Regulation of RNA Polymerase III Transcription by the Oncoproteins ErbB2/Neu, c-Myc and Id2

by

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For Sofia, who spent so many hours hearing the tapping of the computer keys, kicking at the same rhythm.

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Summary

For cells to proliferate, a certain size needs to be reached, which is mainly determined by the rate of translation. Thus, the constituents of the translational apparatus play an essential role in cell growth and proliferation. Because some of the products transcribed by pol III transcription are constituents of the translational apparatus, the rate of pol III transcription will affect cell growth rate. Mitogenic stimulation induces pol III activity [1], while differentiation represses pol III transcription [2].

The retinoblastoma protein (RB) has been implicated in repression of pol III transcription by inhibiting TFIIIB activity through binding of its hypophosphorylated form [3,4]. The oncoproteins ErbB2/neu, c-Myc and Id2 have been shown to block RB function by inducing its phosphorylation (ErbB2 and c-Myc) [5,6], or by binding and sequestrating it (Id2) [7,8]. These oncoproteins have been shown to be involved in the upregulation of cell proliferation, growth and the repression of differentiation, processes in which pol III transcription displays similar regulation. For this reason, their involvement in the regulation of pol III output was investigated. The data presented here demonstrate that all three oncoproteins upregulate pol III-transcribed genes. Although these oncoproteins can inhibit RB function, only ErbB2/neu seems to induce pol III activity disrupting TFIIIB-RB complexes. In addition, consitutive activation of ErbB2/neu also produces several other changes that can affect pol III transcription, including protein modifications of the TFIIIB subunits TBP and Brf, as well as induction of TBP, the pol III subunit BN51 and the transcription factor c-Myc. Furthermore, it was demonstrated that by inhibiting expression of c-Myc, the stimulation of pol III activity by ErbB2/neu could be overriden. Together these results suggest that constitutive activation of ErbB2/neu increases pol III transcription through several mechanisms, though induction of c-Myc appears to play an essential role in this upregulation.

On the other hand, the activation of pol III transcription by c-Myc and Id2 is through an RB-independent mechanism, since their overexpression in the absence

of the entire RB family, still produced pol III upregulation. Instead, they activate directly pol III transcription. Chromatin immunoprecipitation revealed that both endogenous c-Myc and Id2 are present at class III genes in cultured mammalian cells. c-Myc and Id2 induction of pol III activity appears to be through direct mechanisms by interaction of these proteins with TFIIIB. In the case of c-Myc, coimmunoprecipitations demonstrated that it interacts with TFIIIB at physiological ratios. Furthermore, both endogenous c-Myc as well as endogenous Id2 were found to cofractionate with samples containing TFIIIB activity prepared on several columns, such as MonoQ gradients. From these results it can be concluded that endogenous c-Myc binds stably and specifically to TFIIIB. Further analysis has to be performed for to confirm the interaction between Id2 with TFIIIB.

In summary, this work has identified three oncogene products that can increase pol III transcriptional output. These findings have important implications for tumour development in a range of tissue types.

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Natividad Gomez-Roman, Carla Grandori, Robert N. Eisenmann and Robert J. White. Direct activation of RNA Polymerase III Transcription by c-Myc. *Nature*, 421; 291-294, 2003.

Felton-Edkins ZA, Kenetli NS, Brown TR, Daly NL, Gomez-Roman N, Grandori C, Eisenmann RN and White RJ. Direct regulation by RNA polymerase III by RB, p53 and c-Myc. *Cell Cycle*, 3; 181-184, 2003.

Abbreviations

βgal	β-galactosidase
A	Adenine
Arg	Arginine
ARPP-P0	Acidic Ribosomal Phosphoprotein P0
b	basic
bp	base pairs
Brf	TFIIB-related factor
BSA	Bovine Serum Albumin
CAT	Chloramphenicol transferase
cdk	cyclin-dependent kinase
CHO	Chinese hamster ovarian cells
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DSE	Distal Sequence Element
EBERs	Epstein-Barr virus encoded RNAs
EBV	Epstein-Barr virus
EGF	Epidermal Growth Factor

ER	Estrogen Receptor
G1	Gap 1
G2	Gap 2
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
Glu	Glutamate
Gly	Glycine
GST	Glutathione-S-Transferase
HA	Haemagglutinin
HATs	Histone Acetyl Transferase
HBV	Hepatitis B Virus
HPV	Human Papilloma Virus
HTLV-1	Human T-cell leukaemia virus type 1
HDACs	Histone Deacetylases
Her	Herregulins
HLH	Helix-loop-helix
HPV	Human Papilloma Virus
Id	Inhibitor of differentiation
IE	Intermediate Element
Ile	Isoleucine
Leu	Leucine
LZ	Leucine Zipper
M	Mitosis
MAPK	Mitogen Activated Protein Kinase
Max	Myc Associated X protein

MEFs	Mouse Embryo Fibroblasts
MBI	Myc-box I
MBII	Myc-box II
mRNA	messenger RNA
MUC4/SMC	mucin/sialomucin complex
NAF	Neuregulin Activating Factor
NRG	Neuregulin
nt	nucleotide
OHT	Hydroxytamoxifen
P	Phosphate group
PKB	Protein Kinase B
PKC	Protein Kinase C
PI3K	Phosphatidyl Inositol-3-Kinase
Pol	RNA Polymerase
PSE	Proximal Sequence Element
PTF	Proximal Sequence Element Transcription Factor
Py	Poliomavirus
NAF	Neu Activating Factor
NRG	Neuregulins
nt	nucleotide
RB	Retinoblastoma
RNA	Ribonucleic Acid
rRNA	ribosomal RNA
RNase-P	Ribonuclease P

S	Synthesis
SDS	Sodium Dodecyl Sulphate
SINEs	Short Interspersed Elements
snRNA	small nuclear RNA
SV40	Simian Virus 40
T	Thymidine
TAD	Transactivation Domain
TAFs	TBP-Associated Factors
TBP	TATA-Box Protein
TF	Transcription Factor
TKO	Triple Knockout
Tn	Termination
tRNA	transfer RNA
TRRAP	TRansactivation/tRansformation-domain Associated Protein
Tyr	Tyrosine
Val	Valine
YY1	Ying Yang-1

1 Introduction

For a cell to express its genome, it needs to transcribe the message encoded in its DNA to molecules that will perform the functions required for life. To achieve this, the genes are converted (or transcribed) into RNA, in a process called transcription, which is then converted (or translated) into protein or employed as ribosomal or transfer RNA. The work described in this thesis concerns the transcriptional activity of eukaryotic cells, particularly that performed by RNA polymerase III.

Transcription is the process where one strand of DNA is copied by complementary base pairing into one strand of RNA. The RNA sequence is complementary to the template strand, which provides the template for its synthesis, and identical to the coding strand. RNA polymerase (pol) catalyzes the synthesis of RNA. Transcription starts when the enzyme is brought to a region in the DNA called the promoter, and is positioned at the start of the gene. From this point, called the startsite (or +1), RNA polymerase binds the double helix of DNA, separating the strands. The template strand is now available for base pairing with ribonucleotides and the enzyme synthesizes nucleotide bonds in RNA. The DNA helix is unwound as the enzyme moves along the gene, covalently adding nucleotides to the 3' end of the growing RNA chain, forming an RNA-DNA hybrid in the unwound region. This process continues until a terminator sequence is reached. At this sequence, no further bases are added to the RNA chain, the RNA-DNA hybrid is disrupted and both the enzyme and the RNA are released [9]. This is the general mechanism by which transcription is performed.

