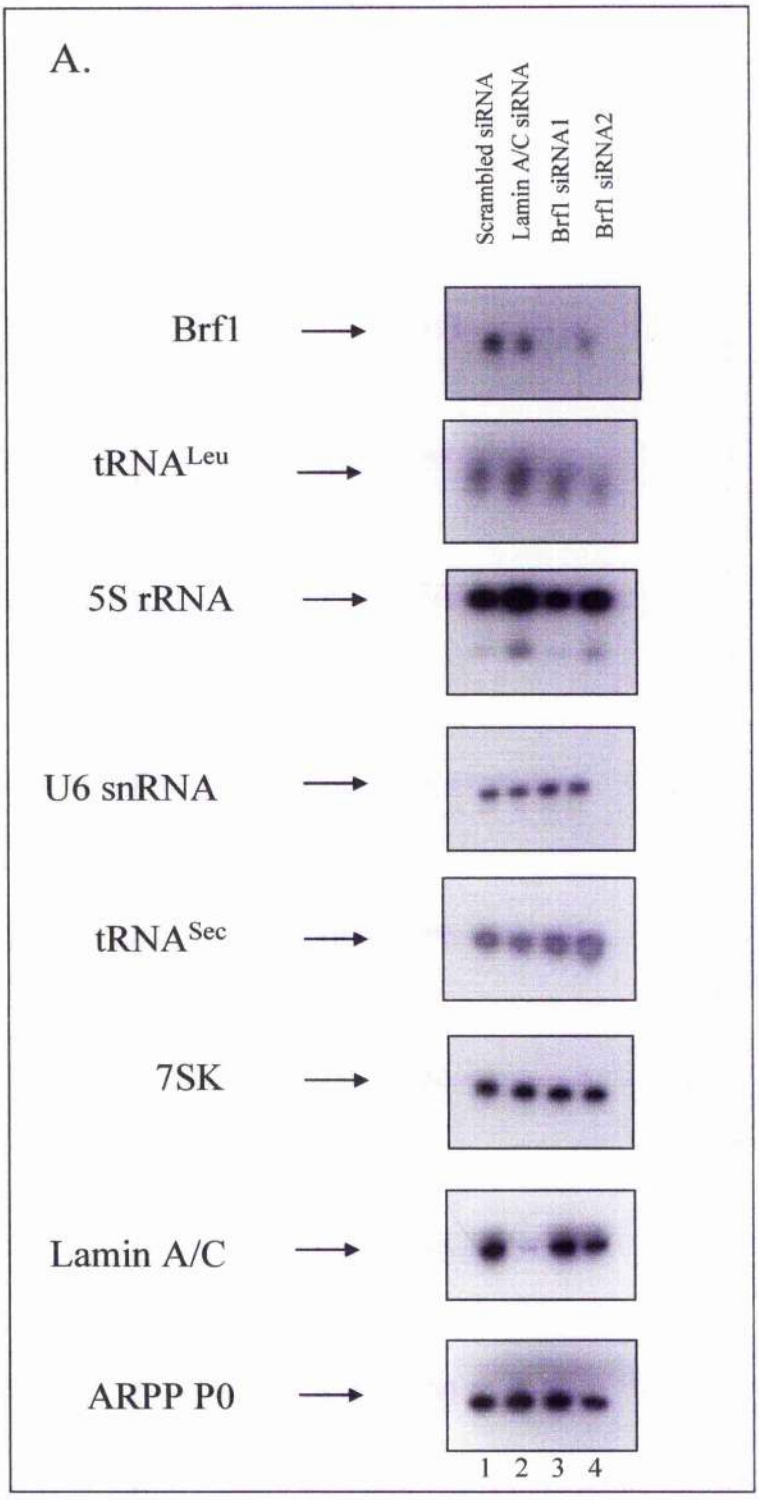
As Brf1 (through TFIIIB) has been shown to be a target for many significant proteins involved in regulating levels of pol III transcription, and pol III transcription is linked to a cells biosynthetic capacity, one could expect that decreasing the levels of Brf1 by siRNA might have an impact on a cell's phenotype. By using siRNA as a tool to decrease levels of Brf1, and hence pol III transcription, effects on intracellular characteristics such as mRNA and protein expression were observed, as well as phenotypic traits, including proliferation.

3.2 Results

3.2.1 siRNA designed to target Brf1 decreases pol III transcription in mammalian cells

siRNA designed to target Brf1 was applied to cervical cancer cells by electroporation in order to assess the consequences of decreasing TFIIIB-subunit Brf1 on pol III transcription. An effect on pol III transcription was observed after 48 hours, as seen by a decrease in pol III transcripts $tRNA^{Leu}$ and 5SrRNA, when compared to Lamin A/C control siRNA (Figure 3.3A). Decreases in pol III transcripts that use a type III promoter (i.e. U6 snRNA, 7SK, $tRNA^{Sec}$) are not seen, as this type of promoter does not utilize Brf1 during transcription (see *Introduction* 1.5.3). Although Brf1 siRNA1 appears to decrease Brf1 mRNA more efficiently than Brf1 siRNA2, levels of $tRNA^{Leu}$ do not reflect this as it would be expected that $tRNA^{Leu}$ levels in cells treated with Brf1 siRNA1 would be more decreased than $tRNA^{Leu}$ levels in siRNA2. The reasons for this are unidentified and considered an anomaly.

Translational effects were observed as a decrease in Brf1 protein levels was recorded after Western blotting when normalized to actin, a protein found in the cell cytoskeleton (Figure 3.3B). Brf1 protein levels in lane 4 (siRNA-1) appear lower than those of lane 5 (siRNA-2), reflecting Brf1 mRNA levels. As Brf1 serves to recruit TFIIIB through interactions with pol III and TFIIIC, reducing Brf1 levels probably hinders the binding of TFIIIB to the promoter, disrupting transcription.


A.
Scrambled siRNA
Lamin A/C siRNA
Brf1 siRNA1
Brf1 siRNA2
Brf1
tRNA^Leu
5S rRNA
U6 snRNA
tRNA^Sec
7SK
Lamin A/C
ARPP P0
1 2 3 4

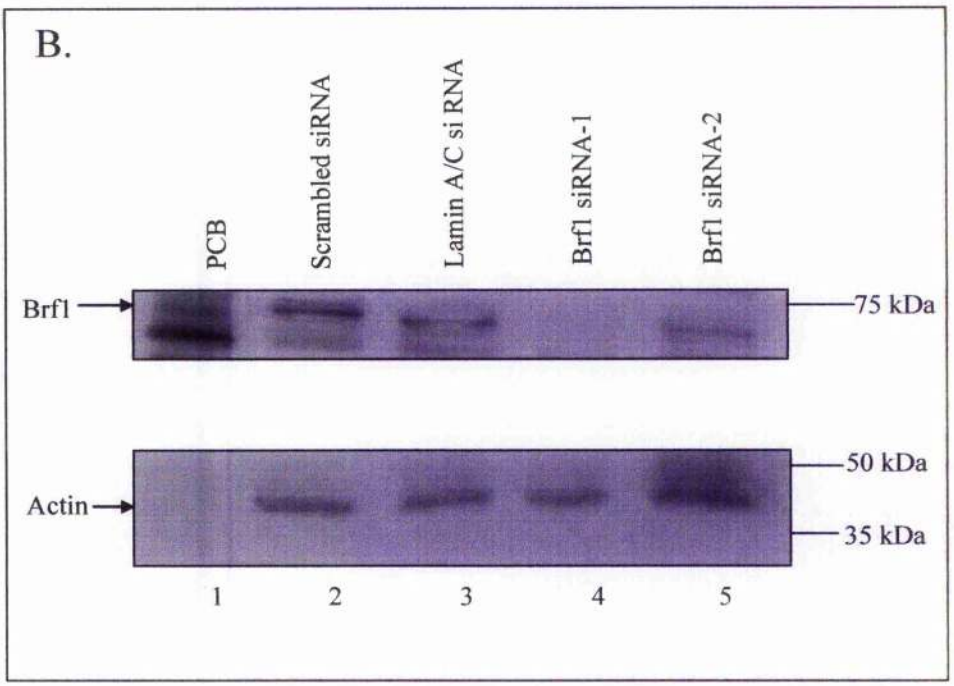


Figure 3.3 Small interfering RNA targeting Brf1 reduces pol III transcription *in vivo*

A. Exponentially growing HeLa cells were electroporated with scrambled control siRNA (lane 1), Lamin A/C siRNA (lane 2), Brf1 siRNA-1 (lane 3) and another Brf1 siRNA-2 (lane 4) targeting a different region to Brf1 siRNA-1. All siRNAs were transfected using 3 ug siRNA into each electroporation. Cells were harvested for RNA extracts 48 hours after transfection. RT-PCR analysis was then performed. Brf1(panel one), $tRNA^{Leu}$ (panel two), 5S rRNA (panel three), U6 snRNA (panel four), $tRNA^{sec}$ (panel 5), 7SK (panel 6), Lamin A/C (panel 7) and ARPP P0 (panel 8) primers were used. Results are representative of three independent experiments. **B.** Protein extracts were harvested at 48 hours after transfection, as above. Whole cell extracts were made and subjected to SDS-PAGE analysis and were immunoblotted with antibodies against Brf1 (SK-2839, upper panel), or actin (lower panel). PC-B (panel 1) is a fraction containing TFIIIB and pol III, used as a marker.

Daley *et al.* found Brf1 to be a limiting factor for pol III transcription in cervical cells when increased concentrations of Brf1 were found to stimulate $tRNA^{Leu}$ and 5S rRNA transcription (2005).

Clearly, decreasing levels of Brf1 by using siRNA would have an opposite effect. Because siRNA targeting Brf1 decreased pol III transcription, we thought it would be interesting to see if this had any effects on the proliferation rate. Deregulation of pol III is a general characteristic of transformed cells (reviwed in White, 2004), so discovering if decreasing pol III transcription through Brf1 siRNA in transformed cells would be sufficient to decrease proliferation would be beneficial.

3.2.2 Brf1 siRNA decreases the proliferation rates of two types of transformed mammalian cells

To access the proliferation rates of both cervical carcinoma cells (HeLa) and a breast adenocarcinoma cell line (MCF-7), cells were electroporated with Brf1 siRNA, harvested and counted at 0 and 72 hours. Proliferation rates of HeLa cells treated with two different Brf1 siRNAs, as well as a pool of these two siRNAs showed a ~2 fold decrease in proliferation rates at 72 hours when compared to cells treated with control siRNA at 72 hours (Figure 3.4).

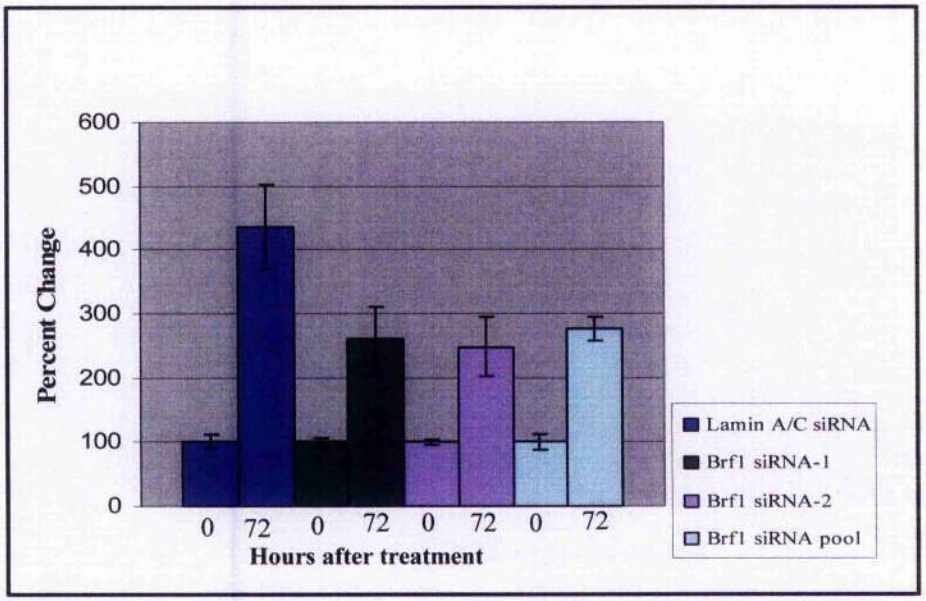


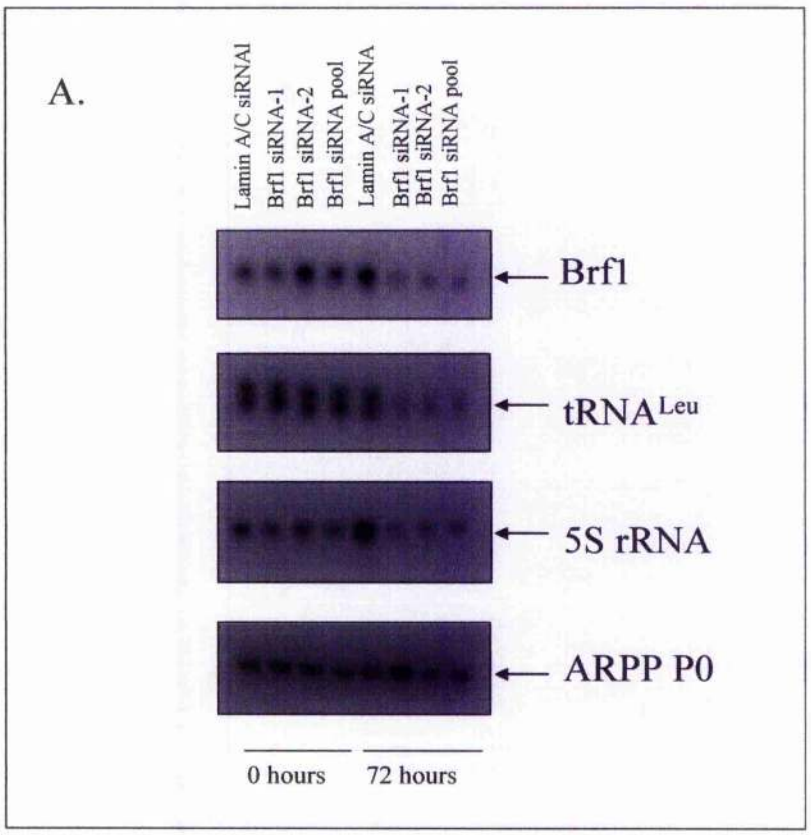
Figure 3.4 Transfection of HeLa cells with Brf1 siRNA decreases the proliferation rate in HeLa cells