The work done in this thesis concerns transcription by RNA polymerase III (pol III). Its characteristics are going to be discussed in detail in the following sections. Pol III products are essential components of the protein synthetic machinery and therefore are essential for viability [10]. Its activity is controlled by a number of proteins involved in cell cycle regulation, like the tumour suppressors p53 and retinoblastoma (RB), by signal transduction pathways like the Ras signalling path-

way and by viral proteins [11]. These proteins are common targets for deregulation in carcinogenesis, which might suggest a tight link between pol III transcription and cancer. Indeed, this appears to be the case since transformed and tumour cells show increased pol III transcriptional activity [12].

In particular, the work presented in this thesis studies the regulation of pol III transcription by three different oncoproteins, ErbB2/Her-2, c-Myc and Id2. These proteins were chosen due to their relevance to cell transformation and oncogenesis, and the cellular changes they induce, like cell growth, where pol III transcriptional activation plays an important role.

The tyrosine kinase receptor ErbB2 is overexpressed in different types of carcinomas (e.g. breast, ovary, stomach, colon and kidney) and is associated with poor prognosis [13]. Its activation causes induction of several signalling cascades that affect gene expression and induce cell proliferation [13]. The third and fourth chapters address ErbB2 regulation of pol III transcription.

c-Myc is another oncoprotein with particular importance in cancer, as its deregulation is considered to contribute to one seventh of U. S. cancer deaths [14]. In normal cells, it is involved in cell cycle and cell growth control [15]. It has been shown to affect RB phosphorylation status and thus it might affect pol III transcription activity. Chapters five and six address c-Myc's regulation of pol III transcription.

And finally, pol III transcriptional regulation by Id2 is addressed in chapter seven. This protein is also deregulated in some cancers. It can bind to and inactivate RB [16], which leaves an open window for pol III activation.

In this first chapter, pol III transcription, the proteins involved in it and its regulation by different factors are going to be described. The following chapters will contain a brief introduction to each oncoprotein and the results obtained concerning their participation in the regulation of pol III transcription.

1.1 Transcription in Eukaryotes

Prokaryotes (organisms that lack nuclei and membranous subcellular organelles) contain one RNA polymerase (pol); eukaryotes (organisms that contain a membrane-enclosed nucleus encapsulating their DNA, as well as membranous subcellular organelles) require three pols to transcribe their nuclear genes: pol I, pol II and pol III [9]. There is a fourth pol in the mitochondria, required for the transcription of mitochondrial genes. However, only nuclear pols are going to be discussed.

Pol I transcribes class I genes, encoding 5.8S, 18S and 28S ribosomal RNAs (rRNAs), from a single type of promoter. rRNA is synthesized at the nucleoli,

where tandem arrays of genes specifying rRNA condenses. In these sites, rRNA is synthesized as well as processed and assembled into immature ribosomes. Then they are exported to the cytosol where the assembly is completed [17]. rRNA genes are contained in repeated clusters with mutliple copies of the genes specifying the 18S, 5.8S and 28S rRNAs invariably arranged in this order; and are separated by intergenic spacers, where the promoter lies. The core promoter lies ~50 base pairs (bp) upstream[1] of the startpoint; however its efficiency is quite low. This efficiency is very much increased by the presence of the upstream promoter element, 150-200 bp upstream of +1. Pol I, as well as pols II and III, lacks specificity for particular DNA sequences, so to initiate transcription accurately and specifically, it requires the assistance of other proteins called transcription factors. UBF1 binds to a GC -rich element in the core promoter and upstream promoter element; then the factor SL1 is recruited by UBF1 and extends the region of DNA that is covered. Finally, these factors recruit pol I to the core promoter and transcription is initiated [18].

Pol II transcribes class II genes encoding messenger RNA (mRNA), which is translated into protein, and almost all small nuclear RNAs (snRNAs) [19]. Transcription by pol II occurs in the nucleoplasm. Usually, there is a single copy of any particular class II genes in an organism's haploid genome [17]. In general, their promoters contain an initiator element, composed of the first base of mRNA being A surrounded by pyrimidines at position -3 and +5, and a TATA box located ~25 bp upstream of +1, composed of an 8 bp A/T rich consensus [9]. As for pol I, pol II requires transcription factors to start transcription. There are around six basal transcription factors involved in initiation of pol II transcription. The order of association at pol II promoters of the transcription factors is as follows: TFIID, TFIIA, TFIIB, TFIIF, which is bound to pol II bringing it to the promoter, TFIIE, and TFIIH [19]. All these proteins constitute the basal apparatus required for pol II transcription.

1.2 RNA Polymerase III Transcription

Pol III transcribes class III genes which encode transfer RNA (tRNA), 5S rRNA, 7SL, 7SK and a variety of other small cellular RNAs like U6 snRNA and viral VA RNA [10]. Pol III transcripts account for 5-10% of all nuclear transcription [20]. The transcripts have the common feature of being short (<300 bases) and not translated.

[1] By convention, if a sequence precedes the startpoint, it is referred to as upstream of it; when it is after the startpoint (in the transcribed region), it is referred to as downstream of it. When bases positions are numbered, the startpoint has the value of +1 assigned; positive numbers increase downstream while negative numbers increase upstream.

Recently, by confocal and electron microscopy of HeLa cells' nuclei, it has been discovered that pol III transcription occurs at discrete locations (~2000 sites) in the nucleoplasm [2] [20]. These sites are specific for pol III, and each site has a diameter of ~40 nm with approximately five active polymerases [20]. To comprehend the results obtained in this thesis, the characteristics of pol III transcription have to be explained in greater detail. Pol III transcription is going to be divided into the following subsections: class III genes, promoters, transcription factors, pol III and transcription. The third and fourth sections will describe how it is regulated by different factors.

1.2.1 Class III Genes

Several pol III products are involved in RNA processing which is paramount for a cell to perform its functions. 5S rRNA is an essential component of the ribosomal large subunit [9]. It is 120 nucleotides long and like other rRNA genes, there are multiple copies of 5S rRNA arranged in clusters. These clusters consist of a 5S rRNA gene followed by an untranscribed pseudogene and a spacer, respectively [17]. In the human genome, 200-300 5S genes are found in tandem arrays, the largest of which has been mapped to chromosome one [22].

Another group of pol III products are transfer RNAs (tRNAs). They serve as translators between the triplet nucleic acid code of mRNA and the amino acid sequence of protein, by recognizing the sequence in mRNA and bringing with it the correspondent amino acid for the synthesis of the peptide chain [9]. There is at least one tRNA per amino acid. tRNAs are small molecules of 74-95 bases long, and have secondary structure as a cloverleaf [9]. In the human genome, there are ~497 tRNA genes which are dispersed throughout the genome. However, more than 25% of these genes cluster at chromosome 6; smaller clusters appear on chromosome 7 and 1, and other chromosomes contain fewer than 10 tRNA genes each (except chromosomes 22 and Y which don't contain any) [23].

7SL RNA is also transcribed by pol III. It is an essential component of the signal recognition particle, involved in protein translocation across the endoplasmic reticulum [24]. It is 305 bases long [25] and provides the structural backbone of the signal recognition particle; without 7SL, the other constituents of the signal recognition particle (proteins) cannot assemble [26].

A fourth pol III product, 7SK, until recently, had no known function. Bensaude and Zhou's groups found that it can modulate pol II activity by binding to and

[2] Pol II "factories" have also been detected in the nucleoplasm; in HeLa cells there are ~8000 of them [21].

inhibiting the activity of P-TEFb, which is responsible for the phosphorylation of pol II's carboxyl-terminal domain that is required for activation of transcription [27,28]. 7SK is transcribed from a single gene mapped to chromosome 6 [23].

Contrasting with other spliceosomal RNAs, which are transcribed by pol II, U6 small nuclear RNA (snRNA) is also a pol III product [19]. It is involved in splicing introns of pre-mRNA [9]. There are multiple copies of U6 snRNA in the human genome (44) dispersed in chromosome 6 [23].

Another group of class III genes are the short interspersed elements or SINEs. The SINEs are repetititive sequences that occur throughout the DNA in segments of 100 to several thousand bp, interspersed with larger blocks of unique DNA [17]. 13% of the draft human genome are SINEs, composed of three families: the active Alu (comprising 10% of the 13% mentioned above) and the inactive MIR and MIR3 [23]. Alus are approximately 300 bp in length [29]. In rodents' genome, the active SINE B1 is analogous to human Alu [17,19]. Rodents also have an abundant SINE called B2 that is rare in humans. Their promoter regions are derived from tRNAs sequences (B2, MIR and MIR3) or 7SL sequences (Alu and B1) [23]. SINEs are often described as junk DNA; however, this conception has to be reconsidered. A recent report by Ferrigno et. al. has shown that B2 also contains an active pol II promoter outside the tRNA-derived region. With this promoter it regulates transcription of mouse Lama3, a class II gene, thus providing mobile pol II promoters [30]. Furthermore, repetitive elements also provide information on genome evolution [23].

Like pol II products, pol III products undergo processing. tRNAs require three processing steps for their maturation: 5' end processing by the ribonuclease P (RNase P), 5' and 3' end processing by the human phosphoprotein La, and intron excision by endonuclease- and ligase-mediated splicing [31]. The RNA subunit of RNase P is transcribed by pol III from the RPR1 gene. This RNA comprises the catalytic activity of RNase P, while protein subunits account for its structure [32].

Pol III also trancribes different viral genes like adenovirus VA1, or Epstein-Barr virus (EBV) encoded RNAs (EBERs) [10]. However, the bulk of pol III products are represented by the genes mentioned in the paragraphs above.

1.2.2 Promoters

Most class III genes' promoters have the characteristic of being localized downstream of the start site, within the transcribed region [10]. The promoters are composed of discontinuous internal elements or blocks within the gene [18]. Internal elements show a considerable degree of sequence conservation. There are three general types

of class III promoters, depending on the blocks present in them. Type I and II lie downstream of +1, while type III contains upstream sequences [10]:

Type I promoters contain three internal regions: an A-block followed by an intermediate element and a C-block (Fig. 1.1). The space between the blocks is quite constant, transcription being affected if it is changed [33]. The transcription factor (TF) IIIA recognizes the blocks and initiates recruitment of the pol III transcription apparatus [34, 35]. These promoters are found only in 5S rRNA genes.

Type II promoters contain two internal regions: the same A-block as in type I and a B-block (Fig. 11.1) [36]. In this case, the A-block is much closer to +1 [37]. TFIIIC is required for direct promoter recognition, which nucleates complex assembly [10, 38]. This is the most common promoter used by pol III, found in tRNA genes, the adenovirus VA genes and the SINEs. Some tRNA genes contain short introns within their coding regions [39, 40], so the location of the B-block can be extremely variable [41].

In contrast to type I and II promoters, type III promoters are external. They are composed of three external elements: a distal sequence element (DSE), recognized by Oct-1 and Staf; a proximal sequence element (PSE) recognized by SNAPc/PTF (PSE-associated TF); and a TATA-box bound by TFIIIB which confers pol III specificity [3] (Fig. 1.1) [42, 43]. Human and mouse U6 and 7SK genes contain this promoter (although some active U6 genes contain type II promoters) [42]. *Saccharomyces cerevisiae* U6 genes lack the DSE and PSE, but contain the A and B-blocks, the latter being in a unique location 100 bp downstream of the transcribed region [44].

Some class III genes with type I and II promoters also contain sequences upstream of +1 that are required for efficient transcription. These sequences are not conserved between organisms nor between different pol III-transcribed genes [45]. For example, *Drosophila's* tRNA genes [46] and *Schizosaccharomyces pombe* class III genes [45] require a TATA-box like element between -25 and -30 bp. Moreover, tRNA genes in *Caenorhabditis elegans* [47], *Neurospora crassa* [48], the silkworm $tRNA^{Ala}$ [49] and human 7SL [50] need upstream sequences for their transcription.

[3] The U class spliceosomal RNA genes share the same promoter organization and can be transcribed by pol II or pol III. The discrimination between pol II/pol III is given by the TATA-box. If a TATA-box is present, pol III transcribes it; if there is none, transcription is performed by pol II [42].

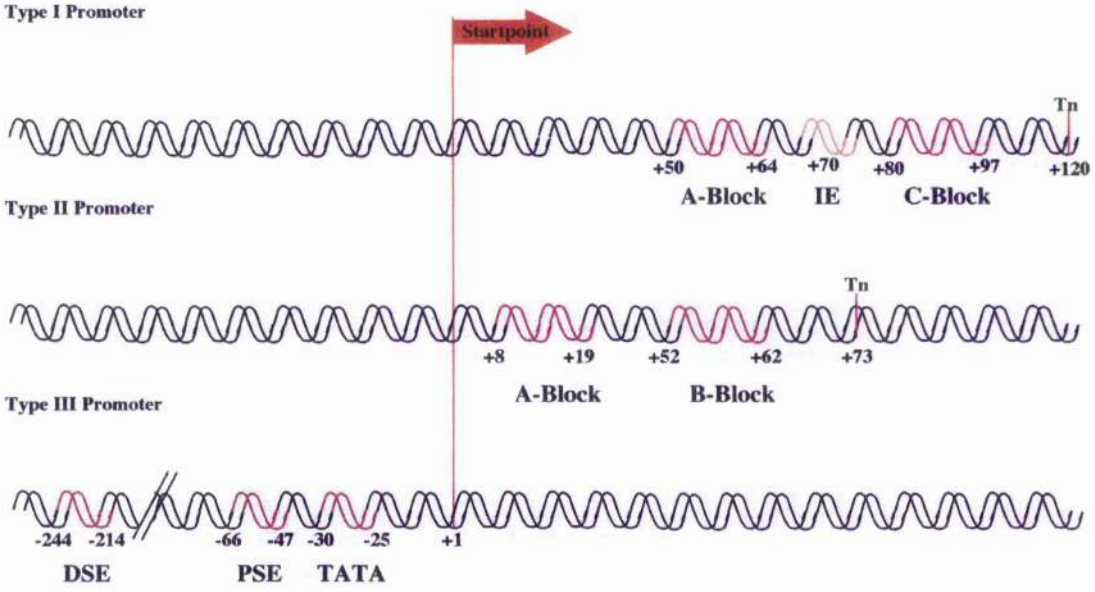

Figure 1.1: Structure of the three general types of promoters used by pol III. Representing type I is the *Xenopus* somatic 5S rRNA genes, type II is from *Saccharomyces* SUP4 tRNA gene and type III is the human U6 snRNA gene. IE stands for intermediate element, PSE is proximal sequence element and DSE is distal sequence element. The site of termination is indicated by Tn. Adapted from ref. [10].

1.2.3 Transcription Factors

Pol III lacks specificity for any particular sequence of DNA, like other pols. To initiate transcription accurately and specifically, pol III needs the assistance of other proteins to position it on the appropriate start site of class III genes. Each type of promoter has its own requirement for different transcription factors. TFIIIB and TFIIIC are required for type I and II promoters; the former also needs TFIIIA [10]. Type III promoters utilise SNAPc/PTF, Oct-1, TFIIIC1 but not TFIIIC2, and a TFIIIB that is separable chromatographically from the form used by types I and II [42].