Cells were electroporated with either 3 ug siRNA targeting Lamin A/C (blue bars), Brf1 (green and purple bars) or a pool of siRNA targeting Brf1 (light blue bars). siRNA-1 and siRNA-2 reflect two separate siRNAs targeting different regions of mRNA within Brf1. The Brf1 siRNA pool represents a mixture of siRNA-1 and siRNA-2 to equal 3 ug total. Cells were counted at 0 and 72 hours. Results are representative of three separate experiments. Mean percentage change of all Brf1 siRNAs is 59.8%. Standard deviations (in percentages) are as follows: Lamin A/C time 0, 10.3; Lamin A/C time 72, 66.0; Brf1 siRNA-1 time 0, 6.8; Brf1 siRNA-2 time 72, 51.8; Brf1 siRNA-2 time 0, 3.3; Brf1 siRNA-2 time 72, 46.4; Brf1 siRNA pool time 0, 11.1; Brf1 siRNA pool time 72, 18.5. p= 0.0014

To verify the siRNA was functioning, pol III transcripts, as well as Brf1 mRNA and proteins levels were checked. Indeed, at 72 hours cells treated with Brf1 siRNA were found to have decreased levels of Brf1 mRNA and protein, and levels of pol III transcripts $tRNA^{Leu}$ and 5S rRNA were also decreased when compared to control Lamin A/C mRNA (Figure 3.5A, 3.5B). Levels of Lamin A/C mRNA increased slightly from 0 to 72 hours, possibly due to non-specific effects. However, because mRNA levels are normalized to the pol II transcript ARPP P0, comparisons between mRNA levels from Brf1 siRNA and Lamin A/C siRNA treated cells can still occur.

Brf1 siRNA-2 decreased Brf1 mRNA levels less efficiently than Brf1 siRNA-1; however, these two siRNAs, as well as the Brf1 siRNA pool, give equally potent anti-proliferative effects. Perhaps this can be explained by the idea that the siRNA system may be saturable. Even though the same concentration of Brf1 siRNA was used, the system may be saturated by the least effective siRNA used (Brf1 siRNA-2). Therefore, any siRNA that decreases Brf1 levels beyond this point may not affect proliferation rates if proliferation is not affected by the further decrease in Brf1 mRNA levels. If this saturable amount of Brf1 is achieved by Brf1 siRNA-2, than all other Brf1 siRNAs would have the same affect on proliferation.

Cell death was counted by staining with trypan blue to check that the decrease in proliferation rate was not due an increase in cell death, but rates were too low to be significant and the same in all samples.


A.
Lamin A/C siRNAI
Brf1 siRNA-1
Brf1 siRNA-2
Brf1 siRNA pool
Lamin A/C siRNA
Brf1 siRNA-1
Brf1 siRNA-2
Brf1 siRNA pool
Brf1
tRNA^Leu
5S rRNA
ARPP P0
0 hours
72 hours

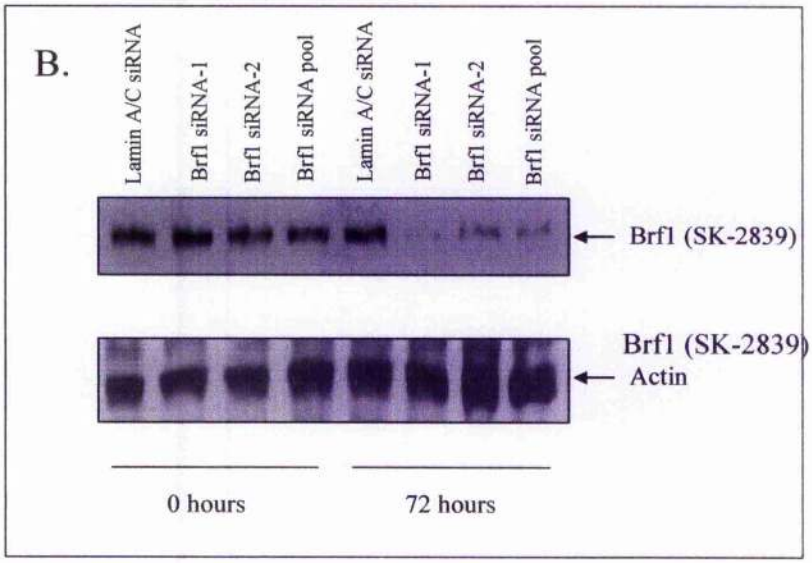

B.
Lamin A/C siRNA
Brf1 siRNA-1
Brf1 siRNA-2
Brf1 siRNA pool
Lamin A/C siRNA
Brf1 siRNA-1
Brf1 siRNA-2
Brf1 siRNA pool
Brf1 (SK-2839)
Brf1 (SK-2839)
Actin
0 hours
72 hours

Figure 3.5 RT-PCR and Western blotting results correlate with decreases in proliferation rates in Brf1 siRNA transfected HeLa cells

A. Cells were electroporated with siRNA as above, in Figure 3.4. After cells were counted RNA extracts and whole cell extracts (B, below) were made. PCR was carried out using primers for Brf1 (panel one), $tRNA^{Leu}$ (panel 2), 5S rRNA (panel 3) and ARPP P0 (panel 4). These results are indicative of two independent experiments.

B. Cell extracts were subjected to SDS-PAGE analysis and Western blotting was applied. An antibody against Brf1 (SK-2839, upper panel), or actin (lower panel) was used for immunoblotting. These results represent two separate experiments.

The proliferation rates of MCF-7 cells after treatment with Brf1 siRNA were also assessed. MCF-7 cells treated with two different Brf1 siRNAs, as well as a pool of these two siRNAs, showed a ~1.5 fold decrease in prolifcration rates at 72 hours when compared to cells treated with control siRNA at 72 hours (Figure 3.6).

Error was higher in the MCF-7 proliferation chart as cells were more difficult to work with, as they needed to be plated at a high density for growth. By the end of 72 hours these cells grew out of a monolayer, making them hard to remove from the culture dish. mRNA and protein levels were not assessed as the RNA and protein extracted from these cells was insufficient to perform adequate analysis. Trypan blue staining and subsequent counting for cell death was also performed, results paralleling that of the HeLa cell line.

The coinciding results of these two experiments show the parallel affects of decreasing Brf1 levels in two different transformed cell lines. The question remains as to how decreasing Brf1 directly affects the proliferation rate of a cell, which will be discussed below.

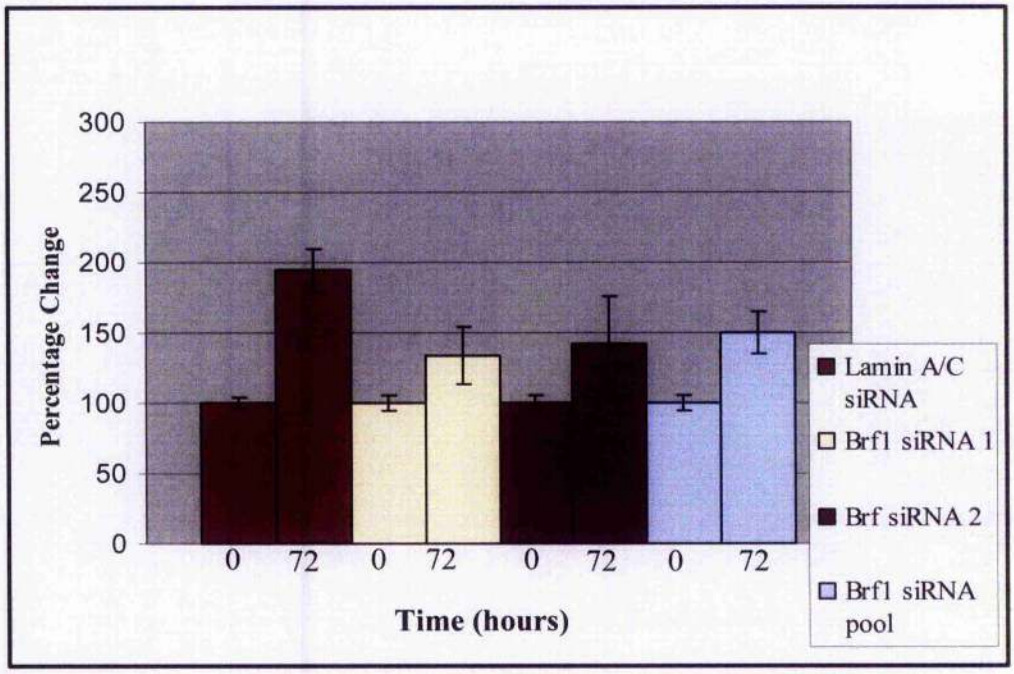


Figure 3.6 Brf1 siRNA decreases proliferation rates in MCF-7 cells

Cells were harvested at 72 hours after electrporation with 3 μg Lamin A/C siRNA (purple), Brf1 siRNA-1 (ivory), Brf1 siRNA-2 (dark purple) or Brf1 siRNA pool (light blue). Brf1 siRNA-1 and Brf1 siRNA-2 represent two different siRNAs targeting different regions of Brf1. Brf1 siRNA pool represents a pool of these two siRNAs, totaling 3 μg. These results are from two independent experiments.

3.3 Discussion

The untranslated RNAs produced by pols I and III are primarily involved in cellular biosynthesis, the major determinant in the rate of cell growth and division. If a cell increases its rate of protein synthesis, proteins accumulate to a point of sufficient protein mass, and a cell divides (Baxter and Stanners, 1978). In this way, cell growth is rate-limiting for cell division (Johnston *et al.*, 1977). As the availability of pol III transcripts tRNA and 5S rRNA are a major factor of protein synthetic capacity, high rates of pol III transcription are essential for rapid growth. This rapid growth is a characteristic of transformed cells. Thus, pol III products are generally found to be overexpressed in transformed and tumour cells, and these results show that both HeLa and MCF-7 cells have increased amounts of pol III products compared with normal cells (Chen *et al.*, 1997; Felton-Edkins and White, 2002). When Brf1 siRNA decreases the amount of Brf1 bound into TFIIIB complexes, less TFIIIB is available to recruit pol III and position it at the start site. Hence, there is a decrease in transcription from type I and II promoters, the products of which are extremely influential upon cellular growth and proliferation. This characteristic becomes significant when increased levels of pol III transcripts in transformed cells can be decreased by techniques such as siRNA, bringing the cells back to a more "normal" physiological state. This research provides evidence of a direct link between the levels of Brf1 in a cell and its ability to influence cell division. Further research might include an experiment such as measuring protein synthesis, and FACS analysis. Cells treated with siRNA targeting Brf1 would probably decrease their protein turnover rate. This could be tested by monitoring incorporation of [^{35}S]-Met in a pulse-chase experiment. FACS analysis would provide an insight into the progression of

these cells through the cell cycle, and how cells treated with control siRNA differed from those treated with Brf1 siRNA.

A more extensive panel of controls could also be applied. Mismatched or scrambled Brf1 siRNA could be used, although a rescue experiment would be the most conclusive. A siRNA vector-based inducible system could be made and used to decrease expression of the Brf1 gene. The inducible system could then be shut off, and proliferation rates could be analyzed to discover if they returned to higher levels once Brf1 levels increased. Titrations of siRNA could also be carried out, to observe the optimal siRNA concentration and to minimize non-specific effects.

Although using Brf1 siRNA to decrease pol III transcription was successful, we decided to use other techniques to directly target pol III transcription. Using siRNA is effective once the system is optimized, but this technique can be laborious and therefore other systems might provide added benefits. The following chapters discuss the targeting of pol III transcription through two different techniques; one through direct application of the drug tagetitoxin, and the other through a recently discovered pol III negative effector Maf1.