TFIIIA

Xenopus laevis TFIIIA was the first eukaryotic TF to be purified to homogeneity [51] and cloned [52, 53]. The nine zinc fingers present in TFIIIA recognise and bind to >50 bp of 5S rDNA. Three fingers (fingers 1-3) in the N-terminal domain recognise the C-block and wrap around the major groove (this interaction contributes ~95% of the total binding energy of full-length TFIIIA) [18]. The A-block is recognised by fingers 7-9 and the middle three fingers (4-6) bind to the interblock region of DNA [10]. TFIIIA is quite divergent between species, sharing similarity only at the zinc finger domains [18]. It is the first protein that recognizes and binds to 5S rDNA and then recruits TFIIIC to the promoter [54].

TFIIIC

Type II promoters are recognized by TFIIIC. In *S. cerevisiae*, TFIIIC or τ is composed of six subunits, TFC1/τ95, TFC3/τ138, TFC4/τ131, TFC6/τ90, TFC7/τ55 and TFC8/τ60, with an aggregate mass of 520 kDa. The subunits arrange in two globular domains: τ_A, which is composed of TFC1, TFC4 and TFC7 and recognizes the A-block, and τ_B, which is composed of TFC3, TFC6 and TFC8 and recognizes the B-block [38, 55, 56]. TFC8 serves as a linker between the two domains. Electron microscopy suggests that TFC8 stretches so that TFIIIC can contact both blocks. However, when the interblock DNA is too long, TFIIIC can loop out the DNA in between to be able to bind both blocks [55].

Human TFIIIC can be separated by ion exchange or sequence-specific DNA affinity chromatography into two components, TFIIIC1 and TFIIIC2 [57, 58]. TFIIIC1 is generally required for transcription of all class III genes whereas TFIIIC2 is only required for class III genes containing type I and type II promoters [59]. A third component, TFIIIC0, has been purified from a phosphocellulose C fraction (fraction with TFIIIC activity purified from HeLa nuclear extracts) [59]. Footprint analyses

showed that TFIIIC0 binds to the termination region of several class III genes and is partially able to substitute for TFIIIC1 on type II promoters *in vitro* [59]. TFIIIC1 enhances the binding of TFIIIC2, TFIIIA and PTF, and is required for transcriptional activity [59]. It binds after TFIIIC2 is already bound to the promoter [60].

As mentioned before, the initial recognition of type II promoters is performed by TFIIIC2, through the B-block. When bound, it recruits TFIIIC1 and TFIIIB [60]. TFIIIC2 is composed of five subunits, TFIIIC220/TFIIICα, TFIIIC110/TFIIICβ, TFIIIC102/TFIIICγ, TFIIIC90/TFIIICδ, and TFIIIC63/TFIIICε (the number corresponds to molecular weight in kDa) [61–64]. TFIIIC2 can be present in two forms: TFIIIC2a, which is transcriptionally active and TFIIIC2b, which lacks the TFIIICβ subunit, and is transcriptionally inactive [65]. Moreover, all the subunits are absolutely required for pol III transcription of class III genes containing type I and II promoters [10]. There is no homology of TFIIICα, and TFIIICδ with any of the yeast TFIIIC subunits, which seems quite striking since the sequences in A- and B-blocks are well conserved between species [63, 66, 67]. Three subunits, TFIIIC220, TFIIIC110 and TFIIIC90, display histone acetyltransferase activity (HAT), relieving chromatin-mediated repression of pol III transcription [68].

Crosslinking experiments have shown that TFIIIC220 is responsible for the binding to the B-block [66]. Its N-terminal domain associates with TFIIIC110 while the C-terminal domain associates with TFIIIC102 and TFIIIC63 (Fig. 1.2) [69]. The TFIIIC220 gene is located at chromosome 16.

Sinn et al, using a cDNA library from a human immature myeloid cell line, isolated the second largest TFIIIC subunit TFIIIC110 [67]. PSI-BLAST revealed that *S. cerevisiae* TFC6 exhibits sequence homology to human TFIIIC110 [70]. Its gene maps to chromosome 2. TFIIIC2b, although transcriptionally inactive, can still bind to DNA, demonstrating that TFIIIC110 is not required for DNA recognition [65].

TFIIIC102 has the highest homology of all the human TFIIIC subunits with its yeast counterpart TFC4 subunit (31% identical) [62]. Its gene is localized at chromosome 9. Its amino terminal domain contains acidic regions, 11 copies of a tetratricopeptide repeat (TPR) and a helix-loop-helix motif [62]. Through its TPRs it interacts with TFIIIC63 and TFIIIC90 [62]. The TPRs also recruit TFIIIB to the promoter by interacting principally with Brf, but also with Bdp1 (Fig. 1.2) [71–73]. In yeast TFC4, the region responsible for the interaction with Brf maps to the first 168 residues [74]. In addition, two-hybrid screens have demonstrated an interaction between TPRs and the pol III subunits C53 and ABC10α [75, 76].

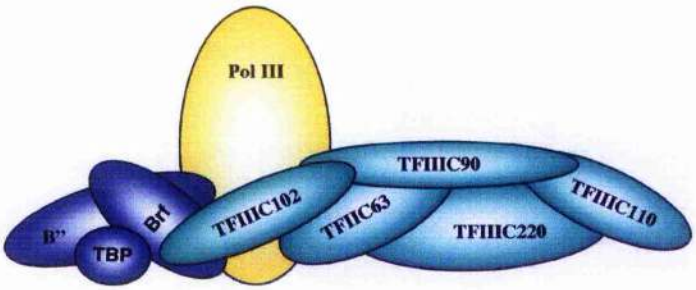

Figure 1.2: Representation of a network of interactions between the different subunits of human TFIIIC (green) and TFIIIB (blue). The different subunits of pol III (in yellow) have been ommitted for simplification, however the text mentions which subunits interact with TFIIIC.

Human TFIIIC90 was cloned by screening human cDNA cell libraries using degenerate PCR primers [63]. It specifically acetylates K14 on histone H3 [68]. Direct interactions with TFIIIC90 in vitro have been seen with pol III subunit C39 (yC34), TFIIIB subunit Brf and TFIIIC subunits 220, 110 and 63 [63]. It can be seen as the linker between the subunits 220-110 that lie on the B-block and 63-102 that lie on A-block and upstream sequences and the homologue to *S. cerevisiae* Tfc8 (Fig. 1.2). The TFIIIC90 gene maps at chromosome 9.

The smallest TFIIIC subunit, TFIIIC63, is homologous to *S. cerevisiae* TFC1 (22% identical) [62]. Its yeast counterpart is responsible for the recognition of the A-Block [77]. The human TFIIIC63 gene is also localized at chromosome 9. As mentioned before, TFIIIC63 interacts with TFIIIC102, TFIIIC90 and with pol III subunit C62 [62]. The protein contains a central helix-turn-helix motif and a C-terminal acidic region [62].

There is not much information concerning the regulation of TFIIIC genes. However, it is known that several conditions, e.g. viral infection or transformation, can induce protein levels and TFIIIC activity. This regulation is going to be addressed in further sections.

TFIIIB

Once TFIIIC has assembled on the promoter, it recruits TFIIIB. In *S. cerevisiae*, TFIIIB is composed of three subunits, the TATA-binding protein (TBP) and two TBP-associated factors, Brf and B". TBP and Brf form a very stable complex called B', while B" is less tightly associated and can be separated chromatographically [78]. Human TFIIIB also contains TBP, Brf and B", and might contain other unidentified

components as well [18].