Chapter 4

Targeting RNA polymerase III with tagetitoxin decreases transcription in mammalian cells

4.1 Introduction

4.1.1 Tagetitoxin: the discovery of a RNA polymerase III inhibitor

Tagetitoxin, a bacterial phytotoxin, is produced in liquid cultures of the plant pathogenic bacterium *Pseudomonas syringae* pv. *tagetis* (Mitchell and Durbin, 1981). This substance was found to causes chlorosis, a condition in which plant foliage produces insufficient chlorophyll in developing, but not mature, plant leaves (Trimboli *et al.*, 1978). The levels of chloroplast ribosomal RNA, as well as mRNAs, are severely reduced in the leaves of tagetitoxin-treated seedlings (Lukens *et al.*, 1987). This led researchers to investigate the mechanism of action of tagetitoxin on chloroplast metabolism. Indeed, tagetitoxin turned out to be a selective inhibitor of chloroplast RNA polymerase, providing an explanation for the reduced chloroplast ribosomal levels, the decreased mRNA levels, and chlorosis all observed in tagetitoxin-treated plants (Mathews and Durbin, 1990). Further studies still included tagetitoxin research in plants, but also broadened to encompass other species.

In 1990, Mathews and Durbin provided the first evidence that *in vitro* RNA synthesis directed by the RNA polymerase from *Escherichia coli* is inhibited by tagetitoxin (Mathews and Durbin, 1990). *In vitro* transcription by RNA polymerase II, isolated from wheat germ, had an extremely decreased sensitivity to the toxin and SP6 and T7 RNA polymerases from bacteriophages were unaffected by tagetitoxin at high concentrations. In the same year, a partnership with a another research group demonstrated the ability of tagetitoxin to preferentially inhibit eukaryotic pol III (Steinberg *et al.*, 1990).

The results in this study further characterised the action of tagetitoxin, as pol III transcription directed from HeLa, *Xenopus laevis* (African clawed frog), *Bombyx mori* (silkworm), *Drosophila melanogaster* (fruitfly) and *Saccharomyces cerevisaie* extracts was inhibited. Tagetitoxin did not inhibit transcription from calf thymus pol II, reinforcing this group's previous findings and establishing a control for tagetitoxin testing (Mathews and Durbin, 1990; Steinberg *et al.*, 1990). Understanding the mechanism of action of tagetitoxin began with this research. The inhibition of silkworm pol III seemed to act directly on the polymerase, rather than by binding a transcription factor (Steinberg *et al.*, 1990).

4.1.2 The mechanism of tagetitoxin: enhanced pausing at discrete sites

Studies were performed in yeast extracts to further determine the mechanism of action of tagetitoxin. Due to the lengthy purification procedures necessary to obtain tagetitoxin from *P. syringae* pv. *tagetis*, the following studies switched to a commercially available partially purified form of tagetitoxin, with the trade name Tagetin™ (Epicentre® Technologies). Tagetin™ was found to contain the same activity as the previously used tagetitoxin (Steinberg *et al.*, 1990).

Steinburg and Burgess treated yeast extracts with tagetitoxin and performed *in vitro* transcription experiments on a tRNA gene, adding varying concentrations of nucleotides (1992). Tagetitoxin inhibition of transcription was more pronounced at low nucleotide levels and created tagetitoxin-enhanced small RNAs, in addition to primary unprocessed pre-tRNA transcripts. These small

and lower molecular weight RNAs result from transcription complex pausing, premature termination, or processing of the full-length transcript. These data showed that the inhibitory effect of tagetitoxin depends, to some degree, on total nucleotide concentration.

To further elucidate the way in which tagetitoxin inhibits pol III transcription, for example, if the short RNAs mentioned above were formed because of the accumulation of stalled complexes or RNA release, multiple round transcription of the $tRNA^{Leu}$ gene in yeast extract was done at a high concentration of tagetitoxin (Steinberg and Burgess, 1992). After 5 minutes the transcription reaction was stopped, fractions collected, and then portions of these fractions were added to nucleotides and $MgCl_2$. Small RNAs created by tagetitoxin treatment and corresponding to the low molecular weight RNAs in the previous experiment are contained in the ternary complex which contains DNA, pol III, and growing RNA chains that migrate distinctly from and ahead of the full-length precursor tRNA. These small RNAs also migrated separately from other small RNAs that result from premature termination and release or processing of the mature transcript. Therefore, these low molecular weight RNAs created from tagetitoxin treatment are created by pausing of the transcription complex, not from RNA release. Further experiments implicated the interference of tagetitoxin with nascent RNA chain elongation (Steinberg and Burgess, 1992). Together, this research suggests that the primary effect of tagetitoxin is increased stability of intrinsic pausing, depending on the frequency and stability of pausing by pol III.

4.1.3 The structure of tagetitoxin

Work focusing on revealing the structure of tagetitoxin began soon after it was reported to be a specific inhibitor of pol III. Early work suggested that tagetitoxin has a molecular weight (MW) of 435, held the molecular formula of $C_{11}H_{18}O_{13}SNP$, and was a highly unusual and novel molecule (Mitchell and Hart, 1983). Later, more intricate studies deduced the inhibitor to consist of two fused 6-membered heterocyclin rings with the molecular formula revised to $C_{11}H_{17}N_2O_{11}PS$ with a MW of 417 (Mitchell *et al.*, 1989) (Figure 4.1).

Most of the work done to further characterize the structure has been done in order to chemically modify the inhibitor for use as a plant growth regulator and as an herbicide (Dent *et al.*, 1999). Recent work on the structural basis for transcription inhibition by tagetitoxin found that tagetitoxin inhibited all catalytic activities of *Thermus theromophilus* RNA polymerase (RNAP) by "freezing" the RNAP catalytic centre in an inactive state (Vassylyev *et al.*, 2005). Mg^{2+} may have a role in the stabilization of an inactive transcription intermediate, remodelling the site of active transcription. Additional analysis of the structure of tagetitoxin, along with mechanistic investigations, are needed to clarify the function of this inhibitor on pol III.

As tagetitoxin was found to be a specific inhibitor of pol III transcription *in vitro*, the aim of my experiments with the commercially available compound, Tagetin™, was to analyse the effectiveness of tagetitoxin when applied to mammalian cells *in vivo*.

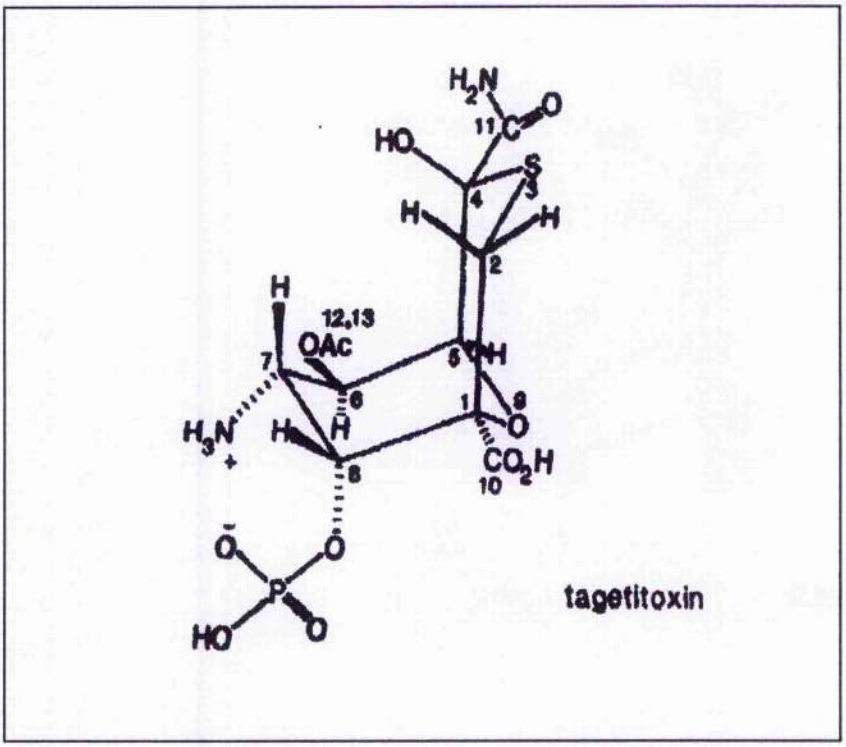
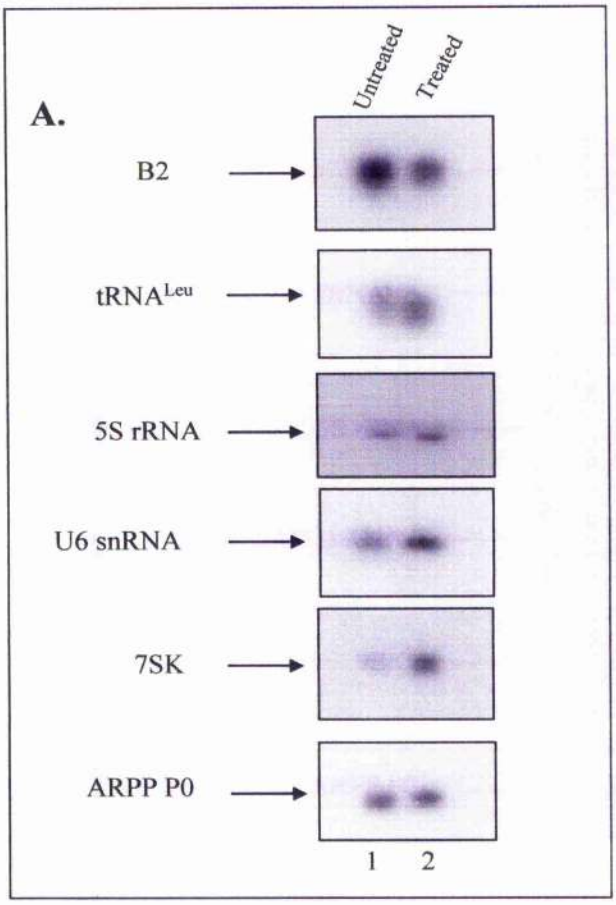


Figure 4.1 The structure of tagetitoxin (Mitchell *et al.*, 1989)

4.2 Results

4.2.1 Tagetitoxin does not serve as a specific pol III inhibitor *in vivo* when directly applied to mammalian cells

To test whether Tagetin™ could inhibit pol III transcription in a mammalian system by transfection, 45 micromoles (μM) of tagetitoxin was added per well to NIH 3T3 cells for 2 hours. Cells were then harvested for RNA and cDNAs were created for RT-PCR analysis. Only the B2 pol III transcript appeared decreased, and all other transcripts, $tRNA^{Leu}$, 5S rRNA, and 7SK increased when compared to the pol II control ARPP P0 (Figure 4.2A). This experiment was performed three times to verify these results. Tagetin™ was active, as *in vitro* transcription reactions showed a decrease in pol III transcripts when the drug was added (Figure 4.2B). Perhaps B2, found only in mouse cell lines, is more sensitive to tagetitoxin than the other pol III transcripts or this could be some sort of artefact from the treatment of cells with a drug. Allen *et al.* used tagetitoxin to do studies on SINE-encoded mouse B2 RNA and heat shock, and one RT-PCR analysis shows a decrease in B2 RNA levels without heat shock, and no effect on 7SK RNA (2004). $tRNA^{Leu}$ and 5S rRNA were not analysed. The results of my studies seem to mimic those of this experiment, although the reason for the increases in $tRNA^{Leu}$ and 5S rRNA still remains unexplained. Contact was made via e-mail to this laboratory, but they reported that there were no attempts at analysing other pol III transcripts. Whatever the case, no previous research has reported the use of tagetitoxin *in vivo* by direct application into culture medium, but the ease of use and specific activity on pol III prompted us to attempt it.


A.
Untreated
Treated
B2
tRNA^{Leu}
5S rRNA
U6 snRNA
7SK
ARPP P0
1
2

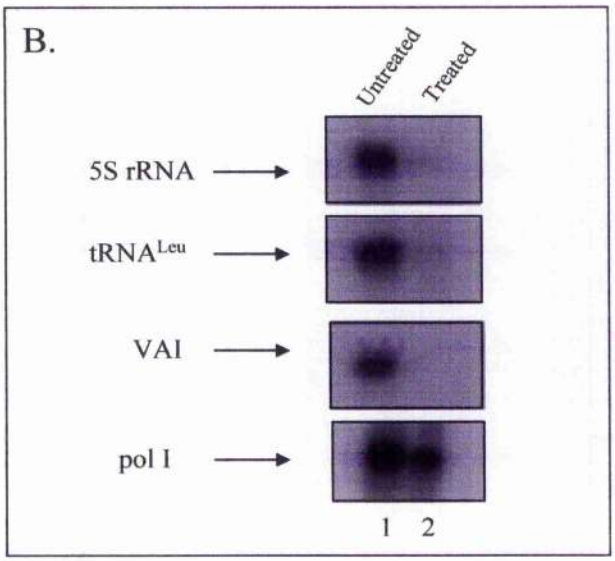

B.
Untreated
Treated
5S rRNA
tRNA^{Leu}
VAI
pol I
1
2

Figure 4.2 Tagetitoxin does not decrease pol III transcription (in NIH 3T3 cells) when applied to culture medium

A. After cells were seeded in 6-well culture plates they were left at normal culture conditions for 24 hours before no treatment with (lane 1) or treatment with 45 μM Tagetin™ (lane 2). After 2 hours incubation cells were harvested for RNA. RT-PCR analysis was then performed using primers for B2 (panel 1), $tRNA^{Leu}$ (panel 2), 5S rRNA (panel 3), U6 snRNA (panel 4), 7SK (panel 5) and ARPP P0 (panel 6). These results are representative of three separate experiments.

B. An *in vitro* transcription assay was done to confirm the activity of the drug Tagetin™. HeLa nuclear extracts were incubated with no Tagetin™ (lane 1) or treatment with Tagetin™ (lane 2) for 10 minutes prior to incubation with a reaction mixture including [α-^{32}P] UTP, etc. and DNA templates 5S rRNA (panel 1), $tRNA^{Leu}$ (panel 2), or VA1 (panel 3). A pol I rRNA primer is used as a control. After a reaction time of 60 minutes the reaction was stopped, protein and DNA was removed and the RNA was extracted. Formamide loading buffer was added, vortexed and then run on a polyacrylamide sequencing gel. These results are representative of two independent experiments.

4.2.2 Treating NIH 3T3 cells after heat shock with tagetitoxin decreases pol III transcription

As experiments demonstrated by Kugel's group with tagetitoxin and heat shock were successful at inhibiting pol III transcription (in select transcripts), *in vivo* attempts were made to duplicate these experiments within our laboratory (Allen *et al.*, 2004). NIH 3T3 cells were treated with tagetitoxin for 60 minutes, heat-shocked for 25 minutes at 45°C, and allowed to recover for 25 minutes before RNA extracts were made. RNA levels were evaluated by RT-PCR. $tRNA^{Leu}$ and 5S rRNA were detected when cells were heat shocked without tagetitoxin treatment, but after application levels of $tRNA^{Leu}$ were not detectable and 5S rRNA levels were decreased when compared to the control (Figure 4.3). Levels of B2 appear to be increased (lane 2), which may reflect B2's response to heat shock and the combined non-specific effect drug treatment. This will be discussed further, below.

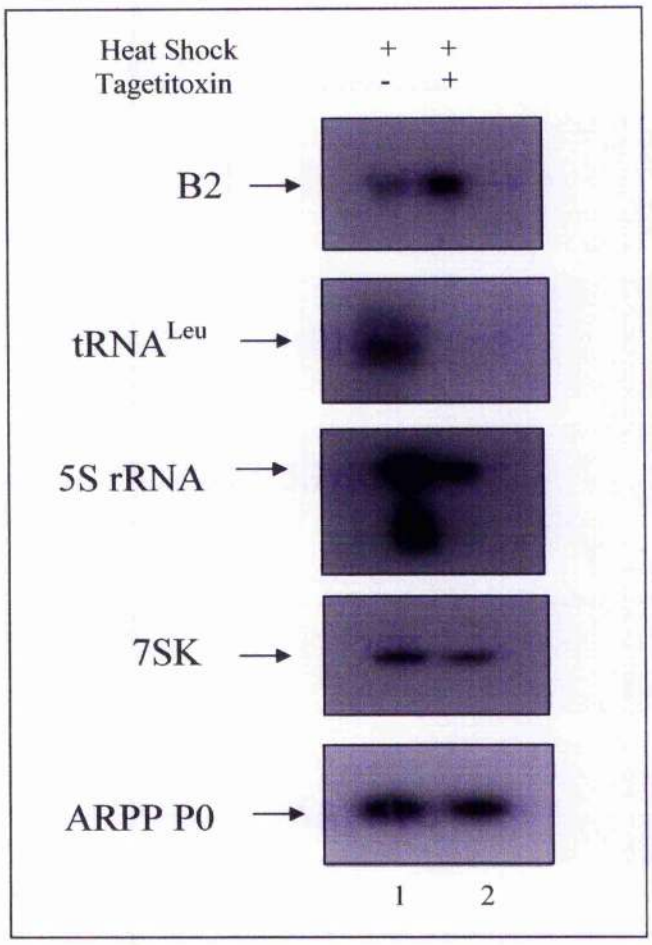


Figure 4.3 Treating NIH 3T3 cells after heat shock with tagetitoxin decreases pol III transcription

NIH 3T3 cells were seeded into a 6-well culture plate. After 24 hours of normal culture conditions cells were either given no treatment (lane 1) or treated with 45 μM Tagetin™ (lane 2) and incubated for 60 minutes at 37°C. Cells were then heat shocked for 25 minutes at 45°C, and then allowed to recover for 25 minutes at 37°C before harvested for RNA. RT-PCR analysis was carried out with primers for B2 (panel 1), tRNA^{Leu} (panel 2), 5S rRNA (panel 3), 7SK (panel 4) and ARPP P0 (panel 5). These results are representative of two independent experiments.

4.2.3 Electroporation of HeLa cells with tagetitoxin decreases pol HI transcription

Electroporation is a significant increase in the electrical conductivity and permeability of the cell plasma membrane caused by an externally applied electrical field. In molecular biology it is used as a way of introducing a substance into a cell, such as DNA or a drug. When voltage is applied, pores are formed across a plasma membrane, the substance enters the membrane, and when electroporation ceases the pores reseal, allowing the substance to move across the membrane into the cell.

One additional method was attempted to introduce Tagetin™ into mammalian cells. HeLa cells were used in this experiment as it was demonstrated in Wang *et al.* that tagetitoxin decreased pol III transcription after microinjection in HeLa cells (2003). In addition, greater quantities of RNA are able to be harvested from HeLa cells compared to NIH 3T3, and this trait was advantageous when harvesting these cells after nucleofection. HeLa cells were electroporated with an Amaxa (Amaxa Biosystems) nucleofector during simultaneous treatment with 45 μM tagetitoxin. After 48 hours, cells were harvested for RNA and cDNAs were created for RT-PCR analysis. Pol III transcripts $tRNA^{Leu}$ and 5SrRNA were greatly reduced, while 7SK was slightly reduced, but not the pol II control, indicating specific targeting of pol III (Figure 4.4). The reason for a slightly decreased level of 7SK is debatable, as previous data differed, showing no change in 7SK mRNA levels in NIH 3T3 cells when treated with tagetitoxin (Allen *et al.*, 2004). However, tagetitoxin inhibition of pol III transcription can be template- dependent, and 7SK uses the unusual pol III type III promoter structure, unlike those of tRNA and 5s rRNA

genes. Studies on the yeast U6 snRNA gene found that about 3-fold more tagetitoxin was required to give half-maximal inhibition of transcription when compared to the tRNA gene, suggesting that the gene template might change the effectiveness of tagetitoxin (Steinberg and Burgess, 1992). 7SK uses the same promoter type as U6 snRNA, and therefore perhaps 7SK RNA levels are influenced differently than those genes transcribed from type I (5S rRNA) and type II (tRNA) promoters.

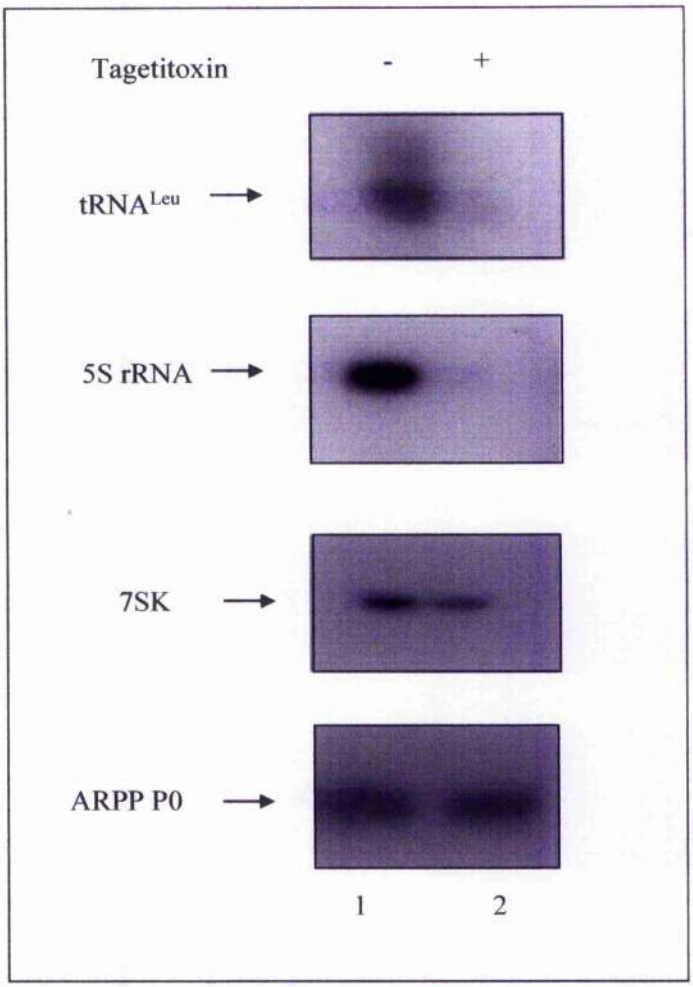


Figure 4.4 Electroporation of HeLa cells with tagetitoxin decreases pol III transcription

HeLa cells were transfected by electroporation (Nucleofector, Amaxa) with either dH_20 or 45 μM Tagetin™ and harvested for RNA 48 hours later. RT-PCR analysis was carried out using primers for pol III templates tRNA^{Leu} (panel 1), 5S rRNA (panel 2), 7SK (panel 3) and pol II control template ARPP P0 (panel 4). These results are representative of three independent experiments.

4.3 Discussion

It is apparent that the direct application of tagetitoxin to cells via culture medium does not decrease pol III transcription (Figure 4.2). This follows the current unpublished research on the inability of tagetitoxin to cross the cell membrane without assistance. When contact was made with Epicentre® personnel via telephone they could not report a successful attempt at inhibiting pol III transcription by the direct application of Tagetin™ to mammalian cells *in vivo,* and they did not recommend it. Introduction of tagetitoxin into HeLa cells by microinjection has been successful, although this technique is not feasible within our laboratory (Wang *et al.*, 2003). This impelled us to find other ways to introduce tagetitoxin into mammalian cells.

The significance of the heat shock results, however, is undetermined. Time permiting, another control would have been beneficial; such as no treatment and no heat shock. Without this control it is hard to conclude if the decrease in the treated and heat-shocked cells are from the tagetitoxin or merely appear to be decreased compared to increased transcripts from heat shock treatment (such as in Figure 2, Allen *et al.*, 2004). A no heat shock, no Tagetin™ control would control for effects from heat shock, allowing for comparisons between this control and the heat shock and Tagetin™- treated cells. If levels of pol III transcripts compared to this control were decreased, compared to the non-treated control, then perhaps deductions could be made as to the performance of tagetitoxin. Indeed, it might suggest that the tagetitoxin was able to gain entry into the cell via a mechanism induced by heat shock. Human heat shock proteins (hsps) have been found to induce ion conducting pores across lipid bilayers, however there are no reports of drugs being introduced into a cell

through heat shock (Alder *et al.*, 1990). This method was not used for further studies as the widespread effects of heat shock on mRNA transcription are not currently known.

Another way to perceive the heat shock results is that the application of tagetitoxin by heat shock did not allow the drug to cross the membrane, but that this was a non-specific response to the drug treatment. Allen *et al.* published that transcription of B2 mRNA increases rapidly after heat shock treatment, but this increase is blocked when cells are treated with both heat shock and tagetitoxin (2004). The experiment shown in Figure 4.3, above, does not parallel this result. Perhaps the effects of both heat shock and tagetitoxin on B2 mRNA levels causes a heightened heat shock response, and the decreases in the remaining pol III transcripts is an anomaly. Allen *et al.* also demonstrated the inhibition of B2 RNA on pol II transcription. mRNA levels in Figure 4.3 were normalized to levels of the pol II transcript, ARPP P0, and normalizing to this transcript may have affected the results.

Treating cells with tagetitoxin during electroporation seems to provide a new tool for the introduction of drugs into HeLa cells, although the effects of electroporation itself have not been taken into consideration. It would be interesting to discover if other drugs could be introduced this way into cells, and if the cells are affected by nucleofection. Another method would be to try transfection of tagetitoxin by the addition of calcium phosphate, a method widely used in the transfer of genetic material and viruses into cells; however, I was unable to find any reports stating the useage of calcium phosphate in the application of drugs in cell culture condition. In any case, electroporation,

above all others mentioned within this chapter, could be fine-tuned and utilized for further studies on the effects of decreasing pol III transcription in mammalian cells.

Chapter 5

Induction, silencing and deletion of Maf1 affects RNA polymerase III transcription in mammalian cells

5.1 Introduction

5.1.1 Discovery of Maf1, a new pol III repressor

In 1997 a new gene, *MAF1*, was identified in a yeast screen for mutations affecting the efficiency of action of a nonsense suppressor tRNA (Boguta *et al.*, 1997). Mutations in this 395 amino acid nuclear protein showed two phenotypic effects: antisuppression and temperature sensitivity in respiratory growth (Boguta *et al.*, 1997; Pluta *et al.*, 2001). These links to both tRNA suppression and growth lead researchers to screen a multicopy gene library for complementation of the temperature sensitive mutant phenotype *maf1*-1. Overexpression of RPC160, a gene which encodes the largest subunit of RNA polymerase III, suppressed the above phenotypes, demonstrating a genetic link between pol III and Maf1. It is this research that spawned additional biochemical analysis to identify the primary function of the *MAF1* gene product.