TBP is a general factor of 38kDa (human) required for transcription by all three nuclear pols [79–82]. Its C-terminal domain is very highly conserved between species (80% identical between yeast and human), while the N-terminal regions are more species specific [56]. The TBP residues involved in the interaction with Brf are located in the N-proximal TFIIA-binding lobe [83]. TBP-Brf complexes are found even if they are not bound to DNA. A general feature of TBP is that it sharply bends DNA; the TBP-Brf complex also bends DNA. Brf must pass accross the concave underside of the TBP-DNA complex in order to connect with sites of TBP lying on opposite sides of bent DNA [54]. In type III promoters, TBP binds to the TATA-box independently of Brf or B" [42]. Brf increases the affinity of binding between TBP-DNA complexes [84].

In promoters that lack a TATA sequence (type I and II), TFIIIB is recruited to the DNA through protein-protein interactions with TFIIIC [60]. The yeast subunit TFC4 (human TFIIIC102) is responsible for this recruitment, by interacting mainly with Brf, but also with B" [62, 71–74]. Brf (or TFIIB-related factor) received this name because of its substantial homology to TFIIB, a basal factor that is essential in transcription by pol II. It is 23% identical and 44% similar to TFIIB in its N-terminal 320 residues [85–87]. Yeast Brf (yBrf) is also referred to as TFIIIB70 since it has a molecular weight of 70 kDa [85]. Human Brf (hBrf) is a 90 kDa protein, of which its N-terminal domain is 24% and 41% identical to human and *S. cerevisiae* TFIIB, respectively [88]. Its C-terminal domain is moderately conserved [88]. TFIIB interacts with TBP; since a TFIIB-like region involved in this interaction is present in Brf, it was expected that this region was responsible for binding between Brf and TBP. Although a weak interaction is observed at this region [89–91], the principal Brf region that regulates binding to TBP is at the less conserved C-terminal domain [83, 90–92]. Brf thus contains two separate TBP-binding domains, allowing the interaction with opposite sites of the TBP-DNA complex [83,90,91]. The C-terminal domain also interacts with pol III and B" [93]. Brf recruits pol III by interacting with pol III's subunit yeastC34/humanC39 [91, 93, 94]. However, effective pol III recruitment to double-stranded DNA requires the entire TFIIIB-DNA complex [95]. The conserved N-terminal domain of Brf, like that of TFIIB, is comprised of a zinc-binding region followed by a conserved block of ~15 residues [96]. This zinc-binding region is not required for pol III recruitment, but its disruption causes a defect in open complex formation [96]. The B'-TFIIIC-DNA complex generates a DNase I footprint that covers the start site of transcription and extends further upstream and

stabilizes binding of TFIIIC to the A-Block [74,97]. So, Brf can be seen as the glue between TBP and B" holding TFIIIB together, as the key for TFIIIB recruitment to TATA-less promoters by interacting with TFIIIC, and as the recruiter of pol III to class III genes.

Once the B'-DNA complex is formed, the last component of TFIIIB, B" binds to it. The entry of B" changes the physical properties of the TFIIIB-TFIIIC-DNA complex [98]. Yeast B" (or TFIIIB90) is a 90 kDa protein with a SANT domain (motif associated with protein-protein and protein-nucleic acid interactions) [99]. The SANT domain is quite conserved among putative homologues (*S. cerevisiae*, *S. pombe*, *Drosophila melanogaster*, and *C. elegans*) and helped to find the human homologue by homology-based PCR [100]. This domain is associated with protein-protein interaction, as well as protein-DNA interactions. Human B" is 160 kDa and is absolutely required for pol III transcription [100]. It also receives the name of Bdp1. Three separate segments are required for viability in yeast: region I (aminoacids or aa 372 to 487 including the SANT domain), II (aa 312 to 269) and III (aa 263 to 158) [101]. In yeast, when B" is incorporated into a TFIIIB-DNA complex, region I and II undergo an extensive burial of surface, while region III is unfolded [98]. The first two regions are required for function and are where the interaction with the TFIIIB components and the TFIIIC subunit TFC4 occur; the third region is not essential for transcription in vitro [98]. As mentioned before, Brf interacts with yC34 of pol III. However the B'-TFIIIC-DNA complex is unable to stably incorporate pol III. B" entry to the complex causes the following changes [98]:

- Nuclease protection upstream of the start site is more complete and extended ~10 bp;
- The transcriptional start site becomes available to DNase I cleavage by the shifting of TFIIIC and presumably leaving this region clear for pol III binding;
- Photochemical cross-linking of DNA to TFC4 and TBP upstream of the transcriptional start site is diminished;
- B'-DNA complex becomes more resistant to high salt and polyanions, making the TFIIIB-DNA complex more tightly bound, and;
- Pol III can stably assemble over the start site of transcription.

All these changes allow pol III transcription to be achieved. A recent article appoints B" to be also involved in tRNA processing. As mentioned before, RNase P trims 5'

ends of pre-tRNA to generate mature tRNA [32], but how this complex is brought into contact with its substrate is not known. Ishiguro et. al. demonstrated that B" interacts directly with RNase P, suggesting the participation of B" in the recognition process [101].

In mammalian cells, TATA-containing promoters (type III) appear to use a variant of the TFIIIB complex. This TFIIIB is composed of TBP, B" and a Brf-related factor of 50 kDa referred to as BrfU [100]. Therefore, to distinguish the two different complexes, the complex composed of TBP, B" and BrfU has been termed TFIIIBα, and the complex composed of TBP, B" and Brf (Brf1) has been termed TFIIIBβ (for simplicity, TFIIIBβ will be referred here as TFIIIB) [100]. The N-terminal domain of BRFU is related to both hBrf and TFIIB, the C-terminal domain is divergent [100]. TBP, through its C-terminal core domain, recruits BrfU to the TATA-box of the snRNA U6 gene, by interacting with the repeat 2 of the BrfU core [102]. Depletion of BrfU debilitates transcription of U6 genes, but addition of recombinant BrfU cannot reconstitute transcription. This might suggest the existence of BrfU-associated polypeptides that are essential for transcription [100]. A different factor called Brf2 has been reported to be required for U6 transcription. Brf2 originates from an alternatively spliced Brf pre-mRNA. This protein lacks the Zn ribbon domain and the first repeat present in Brf and TFIIB [103]. Whether both BrfU and/or Brf2 are required for U6 transcription remains to be determined.

SNAPc/PTF and Oct-1

U6 and 7SK genes, that contain type III promoters, have different TF requirements than those from type I and II promoters. As mentioned before, they utilize TFIIIBα, and TFIIIC1 but not TFIIIC2. In addition to these TFs, the TFs SNAPc/PTF and Oct-1 are necessary for initiation of transcription [42], and a role for the TF Staf has also been described [104]. SNAPc/PTF recognizes and binds to the PSE [100, 105]. It is a complex of five subunits with approximate molecular masses of 190, 50, 45, 43, and 19 kDa. SNAP43 and 19 interact with the N-proximal one-third of SNAP190, while SNAP45 binds to the C-proximal region of SNAP190; SNAP50 is bound to SNAP43 [106]. Oct-1 binds to the octamer sequence of the DSE [107]. It contains activation domains as well as a POU domain. The POU domain is a bipartite DNA binding domain composed of two flexibly joined helix-turn-helix structures, the N-proximal POU-specific (POU_S) and the C-proximal POU homeodomain (POU_H) segments [108]. The SNAPc-DNA and Oct-1-DNA complexes are bound relatively weakly. Each complex is stabilized by interacting cooperatively with one another, and this interaction is mediated by the C-proximal

half of SNAP190 (which occludes the DNA-binding N-proximal domain) and the POU_S domain of Oct-1 [106, 109, 110]. TBP binding to DNA is also regulated by SNAPc. The N-proximal leader domain of hTBP blocks binding to its TATA site. The SNAPc-190_N-43-50 subcomplex interacts with this TBP region, relieving the autoinhibition and allowing TBP binding to the TATA-box. TBP then recruits BRFU and B" [111].