Further studies continued to define Maf1 as a negative effector of pol III. Mutations in RPC160 were shown to mimic the above-mentioned phenotypes, providing more evidence linking pol III and Maf1 physically (Pluta *et al.*, 2001). Co-immunoprecipitations between HA-tagged RPC160 and myc-tagged Maf1 suggested a direct interaction between Maf1 and pol III. Additional evidence suggested a role for Maf1 in the tRNA biosynthetic pathway, Maf1 potentially regulating the level of cellular tRNA in response to external signals (Pluta *et al.*, 2001). After these initial experiments, studies began to focus on the role of Maf1 in pol III's response to nutrient limitation and the mechanism in which Maf1 represses pol III transcription.

5.1.2 Maf1, a common component of multiple signalling pathways

The transcription of pol I and III genes is tightly coupled with nutrient availability and is affected by a wide variety of treatments and conditions including; drugs, secretory pathway defects and DNA damage that disturb the cellular environment (Ghavidel and Schultz, 2001; Stenfanovsky *et al.*, 2001). The disruptions caused by nutrient limitation and treatments/conditions are detected by unique signalling pathways that converge on the transcription apparatus, affecting regulation. Recently, it was shown that Maf1 is a common component of these signalling pathways, providing a critical link between these diverse pathways and the pol III transcription machinery (Upadhya *et al.*, 2002) (summarised in Figure 5.1).

5.1.2.1 The secretory pathway

Initial experiments on yeast were performed to determine the manner in which Maf1 was affecting pol III transcription. By deleting *MAF1* in a *ypt6-1 S. cerevesiae* strain and subjecting the yeast to nonpermissive temperatures to inactivate the secretory pathway, researchers found that the control strain repressed tRNA transcription, while in the *ypt6-1 maf1*Δ strain repression of tRNA transcription was blocked (Li *et al.*, 2000; Upadhya *et al.*, 2002). These results were an indication that Maf1 is essential for the repression on pol III transcription following inactivation of the secretory pathway.

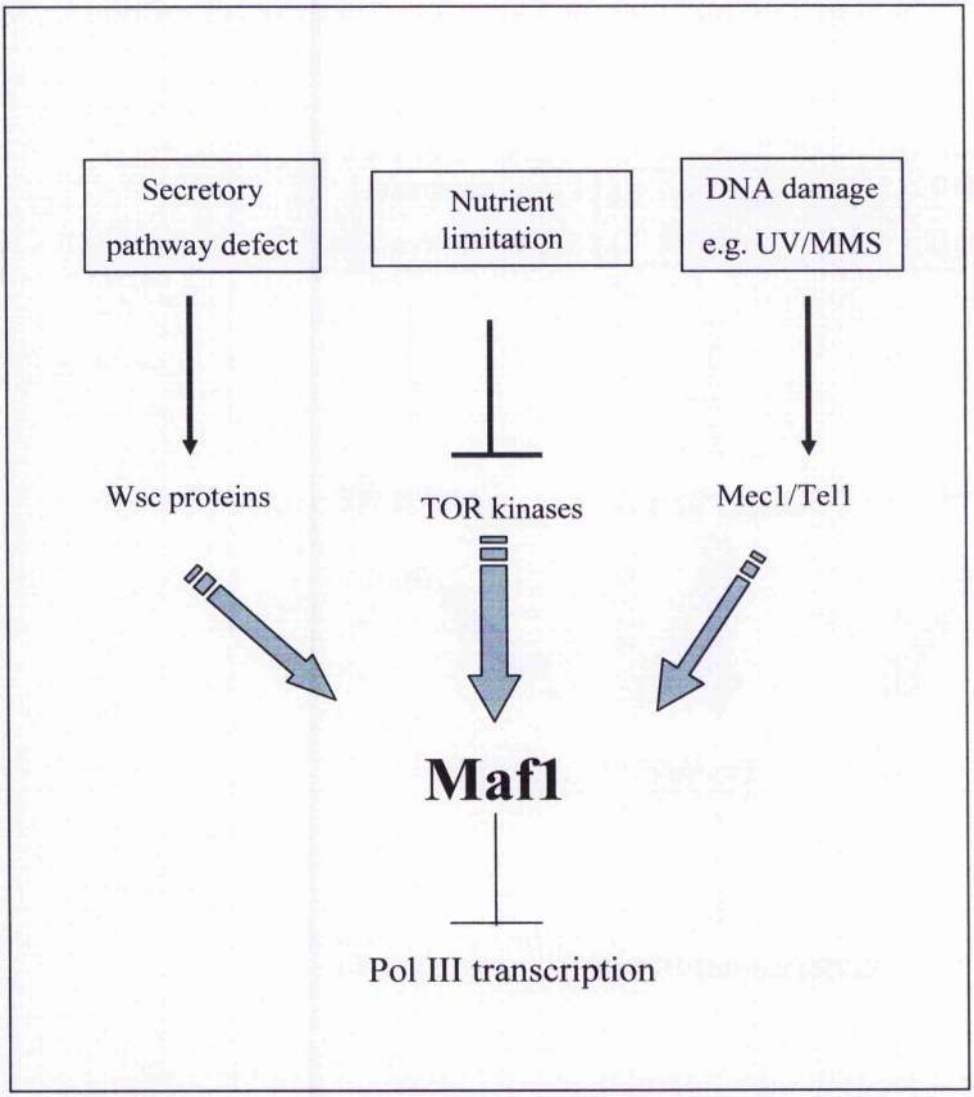


Figure 5.1 Maf1 is a convergent point for regulation of pol III by multiple signaling pathways (adapted from Upadhya *et al.*, 2002).

Conditions that repress pol III transcription are shown in boxes, near top. Preceeding each arrow is the upstream sensor or transducers that distinguish each signaling pathway. The dashed effect of the blue arrows indicate that there are still unknown elements of these pathways, the point(s) of convergence not yet known. Arrows symbolize activation of signaling and bars indicate repression.

To ascertain which proteins within the secretory pathway might be influencing Maf1 repression of pol III, cells were treated with tunicamycin, a drug which inhibits protein N-glycosylation in the endoplasmic reticulum and which requires proteins within the Wsc family (Li *et al.*, 2000). Wsc proteins are found along the secretory pathway and in the plasma membrane, initiating signaling by sensing stressful conditions within the cell. Treatment of cells with tunicamycin reduced transcription of pol III genes in the wild-type strain, however, this repression was abolished in the *maf1*Δ (as above) strain (Upadhya *et al.*, 2002). This provided more definitive evidence that Maf1 is a mediator in the secretory pathway.

5.1.2.2 Nutrient limitation

Another characteristic of Maf1 is its ability to respond to nutrient limitation with repression of pol III transcription (Upadhya *et al.*, 2002). Rapamycin, a macrolide antibiotic which inhibits the conserved target of rapamycin (TOR) kinases, can be used to induce the effect of nutrient limitation on transcription, mimicking the nutrient starvation phenotype (Barbet *et al.*, 1996; Rohde *et al.*, 2001). TOR kinases are a very important set of proteins within the cell, beginning a signaling cascade that starts when decreases in nutrients are sensed, and ending with changes in cell growth and proliferation (Schmelzle and Hall, 2000). Inhibition of TOR results in the rapid repression of tRNAs and rRNA genes transcribed by pol I and pol III (as well as some pol II genes), altering ribosome biogenesis (Cardenas *et al.*, 1999). Temperature studies using yeast with a mutation in the TOR pathway provide further evidence of a direct connection between the TOR pathway and pol III transcription (Zaragoza *et al.*, 1998). Experiments performed by Upadya *et al.*

demonstrated that it was Maf1 which was receiving a signal from the TOR kinase pathway, in turn repressing pol III transcription (2002).

5.1.2.3 DNA damage

Maf1 has a role in yet another signaling pathway- the DNA damage pathway, probably through checkpoint protein kinases Mec1 and Tel1 (ATM and ATR, respectively, in humans) (reviewed in Nyberg *et al.,* 2002). The DNA damage surveillance system has a series of checkpoints that are responsible for genome integrity through proper detection and repair of DNA damage caused by environmental stresses or irregularities during DNA metabolism. Mec1 and Tel1 function by regulating an important group of proteins that detect, signal and repair DNA damage (Rouse and Jackson, 2002). Not all components of this pathway have been identified, and the precise manner in which Mec1 and Tel1 signal pol III repression through Maf1 remains elusive.

Although the exact mechanisms by which Maf1 is signalled by the multiple pathways above are currently not known, research regarding how Maf1 affects the pol III transcription apparatus has provided enlightenment to the downstream effects of these various pathways.

5.1.3 Maf's mechanism of action

Initial work on the way in which Maf1 functions to repress pol III transcription was performed in yeast, although recent research in mammalian systems has provided analogous information. Studies involving the complex mechanism of action of Maf1 on the pol III transcription apparatus have made headway

recently, although some mysteries remain. Even so, Maf1's activity seems to be based around three main characteristics; nuclear localization, phosphoregulation, and direct targeting of TFIIIB subunit Brf1.

5.1.3.1 Maf1 is a nuclear protein

Maf1 was initially found within the nucleus when HA-tagged Maf1 was detected by indirect immunofluorescence microscopy (Pluta *et al.*, 2001). Further studies in *S. cerevisiae* showed that Maf1 is found in both the cytoplasm *and* the nucleus, although localization to the nucleus only occurs during nutrient starvation, growth to stationary phase, or with treatment by rapamycin (Roberts *et al.*, 2006). Under active growth conditions, Maf1 is largely excluded from the nucleus, although residual amounts remain (Moir *et al.*, 2006). This localization is required for transcriptional repression (Roberts *et al.*, 2006). Nuclear accumulation is regulated by a two nuclear localization sequences (NLSs) within the protein, one of these sequences (an N-terminal NLS, NtNLS) being functionally tied to the regulation of pol III by protein kinase A (PKA).

5.1.3.2 Maf1 repression of pol III: a balance between PKA and protein phosphatase 2A (PP2A)

Maf1 contains six consensus PKA phosphorylation sites, in addition to the two localization sites mentioned above. One of these sites, the NtNLS, overlaps two PKA sites. Recent studies by Moir *et al.* have shown that Maf1 is phosphorylated by PKA at the NtNLS, and high phosphorylation activity blocks repression of pol III transcription (2006). Both NLS motifs were found

to independently direct Maf1 to the nucleus under normal conditions, however, the NtNLS activity is required for efficient relocation under repressing conditions and is regulated by PKA. Therefore, under conditions of active growth, Maf1 is phosphorylated by PKA and mostly excluded from the nucleus. When growth-limiting conditions occur, Maf1 is localized to the nucleus, suggesting that Maf1 activity may require a PKA-independent activation step in order for nuclear Maf1 to repress pol III transcription. This activation step was found to be performed by PP2A, functioning to dephosphorylate Maf1, Maf1 then undergoing import to the nucleus (Oficjalska *et al.*, 2006). After Maf1 is dephosphorylated by PP2A, Maf1 can interact with pol III's largest subunit, C160, within the nucleus. Thus, the phosphorylation states of Maf1 are kept in balance by PKA and PP2A, functioning to communicate the cells nutrient state to Maf1, which can then interact with the pol III apparatus to regulate transcription (see Figure 5.2).

There is one caveat to this, however. Recently, experiments with a mutant form of Maf1 lacking all PKA sites has revealed an interesting discovery. The Maf1 mutant protein is nuclear under all growth conditions, yet pol III transcriptional activity was not diminished (Moir *et al.*, 2006). Pol III transcription was only decreased after treatment with rapamycin. This suggests that there must be an additional factor/step involved in transforming Maf1 to its repressive state, as dephosphorylation and nuclear accumulation alone are not sufficient for Maf1-mediated repression.

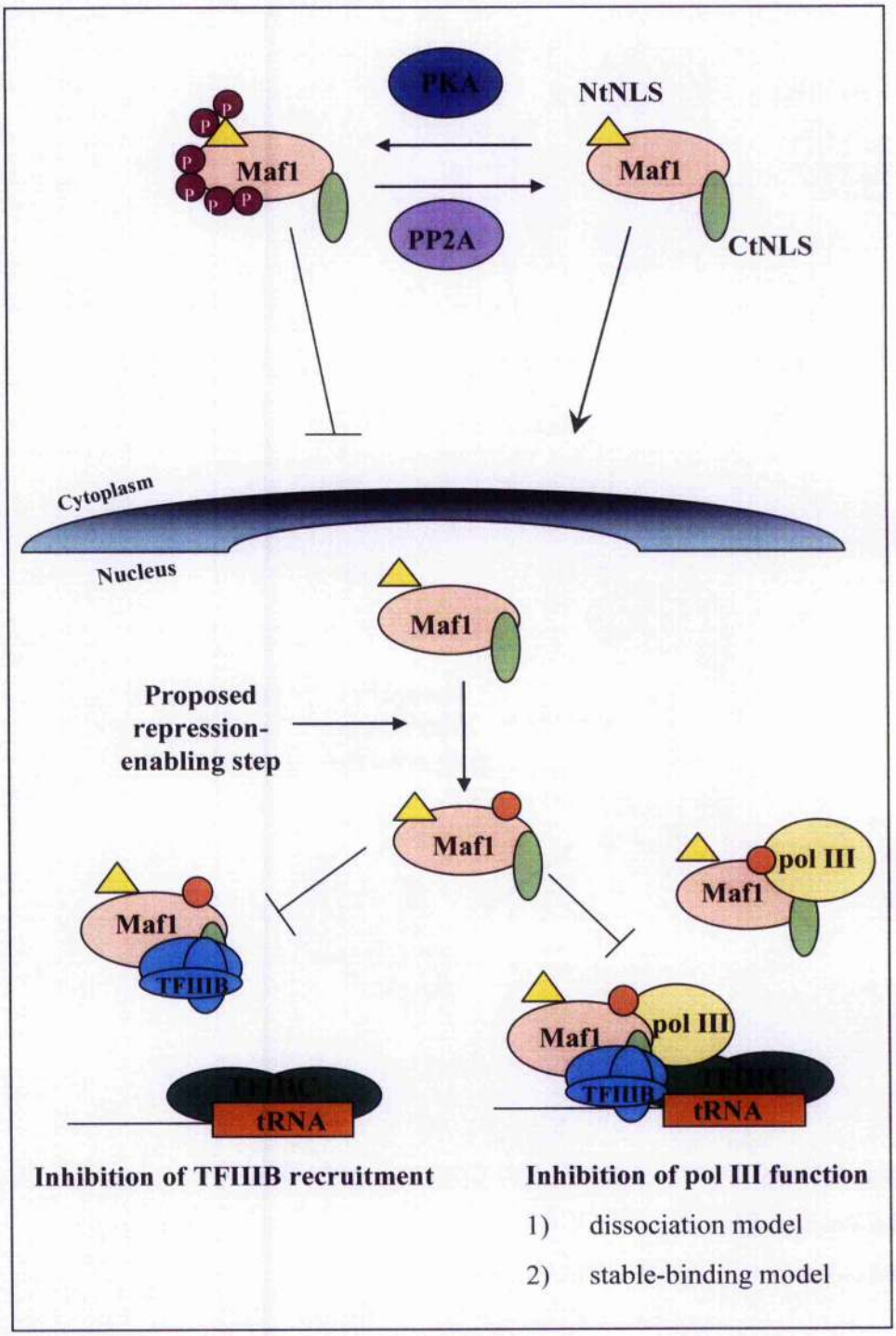

PKA
PP2A
NtNLS
CtNLS
Maf1
Cytoplasm
Nucleus
Proposed repression-enabling step
pol III
TFIIIB
tRNA
Inhibition of TFIIIB recruitment
Inhibition of pol III function
1) dissociation model
2) stable-binding model

Figure 5.2 Model of the functions of Maf1 as negative regulator of RNA polymerase III (adapted from Geiduschek and Kassavetis, 2006).

During normal growth in nutrient-rich media, Maf1 is phosphorylated on consensus PKA sites and is found mostly within the cytoplasm. The NtNLS (triangle) and CtNLS (oval) direct the nuclear import of Maf1. Phosphoryation at consensus PKA sites represses nuclear import by the NtNLS. Many conditions lead to the dephosphorylation of Maf1 which results in nuclear accumulation. The opposing actions of PKA and PP2A in response to nutrients determines the regulation of Maf1 localization. PKA is regulated by the RAS/cAMP pathway and PP2A by the TOR pathway. Because nuclear accumulation is not sufficient to repress pol III transcription, an additional unknown activation step (orange circle) might allow nuclear Maf1 to effect inhibit pol III transcription. Maf1 represses two separate steps of transcription: 1) *de novo* assembly of TFIIIB by TFIIIC; and 2) the disruption of transcription from pre-assembled TFIIIB-DNA complexes. Two alternative mechanisms are proposed for inhibition of step two. Arrows symbolize activation of signaling, and bars indicate repression.

5.1.4 Maf1 associates with pol III and TFIIIB

5.1.4.1 Pol III

Studies in *Saccharomyces cerevisiae* were first to identify the interaction between both pol III and TFIIIB with Maf1 (Desai *et al.*, 2005; Murawski *et al.*, 1994; Pluta *et al.*, 2001; Upadhya *et al.*, 2002). Genetic studies suggested an interaction between Maf1 and pol III when elevated levels of tRNA, caused by decreased Maf1, was suppressed by alterations of pol III subunit C160 (Murawski *et al.*, 1994; Pluta *et al.*, 2001). Coimmunoprecipitation experiments proved this supposition when C160 was found to bind to Maf1 *in vitro*. Pluta *et al.* also reported a slight increase of TFIIIB (Brf1) signal in their Western blot above background, hinting at the possibility of a Brf1-Maf1 interaction, although the data were not shown (2001). The least phosphorylated form of Maf1 preferentially binds to pol III in both pol III-active and pol III-repressed cells. However, the interaction between Maf1 and pol III in repressed-cell extracts is greater, which reinforces the hypothesis that Maf1 is dephosphorylated and transferred to the nucleus upon repression (Oficjalska *et al.*, 2006; Roberts *et al.*, 2006).

5.1.4.2 TFIIIB

Research stemming from Ian Willis's laboratory proved that Maf1 interacts with TFIIIB, namely Brf1, and shortly thereafter provided evidence of direct binding using recombinant proteins (Desai *et al.*, 2005; Upadhya *et al.*, 2002). The discovery of TFIIIB-Maf1 binding was significant, as TFIIIB is a major regulatory target for transcription by pol III, and this discovery provided insight into possible mechanisms in which Maf1 could apply its transcriptional control.

5.1.5 The mechanism of action of Maf1 on the pol III transcription apparatus

5.1.5.1 Maf1 inhibits two distinct steps in transcription

Maf1 repression of pol III transcription occurs in two separate steps; one focusing on the inhibition of TFIIIB recruitment and the other on the inhibition of pol III function.

Inhibition of TFIIIB recruitment

Once Maf1 was established as a common component of multiple signalling pathways that repress pol III transcription, research turned towards discovering the mechanism by which Maf1 asserts its repressive activity on pol III. Upadhya *et al.*, used treatment of yeast cells with chlorpromazine (CPZ) to repress pol III transcription and established that this effect was absolutely dependent on Maf1 (2002). To examine the possibility that CPZ-repression of pol III transcription through Maf1 could be due to CPZ-targeting of a component of the pol III transcriptional machinery, extracts of cells treated with CPZ were supplemented with partially purified yeast TFIIIB, TFIIIC, or pol III. Only the addition of TFIIIB, and not TFIIIC or pol III, restored transcription levels to that of the control extract. These experiments implicated TFIIIB as the target for repression of pol III transcription by CPZ. Further biochemical data suggest that CPZ-induced repression of TFIIIB activity results from a defect in its recruitment to DNA (Desai *et al.*, 2005). This established a link between Maf1-dependent transcriptional repression and de novo assembly of TFIIIB onto DNA (Desai *et al.*, 2005; Upadhya *et al.*, 2002).

More recent experiments performed by Desai *et al.* further clarified the manner in which Maf1 acts through TFIIIB to repress pol III transcription (2005). By executing supplementation experiments with all three subunits of TFIIIB (TBP, Brf1 and Bdp1) to CPZ-treated cells, Brf1 was found to be the target for repression. However, the differential between the control and CPZ-treated extracts was lessened, but not eliminated, following the addition of Brf1, suggesting that there may be another factor/step affected under repressing conditions.

To determine at which assembly point Maf1 asserts its repressional activity, Desai *et al.* added Maf1 to pre-assembled TFIIIB-TFIIIC-DNA complexes (2005). Addition of Maf1 did not have an effect on the amount of the TFIIIB-TFIIIC-DNA complex. This suggests that the inhibitory effect of Maf1 on transcription from preassembled complexes involved a post-TFIIIB recruitment step. By adding Brf1 to mixtures containing Maf1 proteins and TFIIIC-DNA, it was found that Maf1 inhibits Brf1's recruitment onto TFIIIC-DNA complexes. The associations between Maf1, pol III and Brf1 did not change quantitatively in repressed cell extracts, suggesting that the effect on pol III transcription by Maf1 is non-stiochiometric. Maf1 is not simply sequestering away Brf1 or pol III from the transcription complex. The data above implicate Maf1 in a biochemical process that inhibits TFIIIB-DNA complex assembly and transcription. Brf1 is a likely target of repression in yeast, as the activity of Brf1 decreased significantly in the TFIIIB fraction treated with CPZ (Desai *et al.*, 2005).

Inhibition of pol III function

Chromatin immunoprecipitation experiments conducted by Oficjalska-Pham *et al.* and Roberts *et al.* discovered a weak association of Maf1 with pol III genes in exponentially growing cells (2006). Under repressing conditions, the association of Maf1 and pol III increased with the nuclear concentration of Maf1 (Oficjalska *et al.*, 2006; Roberts *et al.*, 2006). However, levels of the polymerase at pol III promoters decreases in a Maf1-dependent manner (Desai *et al.*, 2005; Oficjalska *et al.*, 2006; Roberts *et al.*, 2006). These results combined are interesting as they lead to two distinct models of the inhibition of pol III function. In one model, Maf1 is only associated with pol III genes ephemerally and its action causes the dissociation of the polymerase. In the alternative model, Maf1 is stably bound to pol III genes, where its capacity to disengage (but not necessarily separate) DNA from the polymerase inhibits transcription (Roberts *et al.*, 2006). Clearly, more efforts are needed to further resolve these models.

In summary, Maf1 inhibits two distinct steps in transcription: *de novo* assembly of the initiation factor TFIIIB by promoter-bound TFIIIC, and transcription from pre-assembled TFIIIB-DNA complexes (Desai *et al.*, 2005; Upadhya *et al.*, 2002) (these conclusions and those above are summarized in Figure 5.2).

5.1.6 Mammalian cells

Maf1 was found to be a conserved protein from yeast to humans in early studies and it was recently demonstrated that Maf1 plays a central role in pol III repression of mammalian cells (Pluta *et al.*, 2001; Reina *et al.*, 2006).

Paralleling yeast studies, human Maf1 was found to bind to Brf1 and the largest subunit of pol III (RPC1 in humans), but was also shown to bind to another pol III subunit, RPAC2 (Reina *et al.*, 2006). Experiments using extracts from rapamycin or methanemethylsulfonate (MMS) -treated cells incubated with phosphatase inhibitors determined that human Maf1 is phosphorylated and becomes largely dephosphorylated after stress, the dephosphorylated form associating with pol III (Reina *et al.*, 2006). Therefore, yMaf1 acts similarly to hMaf1 when taking into account the above binding properties and phosphorylation. However, there are a few differences between species. Further advancements have also been made, allowing a deeper understanding of Maf1's role in mammalian cells.

In human cells, Maf1 represses transcription not only from type 1 and type 2 promoters, but also type III promoters, which are absent in yeast (Reina *et al.*, 2006). Maf1 did not affect assembly of any of the transcription components (i.e. $SNAP_c$, TBP, Brf2 or Bdp1) onto the type III human U6 promoter. Only associations with pol III were noted. These results suggest that in mammalian type III promoters Maf1's function occurs when it is bound to pol III and does not act on the formation of the promoter-bound pol III-recruiting complex. Maf1's actions on human type I and type II promoters probably parallel that of yeast, as human Maf1 has been shown to bind to Brf1 and it is these two promoter types that utilize Brf1.

Reina *et al.* also examined the regions in which Maf1 associates with pol III and Brf1 (2006). Maf1 contains three regions of highly conserved sequences, deemed A-, B- and C- boxes, followed by an acidic tail. By generating

truncated versions of the protein, both pol III subunits (RPC1 and RPC2) associated with the A box, whereas Brf1 only associated with the A- and B-boxes of Maf1. Thus, pol III and Brf1 both need the Maf1 A- box to bind, but Brf1 also needs the additional B- box. This indicates that Maf1 interacts with pol III and Brf1 at overlapping binding domains.

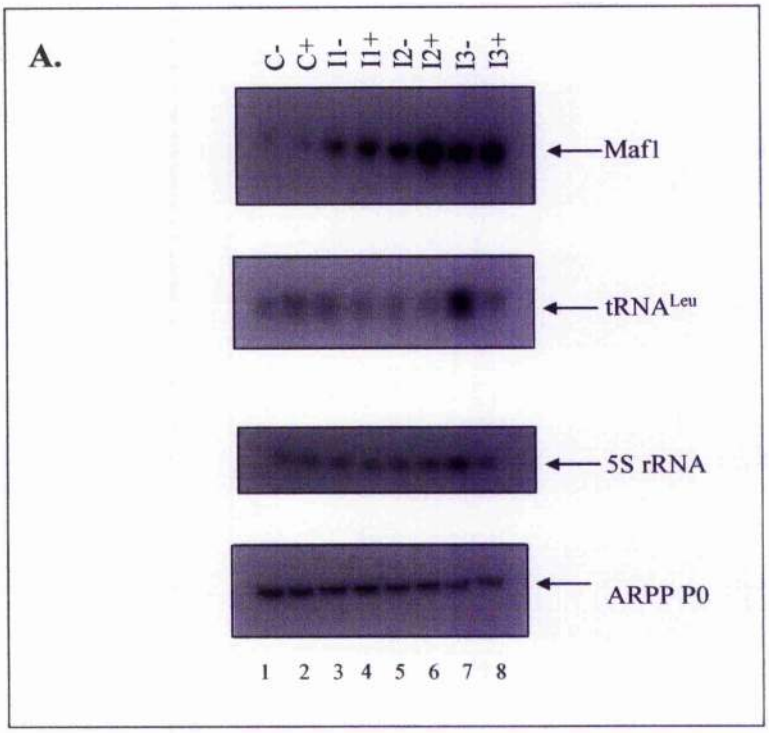
As Maf1 was found to be involved in at least two repression pathways in humans (DNA damage and mTOR) which directly affect pol III transcription, this chapter focuses on the ability of Maf1 to repress pol III transcription in mammalian cells as another technique to decrease pol III transcription. When these experiments were performed, the information contained within this chapter was yet unpublished; however, similar work is now published within Reina *et al.*, although this chapter does contain select novel results (2006).