1.2.4 RNA Polymerase III

In yeast, pol III is composed of 17 subunits; 14 homologuous subunits have been found in human [56]. Some subunits are shared by pol I (referred to as A), pol II (referred to as B) and pol III (referred to as C); others have homology to subunits present in other pols and some are specific to each enzyme. Of the 17 subunits of pol III in *S. cerevisiae*, ABC10α, ABC 10β, ABC14.5, ABC23 and ABC27 are shared by all three pols, AC19 and AC40 are shared with pol I, C160, C128, C25 and C11 are homologous to either pol I or II, and C82, C53, C37, C34, C31 and C17 are unique to pol III [19, 56]. The core is composed of five subunits: C160, C128, AC40, AC19 and ABC23 (shown in dark yellow in Fig. 1.3). Most of the unique subunits (except C37), in addition to ABC10α, appear to function in the recognition of the TFIIIC-TFIIIB-DNA initiation complex and pol III recruitment to its promoters (Fig. 1.3) [45]. Specifically, two hybrid experiments have shown that the subunits C34 and C17 interact with the TFIIIB subunit Brf, while ABC10α and C53 (human ortholog BN51) interact with the TFC4 (TFIIIC102 in humans) subunit of TFIIIC that lies over the transcriptional start site (Fig. 1.3) [75,76]. C34 forms a sub-complex with C31 and C82 (represented in orange in Fig. 1.3). The whole sub-complex is important for initiation, since a pol III mutant lacking it is unable to initiate transcription specifically [112].

A human holopol III has been prepared by immunoselection from extracts of HeLa cells with a FLAG-tagged gene for the C53 subunit. It contains TFIIIC2, TFIIIC1 (as judged by DNA footprint), and Brf, TBP and La protein [113–115]. In addition, nuclear factor 1, PC4 and topoisomerase I have been reported to be associated with TFIIIC2, although its relevance still remains to be determined.

1.2.5 Transcription

Initiation

Several steps are involved in the initiation of transcription by pol III. As mentioned before, the enzyme has to be recruited through protein-protein interactions to the

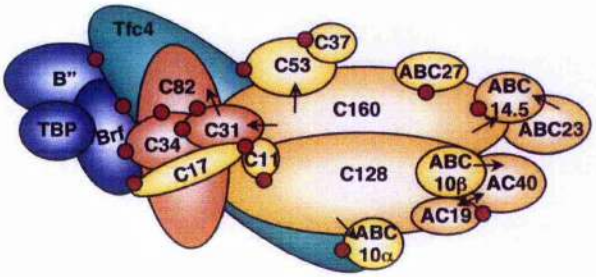

Figure 1.3: Network of interactions in *S. cerevisiae* pol III, TFIIIB and TFIIIC subunit Tfc4. Orange subunits represent a pol III sub-complex required for initiation; the core subunits are in dark yellow. Red dots mark interactions deduced by two-hybrid analysis and black arrows mark interaction inferred from multicopy suppression of temperature-sensitive mutations (the arrowhead pointing to the suppressed mutation (Adapted and edited from [45])

start site. In type III promoters, Oct-1 and SNAPc bind separately to its respective sequence, while TBP binds to the TATA-box. Then, TBP recruits the B" and BrfU and the latter recruits pol III to the start site. It is not known how TFIIIBα recruits pol III [42].

Pol III recruitment to TATA-less promoters is better characterized for the yeast tRNA and 5S rRNA genes. The steps involved are:

1. ***TFIIIC recognition and binding to the promoter.*** TFIIIC binds to the A- and B-Block (or in the case of 5S rRNA genes is brought by protein-protein interactions with TFIIIA which binds to the A and C-block) (Fig. 1.4, first panel). The location of the different TFIIIC subunits on the SUP4 tRNA gene has been found through photocrosslinking to chemical probes [38]. TFC3 and TFC6 interact around the B-Block ensuring primary binding to tDNA [116]. TFC1 and TFC7 bind in the vicinity of the A-Block on opposite sides of the DNA helix [38]. TFC8 does not appear to be bound to DNA and finally, TFC4 is cross-linked upstream of +1, in a region occupied by TFIIIB, and downstream between the A- and B-Block [38].

2. ***TFIIIC recruitment of TFIIIB.*** Once TFIIIC2 is bound to tDNA, it can recruit TFIIIB. First, Brf is brought to the DNA by interaction with TFC4 [71, 74,97,]. TBP is already bound to Brf, so it is brought by Brf, although TFC8

also participates in this recruitment [117]. This entry causes stabilization of the TFIIIC-DNA complex, increasing the cross-linking of TFC4 to upstream DNA [97]. Finally, once the B'-TFIIIC-DNA complex has formed, B" enters the complex. B" is recruited by TFC4 and Brf [73, 101]. As mentioned before, it increases stability of the complex and shifts TFIIIC leaving the DNA at the start site free for pol III binding [98]. The location that TFIIIB occupies in the tDNA is determined mainly by TFIIIC; however, TBP can also scan the region around -30 to choose an optimal site [118]. It occupies a region of ~40 bp immediately upstream of the transcription start site [54, 90, 119]. In the SUP4 tRNA gene, TBP cross-links in the minor groove at bp -30 to -20 in the coding strand and from -28 to -24 in the template strand [119]. B" and Brf are positioned on opposite sides of the TBP-DNA core (Fig. 1.4, panel 2) [119]. Brf binds the TBP-DNA complex through the C-terminal region, requiring 15 bp downstream of the TATA-like sequence and just 1 bp upstream of it [93]. The N-proximal segment of B" cross-links to the upstream end of the TFIIIB-DNA complex (approximately 8 bp of the TATA-like sequence), and the C-terminal domain is located downstream of the TATA-like sequence [120].

3. ***TFIIIB recruitment of Pol III.*** When the TFIIIC-TFIIIB-tDNA complex has formed, pol III is recruited to the start site (Fig. 1.4, panel 3). TFIIIB alone is responsible for pol III recruitment to the start site. This was demonstrated due to the extreme stability of the TFIIIB-DNA complex in yeast, since stringent conditions like high salt concentrations (1 M KCl) or polyanions (heparin) cannot dissociate the complex, while TFIIIC and TFIIA are readily removed [121]. After exposure to these treatments, pol III can still be recruited to class III genes by TFIIIB[4] [121]. Pol III is recruited mainly through Brf-C34 (human ortholog C39) interaction, although Brf-C17 and TBP-C34 interactions have also been observed [112, 125, 126]. C53 (human ortholog BN51) might also be involved in pol III recruitment, through its interaction with TFC4, although this remains to be determined [76]. At the tDNA, C34 cross-links at the furthest upstream end (bp -17 and -3/-2) from the major groove [127], C31 and C82 lie just upstream (bp -3/-2) of the start site in vicinity of the non-transcribed strand [128], and C53 contacts it near the downstream end of the transcription complex [129].

4. ***DNA melting.*** Upon binding of pol III, the double-stranded DNA is melted

[4] In the human, the dissociation pathway is the reverse from that of the yeast. TFIIIB is lost first, whilst TFIIIC-DNA complexes are more stable [122–124].