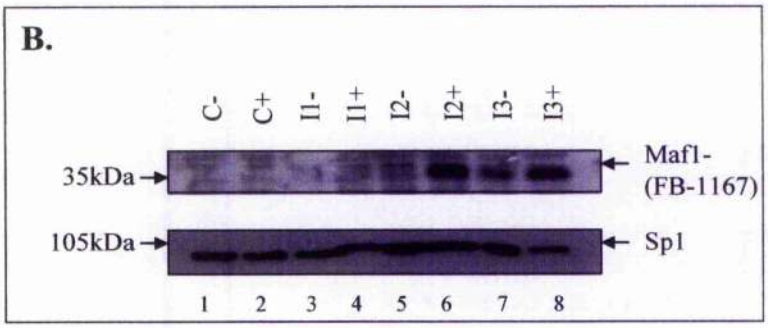
5.2 Results

5.2.1 Induction of Maf1 represses pol III transcription in human cells

To study the effect of overexpressing Maf1 in cervical carcinoma (HeLa) cells, I created a Maf1-inducible HeLa cell line using the BD™ Clontech Tet-On system (hMaf1 cDNA acquired from Olivier Lefebvre, Saclay, France). Maf1 was tagged with hemagglutin (HA) by sub-cloning hMaf1 into pCDNA3HA for better visualization by Western blot. Experimental (pTRE2.HA.Maf1.hyg) and control cells (pTRE2.hyg) were induced with 1 mg/ml doxycycline and harvested after 48 hours for both RNA and protein extracts. cDNAs were prepared from the RNA to utilize in PCR analysis, while the protein extracts

were analysed in SDS-PAGE gels followed by Western blotting. RT-PCR results showed decreases in pol III transcripts $tRNA^{Leu}$ (although very slight in I1 and I2) and 5S rRNA , although the decreases in 5S rRNA levels were not as significant and also varied (discussed below) (Figure 5.3A, panels 2 and 3). As expected, Maf1 mRNA was increased in the pTRE2.HA.Maf1.hyg cells when compared with the control cells (Figure 5.3A, panel 1). The effect of Maf1 in these cells is specific for pol III, as there was no change in the level of the class II gene product ARPP PO mRNA (Figure 5.3A, panel 4). To determine if induction of Maf1 mRNA was also influencing the level of Maf1 protein, and therefore translation, protein extracts were run on SDS-PAGE gels and immunoblotted with an antibody against Maf1 (FB-1167), as well as an anti-HA antibody. Indeed, both the exogenous (detected by the anti-HA antibody) and endogenous levels (detected by the FB-1167 Maf1 antibody) of the Maf1 protein increased in concordance with the RT-PCR results (Figure 5.3, B and C). An anti-Sp1 antibody (Figure 5.3 B and C, lower panels) is used as a loading control. Within the cell, Sp1 functions as a transcription factor, the protein running at approximately 95 kDa on an SDS-PAGE gel. This experiment demonstrates that the induction of Maf1 in a stable human cell line represses pol III transcription.


A.
C-
C+
I1-
I1+
I2-
I2+
I3-
I3+
Maf1
tRNA^Leu
5S rRNA
ARPP P0
1 2 3 4 5 6 7 8


B.
C-
C+
I1-
I1+
I2-
I2+
I3-
I3+
35kDa
Maf1-
(FB-1167)
105kDa
Sp1
1 2 3 4 5 6 7 8

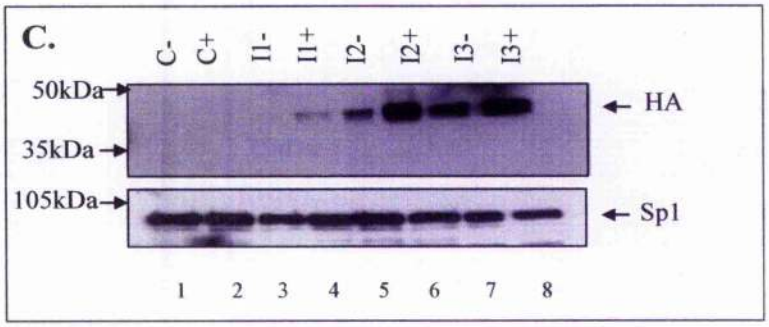

C.
C-
C+
I1-
I1+
I2-
I2+
I3-
I3+
50kDa
35kDa
HA
105kDa
Sp1
1 2 3 4 5 6 7 8

Figure 5.3 Induction of Maf1 represses pol III transcription in human cells

A. HcLa pTRE2.hyg and pTRE2.HA.Maf1.hyg. cells were plated in 10 cm culture dishes and grown under normal conditions for 24 hours. Half the cells were then induced with 1 mg/ml doxycycline and incubated for a further 48 hours, while the other half received fresh media only. At this point cells were harvested for RNA extracts and RT-PCR was applied. Maf1, $tRNA^{Leu}$, 5s rRNA, and ARPP P0 were analyzed. "C" stands for the control cell line pTRE2.hyg, while "I" stands for the inducible cell line pTRE2.HA.Maf1.hyg. "-" symbolizes uninduced and "+" induced. These results are representative of three separate experiments.

B. Cell extracts were also made from uninduced and induced cells, above. Lysates were subjected to SDS-PAGE analysis and then immunoblotted with either antibody against endogenous Maf1 (FB-1167) or loading control Sp1. These results are representative of two separate experiments.

C. The same cell lysate from (B) was ran on a separate SDS-PAGE gel and immunoblotted with either HA antibody, to detect the HA-tagged Maf1, or Sp1, used as a loading control. These results are representative of three independent experiments.

As previously shown, decreasing pol III transcription by using siRNA targeting of Brf1 in mammalian cells decreases proliferation rates (*Chapter 3*, Figures 3.4 and 3.6). Therefore, one might postulate that decreasing pol III transcription by inducing Maf1 might have the same affect. The same clones (as above) were induced for 96 and 120 hours, and cell numbers were counted. When compared to the empty vector cells, the inducible cells overexpressing Maf1 ended at 120 hours with fewer cells (Figure 5.4, bar set C+, compared with bar sets I1+, I2+ and I3+).

The inducible cells all have a proliferation rate that is ~ 2 fold less than that of the empty vector cells. However, when inducible clones are compared as non-induced and induced (such as I1- compared with I1+), only the I1 clone seems to have a reduced proliferation rate at 120 hours. The other clones, I2 and I3 have almost equal proliferation rates as each time point, non-induced and induced. The proliferation data does not correlate with the RT-PCR and Western blotting analysis, as one would expect the clones with higher expression levels, I2 and I3, to produce the largest decreases in proliferation rates.

These results maybe at least partially explained by the fact that these inducible cell lines have leaky expression (i.e. a high background). This is apparent when non-induced cells are compared with induced cells in all clones, as non-induced cells still have a relatively high level of expression (Figure 5.3A, B and C, lanes 3, 5 and 7).

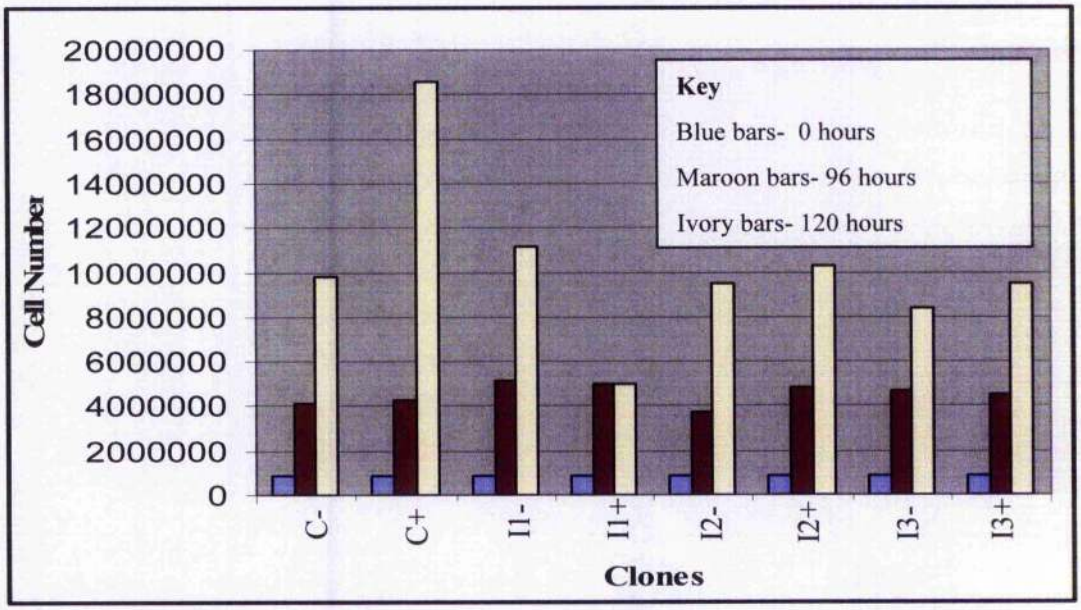


Figure 5.4 Expression of Maf1 in HeLa cells leads to variable proliferation rates

Cells were induced and counted at 0 hours, 96 hours and 120 hours after equal plating on 10 cm dishes and induction by 1 mg/ml doxycyclin. Results are representative of two separate inductions.

The only exception to this may be clone I1, as the signal of the non-induced clone is not apparent and there is a signal for the induced clone (Figure 5.3C, lane 3, compared to lane 4). However, increased exposures time of this film showed a slight background level as well, demonstrating that this clone also lacks tight regulation. This also explains why the levels of pol III transcripts were not as decreased as expected. In a system such as this with high background, levels of pol III transcripts of induced cells will not appear as decreased as they would if the system was under tighter control. The loose control of this Maf1-inducible system was not due to the presence of doxycycline in the media, as Tet system approved FBS (BD Biosciences Clontech) was used. Levels of the $tRNA^{Leu}$ transcript appear to be affected more than 5S rRNA as the primers for $tRNA^{Leu}$ are designed to monitor actively produced transcripts, while the 5S rRNA primers show steady state levels. Further screening experiments were performed to try and discover clones which had lower background, but to no avail. Therefore, more direct and less laborious approaches were commenced to look at the effects of Maf1 on pol III transcription.

5.2.2 Endogenous Maf1 inhibits pol III transcription in human and mouse cells

As discussed in *Chapter 3*, siRNA can be a useful tool for gene knockdown, and it is this technique that was employed to decrease pol III transcription by targeting Maf1 in HeLa cells. Cells were nucleofected with 3 μg of siRNA duplex, plated, and harvested 48 hours later for RNA. cDNAs were made from the RNA and PCR analysis was completed to determine mRNA levels.

As expected, Maf1 mRNA levels were decreased in the cells treated with Maf1 siRNA when compared to Maf1 mRNA levels in cells treated with control siRNA (Lamin A/C) (Figure 5.5, top panel). Levels of pol III transcript $tRNA^{Leu}$ were increased, reflecting the function of Maf1 as a repressor, as decreases in Maf1 would relieve pol III of repression by Maf1, increasing transcription and therefore pol III transcripts. Paralleling that of the above experiment (Figure 5.3), levels of 5S rRNA showed an increase of a lesser degree, reflecting the fact that these PCR primers are recognising a stable transcript. These results are specific, as there is no change in the pol II gene product ARPP P0 (Figure 5.5, last panel).

By knocking down Maf1 with specific siRNA, pol III repression by Maf1 is released, increasing pol III transcription. This method directly targeted and reduced Maf1 levels within the cell, providing a "pseudo-Maf1 knockout." To extend these results, extracts were harvested from a mouse embryonic stem (ES) cell line which had one *maf1* allele deleted (obtained from Olivier Lefebvre, Saclay, France). cDNAs were made from RNA and RT-PCRs were performed to detect pol III transcription. Increased expression of pol III transcripts $tRNA^{Arg}$ and 5S rRNA was demonstrated, although there was little change in $tRNA^{Leu}$ (Figure 5.6). The reason why $tRNA^{Leu}$ did not change is unclear. Maf1 mRNA expression is diminished in the heterozygous cells compared with the homozygous cells, consistent with the expression of only one allele. Figures 5.5 and 5.6 combined show that by depleting Maf1 by using siRNA or by deleting one Maf1 allele, pol III transcriptional output is increased.

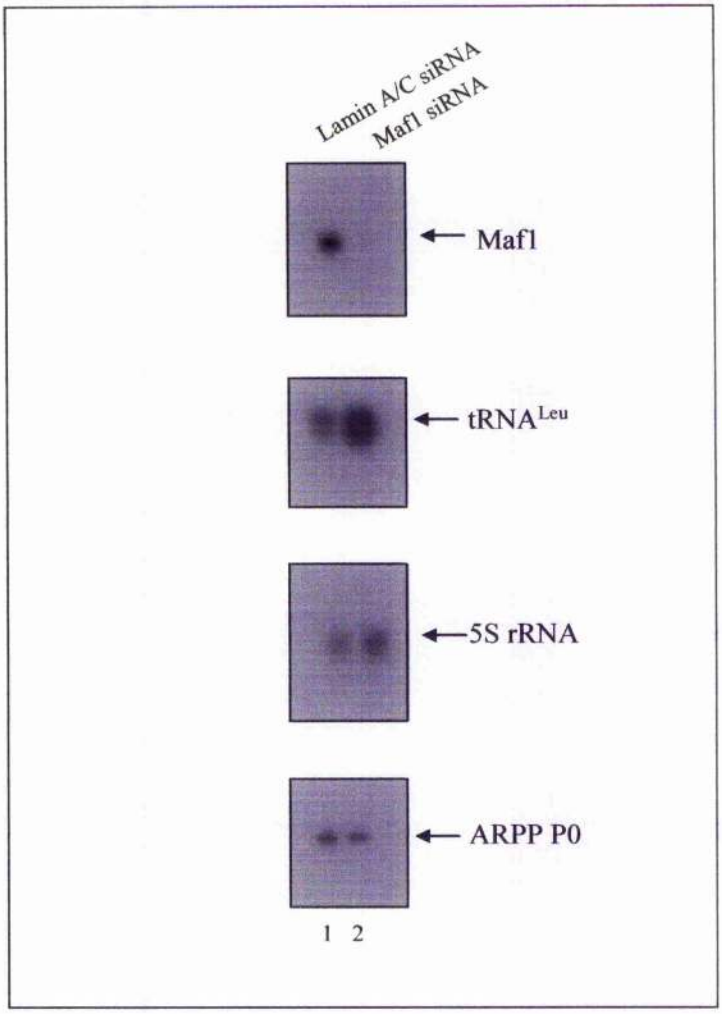


Figure 5.5 siRNA targeting Maf1 inhibits pol III transcription in HeLa cells

HeLa cells were transfected by electroporation with 3 µg siRNA targeting either Lamin A/C (control) or Maf1 mRNAs. 48 hours post transfection, RNA was extracted and analysed by RT-PCR using the gene-specific primers Maf1 (panel 1), tRNA^{Leu} (panel 2), 5S rRNA (panel 3), and ARPP P0 (panel 4). Results are representative of three independent experiments.

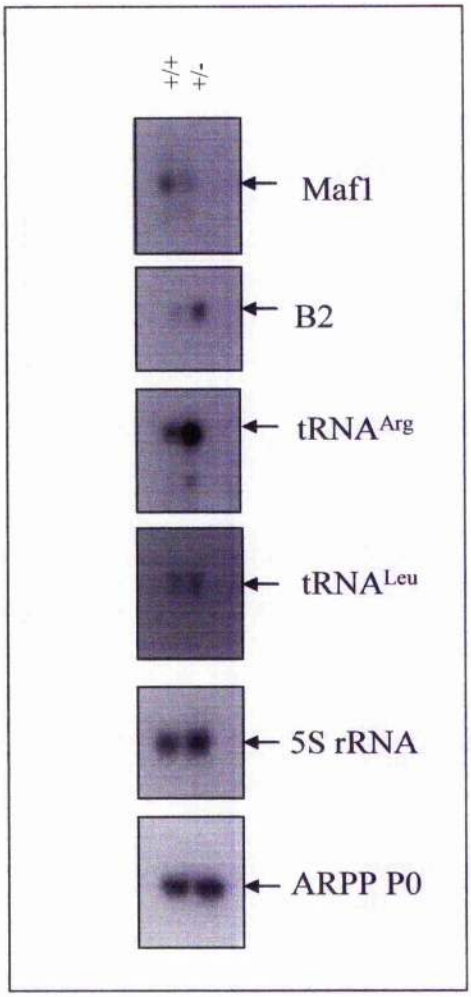


Figure 5.6 Endogenous Maf1 inhibits pol III transcription in mouse cells.

RNA was analysed from matched $Maf1^{+/+}$ or $Maf1^{+/-}$ ES cells by RT-PCR using primers for Maf1 (panel 1), B2 (panel 2), $tRNA^{Arg}$ (panel 3), $tRNA^{Leu}$ (panel 4), 5S rRNA (panel 5) and ARPP P0 (panel 6). Results are representative of three independent experiments.

5.3 Discussion

The methods described above were all successful in affecting pol III transcription by either *increasing* Maf1 levels within the cell using an inducible cell line or by *decreasing* Maf1 levels by utilizing siRNA or by deleting one allele. Depending on the information that one is looking for, different techniques might be used for different experiments to optimize results. For example, using an inducible cell line is clearly more beneficial than using siRNA when proliferation rates are the aim of the study. Inducible cell lines often allow long-term phenotypes to be observed, which may not be possible when adding synthetic siRNAs, as turnover rates may vary and therefore repeated transfections may be necessary. Inducible cell lines have problems as well, when long-term expression of an essential protein kills the cell, or when expression systems are loosely regulated (as above). The production of knockout mice is labour extensive and not always possible, but if achieved, the knowledge from knockout or even heterozygous cells is invaluable. Recently, experiments conducted by Johnson *et al.* showed a correlation between the overexpression of Maf1 and the suppression of anchorage-independent growth (a hallmark of transformed cells) (2007). Ideally, it would have been interesting to perform anchorage-independent growth assays on the Maf1 Tet-On inducible cells, but again, these cells would need to be selected again for minimal background expression levels.

Taken together, the results in this chapter have shown that induction, suppression and deletion of Maf1 affects pol III transcription in mammalian cells. Maf1 was proved to be a potent general repressor of pol III transcription in mammalian cells by three different techniques.

Chapter 6

Summary

6.1 Small interfering RNA targeting Brf1 decreases RNA polymerase III transcription

In *Chapter 3,* pol III transcription was decreased by transfecting HeLa cells with siRNA targeting TFIIIB subunit Brf1. Brf1 was previously shown to bind directly to TBP, TFIIIC and pol III, making it an instrumental component of the basal pol III transcription apparatus (Hsieh *et al.*, 1999b; Hsieh *et al.*, 1999a; Kassavetis *et al.*, 1992; Kassavetis *et al.*, 1998). Brf1 was also found to be an influential binding site for tumour suppressors, oncogenes and kinases, further stressing the significance of Brf1 as a hub for influencing pol III regulation within the cell (Crighton *et al.*, 2003; Felton-Edkins *et al.*, 2003a; Gomez-Roman *et al.*, 2003; Johnston *et al.*, 2002; Larminie *et al.*, 1997; Schmidt, 1999).

siRNA technology has been an innovative and invaluable research tool since its discovery and applications within mammalian systems have made it more applicable in the study of human disease. The proper controls when using siRNA are of the utmost importance; the level of significance of the results relies heavily upon this and control standards within the field must be monitored and updated regularly. Although the mechanism behind siRNAs effectiveness lacks some understanding, future experiments might clarify present knowledge, and perhaps even change the types of controls used. With proper controls in place, siRNA proves to be a powerful research tool in mammalian systems.

Using Brf1 siRNA to decrease pol III transcription *in vivo* proved to be successful within the experiments contained in *Chapter 3*. Although controls could have been more stringent (discussed on pages 48-51, 62), sufficient controls were in place to deduce that the Brf1 siRNA was depleting the cells production of Brf1 mRNA and protein (Figures 3.3A and 3.3B). Further analysis showed that the reduction of pol III transcripts in response to this silencing affected the cell's ability to proliferate at a normal rate (Figures 3.4 and 3.6). This result is substantial, as one of the classifications of cancer involves unchecked proliferation and therefore decreasing proliferation rates may provide insight into growth controls. An increase in pol III transcription has been linked to many types of cancers, therefore the reduction of Brf1, and hence pol III transcription, may provide not only a valuable research tool, but also a glimpse into possible therapeutics.

6.2 Tagetitoxin decreases RNA polymerase III transcription in mammalian cells

Past studies found tagetitoxin to be a potent and specific inhibitor of pol III transcription and that this inhibition was due to enhanced pausing at isolated sites and was template-dependent (Steinberg and Burgess, 1992; Steinberg *et al.*, 1990). Tagetin™, the commercially available form of tagetitoxin, was used in the experiments contained in *Chapter 4*, to attempt inhibition of pol III transcription in mammalian cells by *in vivo* application. When applied to mouse cells by direct application, pol III transcripts did not decrease (Figures 4.2A, or decreases were unexplained when compared to other transcripts which did not decrease (Figure 4.2A). Using heat shock to induce membrane pores

possibly allowed tagetitoxin to repress pol III transcription, but the unknown effects of heat shock would make this technique unreliable (Figure 4.3). Treatment of human cervical cells with tagetitoxin by electroporation was the most successful attempt at decreasing pol III transcription *in vivo*.

There is not a great quantity of research that has been published to date of the successful introduction of a drug in mammalian systems via electroporation, although the research seems promising (Bleomycin, 2004; Hui, 2002; Kambe *et al.*, 2006). Electroporation has been used to introduce anti-cancer drugs across the skin, although further research needs to occur to perfect this technique as the optimal conditions such as voltage, along with problems caused by the size of the drug, are difficult to discern (Hui, 2002). Electroporation holds the most promise for further research with tageitoxin in the laboratory, although the method of application will limit its uses. For example, long-term studies of cells treated with the inhibitor would be difficult as the amount of cells used during transfection with the nucleofector is quite high and therefore confluency is reached by 48 hours. If the cells were split into a larger vessel they would possibly need to be treated again, with both tagetitoxin and nucleofection, and this treatment might prove toxic. Nevertheless, if a technique was found to successfully apply tagetitoxin directly to culture media, then long-term effects on targeting pol III transcription could be observed. It would be interesting to perform proliferation curves with tagetitoxin in transformed cells, to see if the effect mimicked that of the proliferation curves in *Chapter 3*. If the proliferation rates were also decreased by tagetitoxin it would hint at possible therapeutic options for cancers that show an increase in pol III transcription.

6.3 Targeting Maf1 by silencing, deletion and induction influences pol III transcription

Maf1 is a common component of multiple signalling pathways that affect pol III transcription (Upadhya *et al.*, 2002). Research has recently uncovered some of the mechanisms behind these pathways, and the manner in which Maf1 represses pol III transcription. Nuclear localization, phosphoregulation, and direct targeting of TFIIIB subunit Brf1 are characteristics that apply to Maf1's repressive influence over pol III transcription (Murawski *et al.*, 1994; Moir *et al.*, 2006; Pluta *et al.*, 2001; Roberts *et al.*, 2006; Upadhya *et al.*, 2002). Although most of the knowledge of Maf1's repressive nature was acquired in experiments performed in yeast, current work also includes at least a partial understanding of the mechanism of Maf1 in mammalian systems.

The induction of Maf1 in a stable cell line decreased pol III transcription, but to varying degrees (*Chapter 5*, Figure 5.3A). This was probably due to the leaky nature of the stable cell line. Perhaps some of the cells were not killed off by selection and therefore created a high background Maf1 signal. However, this seems unlikely as the drug used for selection was used at a high concentration and therefore the cells would have to adapt to these high levels. The control for resistance to the selection drug (hygromycin), and the induction drug (deoxycyclin), were located on the same plasmid within the cell, making it improbable that the leakiness was do to the loss of one plasmid. The results of the proliferation curve reflect the loose control of Maf1 expression by these

cells (Figure 5.4). In order for any deductions to be made with confidence, cells with a tighter control of Maf1 expression would have to be created.

The experiments focusing on the silencing and deletion of Maf1 were more successful. siRNA targeting Maf1 in HeLa increased pol III transcription, releasing the transcription apparatus from repression (Figure 5.5), while the deletion of one *maf1* allele from ES cells also positively affected transcription (Figure 5.6). Combined, these data provide further evidence that Maf1 represses pol III transcription in two different mammalian cell types.

6.4 Final synopsis

Three different methods were attempted to decrease pol III transcription in mammalian cells; small interfering RNA against Brf1, treatment with the specific pol III inhibitor tagetitoxin, and the induction of a negative effector of pol III transcription, Maf1. These techniques vary considerably, and even the matter in which each individual technique was applied differed (i.e. the direct application of tagetitoxin versus nucleofection, etc.). Whatever the case, each technique has both desirable and disadvantageous qualities.

Finding techniques for decreasing pol III transcription has importance as pol III transcription has been found to be increased in a variety of cancers. Even a subunit within the transcription apparatus, Brf1, has been found to have increased levels in cervical cancer tissue. Pol III is also able to influence a cell's biosynthetic capacity, which must be increased for cell growth and proliferation. Clearly, transcription by pol III has an important role in the outcome of a cell toward normal or transformed growth. Therefore,

discovering techniques which decrease pol III transcription in mammalian cells is beneficial not only for further knowledge in mechanistic purposes but could also be a useful tool as therapeutics for the future.

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