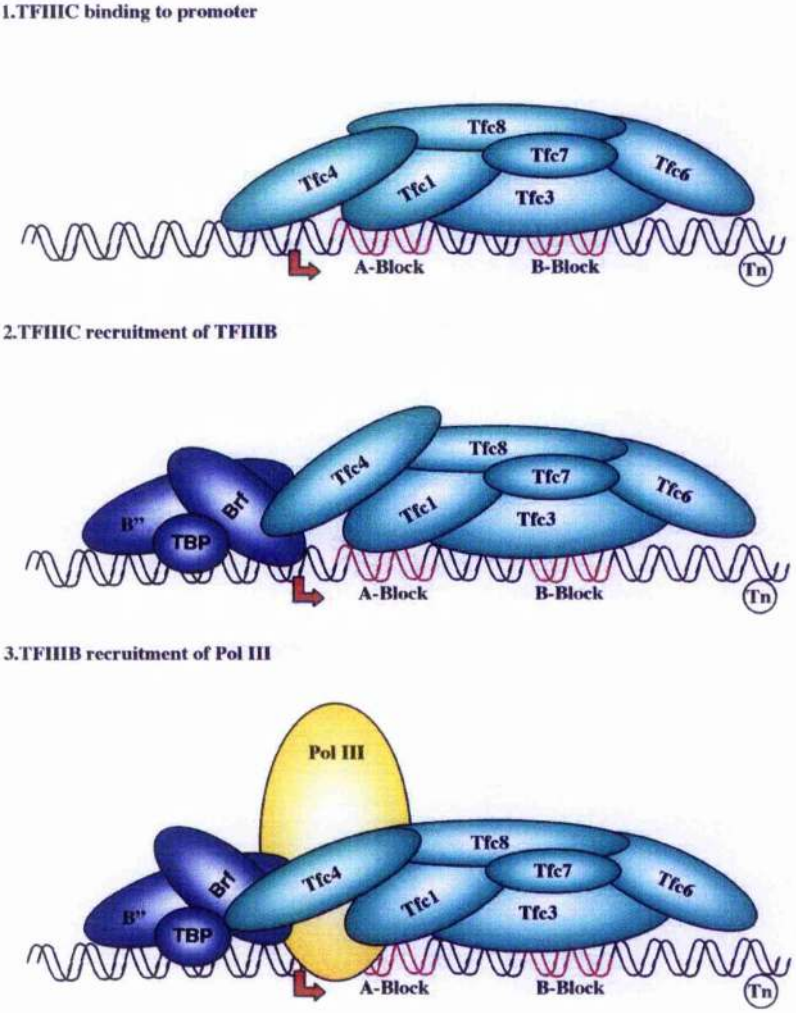

Figure 1.4: Assembly pathway used by genes containing type II promoters for initiation of transcription in *Saccharomyces cerevisiae*. TFIIIC recognises directly the sequences in the promoter and binds to it. Once bound, it recruits TFIIIB by protein-protein interaction, which is positioned upstream of +1. Finally, TFIIIB mediates pol III recruitment to the start site (+1). Adapted from ref. [10].

and a bubble forms which extends from -11 and propagates downstream of the start site. This separation occurs spontaneously, without ATP hydrolysis, unlike pol II's melting, which requires $\beta\gamma$ bond hydrolysis [130]. Brf and B" play a role in this process since certain mutations in these prevent the formation of a strand-separated open promoter [92]. B" is involved in unpairing of the upstream segment, while Brf unpairs DNA around the transcriptional start site [131]. The mechanism of action still remains to be determined.

Elongation, Termination and Reinitiation

Once the DNA is melted, pol III dissociates from the TFIIIB-DNA complex without significant pausing or arrest after several ribonucleotides have been incorporated [130, 132]. As it moves along the gene, the bubble of melted DNA moves with it [130]. TFIIIC and TFIIIA occupy regions that are going to be transcribed by the enzyme, but these potential obstacles do not seem to affect elongation of pol III transcripts. The presence of TFIIIC delays polymerase by just 0.2 s at a single site upstream of the B-Block making no difference in overall transcription rates (if pol III transcribes in the antisense direction, TFIIIC causes a pause of 9 s, while TFIIIB of >1 h) [133]. tRNA elongation in yeast at 20°C occurs at an average of ~20 nt/s; however, it does not proceed evenly, pausing at internal sites [134]. This pausing is associated with the subunits C11 and C128 [135, 136]. C11 affects repair of nucleotide misincorporation and the efficiency of termination, C128 is involved in catalyzing RNA cleavage, as well as C160 [135–137].

Pol III transcription generally terminates at a run of four or more thymidine (T) residues; the efficiency of termination is influenced by flanking sequence and increases with the length of the T-run [138–140]. Although pol III is sufficient for termination [140], additional factors have also been implicated in human cells. The phosphoprotein La may be required for release of RNA chains at the terminator and for execution of additional rounds of transcription [141, 142]. Moreover, the protein NF1 in conjunction with TFIIIC, is claimed to promote accurate termination and also reinitiation of transcription [143].

Since class III genes are small units, pol III never leaves the vicinity of the promoter, allowing multiple rounds of transcription to occur. The bending of DNA feature of pol III TFs facilitates pol III re-attachment to the same transcriptional unit. Subsequent cycles of transcription occur more rapidly (~35 s) than the first one (~5 min) [144].

1.3 Activities Regulating RNA Polymerase III Transcription

Since the genes transcribed by pol III, like tRNA and 5S rRNA, are an essential component of cellular metabolism and the translation machinery, the physiological relevance of regulating its transcription is readily appreciated. A substantial part of metabolic energy consumed by growing cells goes to the biogenesis of ribosomes; the activity of pol I, responsible for the transcription of large ribosomal RNA, is closely correlated with cell growth and proliferation. Since pol III products are also core components of ribosomes, it would be a waste of energy to regulate only pol I transcription and leave pol III activity unfettered. In fact, increasing amounts of data show that pol III transcription is strongly regulated by a variety of conditions, like cell growth, differentiation and proliferation, viruses, transformation and cancer [11, 145–147]. In no case of regulation has the polymerase itself been found to be controlled; instead the activities of TFIIIB and TFIIIC have been implicated. Due to its significance for the work produced for this thesis, the regulation of pol III transcription during cell growth and proliferation are going to be addressed in detail in another section.

Differentiation

In early mouse embryogenesis, pol III transcript levels are drastically reduced in the parietal endoderm [148]. Using F9 embryonal carcinoma cells, this reduction has been attributed to down-regulation of TFIIIB, through a decline in abundance of TBP and more dramatically of Brf as well as TFIIIC1 [2,149,150]. It is interesting to note that c-Myc's mRNA and protein levels decline and are virtually undetectable in differentiated F9 cells [151]. In addition, Id2 levels are also reduced by differentiation [152]. These data might suggest a link between these oncoproteins and pol III transcription.

Viruses

There is a lot of evidence showing that several viruses can stimulate pol III transcription [11]. Viruses often require an increased cellular biosynthetic capacity, which is in part achieved by stimulating pol III transcription. Moreover, some of them also contain genes transcribed by pol III [153]. These viruses, and/or their products, are able to transform cells and participate in the process of carcinogenesis [145]. Studies using cell lines transformed with viral oncoproteins have shown abnormally elevated levels of pol III transcripts [154, 155].

Some tumour viruses, like adenovirus and Simian Virus 40 (SV40), have similar mechanisms of increasing pol III transcription in HeLa cells or rodent fibroblasts, respectively. When human cells are infected by them, pol III transcripts like Alu elements [156,157], as well as tRNA, 5S rRNA and U6 snRNA are increased dramatically [158-160]. Adenovirus contains two class III genes, VAI and VAII [153]. These are synthesized at high levels during late stages of infection, and are required in subverting the host cell translational apparatus into synthesis of viral proteins [161]. Adenovirus protein E1A and the large T antigen of SV40 activate pol III transcription by increasing the availability of TFIIIC2a, through the overexpression of TFIIIC110 [67,159,160,162,163]. Moreover, SV40-transformed fibroblasts overproduce other subunits of TFIIIC2 [162]. Herpes simplex virus type 1 also activates pol III transcription by increasing the activity of TFIIIC [156,164].

Another mechanism of pol III's activation is by inactivating the retinoblastoma protein (RB). Adenovirus E1A and SV40 large T antigen bind to RB, relieving TFIIIB from RB's repression (explained further in the next section) [3,162,165]. Moreover, the viral protein E7 from human papillomavirus can also activate pol III transcription by binding and inactivating RB [162]. Furthermore, SV40 large T antigen can also interact with other components of the pol III preinitiation complex. Damania et al. have shown by coimmunoprecipitation and cofractionation that SV40 large T antigen binds to the TFIIIB subunit TBP [158]. In addition, the human T-cell leukaemia virus type 1 tax protein stimulates pol III transcription by increasing the effective concentration of active TFIIIB molecules [166].

Hepatitis B virus (HBV) utilises a different mechanism for inducing pol III transcription. Its oncoprotein X is essential for viral replication and is also implicated in the development of HBV-mediated carcinogenesis, since it can induce liver tumours in some transgenic mice [167]. It activates protein kinase C (PKC) and Ras-Raf-Mek-mitogen activated protein (MAP) kinase signal cascades [168]. All three classes of pol III promoters are transactivated by X. The mechanism involved appears to be through an increase in the cellular levels of TBP and therefore TFIIIB activity [169,170].Wang et. al. demonstrated that the increase in TBP and enhancement of pol III transcription are dependent on the ability of X to activate the Ras/Raf-1 signal transduction pathway [168]. In this study, they showed that if Ras activity is blocked by transient expression of a dominant-negative mutant Ras gene in a *Drosophila* stable cell line or by incubation with a Ras farnesylation inhibitor, both X-dependent activation of pol III transcribed genes and the increase in cellular TBP are blocked. The ErbB2 receptor can induce activation of the Ras-MAPK

pathway [13]. It will be interesting to study if ErbB2 uses a similar mechanism as HBV protein X to activate pol III transcription.

Transformation and cancer

The mechanisms by which viruses contribute to the formation of tumours may involve the deregulation of pol III transcription. Some viral proteins have the feature of transforming cells when transfected. As described above, transformed cell lines with viral oncoproteins express abnormally elevated levels of pol III transcripts. Indeed, transformed clones that are more malignant in nude mice display the highest abundance of pol III products [160]. As well, cell lines transformed with chemical carcinogens (e.g. the tumour-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate) express high levels of pol III products [10]. Moreover, different types of cancers, like breast carcinoma, colon adenocarcinoma and skin fibrosarcoma, display an increase of pol III transcripts compared to the surrounding tissue [171, 172]. A link between pol III activation and transformation is also suggested by experiments using two fibroblast lines transformed with temperature-sensitive mutants of the SV40 large T antigen. At the non-permissive temperature, pol III gets downregulated and the cells revert to normal morphology and phenotype [154].

At the moment, there is little information on how pol III transcription gets deregulated *in vivo*. Using ovarian tumors, Winter et. al. found that the activation of pol III transcription is due to an increase of TFIIIC activity [12]. These data provide the first direct evidence of a pol III TF being deregulated in malignant human cells. In addition, the group of Grzeschik determined the physical localization in the human genome of the TFs involved in pol III transcription. They discovered, by chromosomal in situ hybridization, radiation hybrids and somatic cell hybrid analysis, that the genes for SNAPc1, SNAPc2 and a TFIIIB-associated protein (GTF3BAP2), are localized within chromosomal regions where recurrent, cytogenetically detectable mutations are seen in specific neoplasias, such as carcinoma of the salivary glands and hemangiopericytoma, or where mutations causing inherited genetic diseases map, such as Peutz-Jeghers syndrome, making them interesting candidates for involvement in these pathologies [173].

Finally, products transcribed by pol III have been shown to be oncogenic. EBV is a human herpesvirus that is associated with >90% of Burkitt's lymphoma (BL) [174]. As mentioned before, pol III synthesizes EBER RNAs, and these products have been shown to have a role in tumor development and oncogenicity when transfected into human cell lines [174]. The present data exposes a role for pol III transcription in carcinogenesis, so it will be very interesting to study what other factors and/or

oncoproteins can regulate its activity.

1.4 Proliferation

For a cell to proliferate (increase in cell number), it requires both to double its mass (cell growth) as well as its DNA content. For this to be accomplished, all eukaryotic cells must follow a correct program, called the cell cycle. The cell cycle is defined as the period between two mitotic divisions. While cell growth is a continuous process, replication of the DNA occurs only during a specific period.

1.4.1 The Cell Cycle

The cell cycle can be divided into four phases: M phase, or mitosis, where a cell is divided into two daughter cells; G1 phase, which follows M phase and is when RNAs and proteins are synthesized; S phase, where the DNA is replicated, marking the transition from G1; and G2 phase, where the cell continues to grow until the next mitosis starts. The different phases of the cell cycle are intimately regulated through proteins that are periodically expressed within an appropriate window of the cycle, and this regulation is at the transcriptional level [9]. Only the cell cycle regulation of pol III transcription is going to be described.

1.4.2 Cell Cycle Control of RNA Polymerase III Transcription

During mitosis, all transcription appears to be globally repressed [175]. In the case of pol III transcription, this is due to the phosphorylation and inactivation of TFIIIB (Fig. 1.5) [146, 176, 177]. Specifically, using *Xenopus* eggs, it was shown that the mitotic kinase cdc2/cyclin B phosphorylates TBP and a 92 kDa TAF (possible *Xenopus* homologue of Brf) [177]. Since *Xenopus* eggs only display S and M phases, somatic cells (with G1, S, G2 and M phases) were used to study if these mitotic changes also happen in cells with normal cycles. For this, White et. al. utilised HeLa cells synchronized at mitosis. In these cells, it seems that TBP is also hyperphosphorylated, however this phosphorylation is not through cdc2/cyclin B kinase and is not limiting for transcription of TATA-less templates. The decrease in TFIIIB activity appears to be through reduced activity of one or more TFIIIB TAFs, but the details still remain to be determined [146].

After exiting mitosis, pol III transcription remains low but increases through G1, being at its maximal expression during S and G2 phases [147]. Again, the limiting factor responsible for this low activity is TFIIIB. Although it loses its phosphoryla-

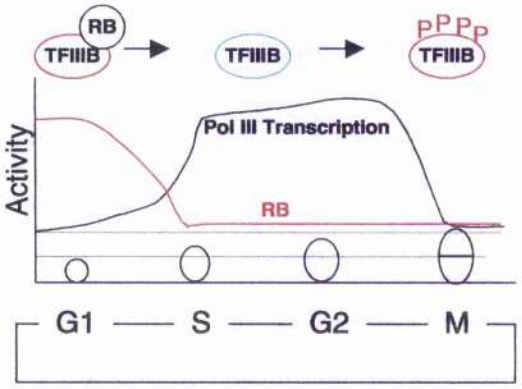

Figure 1.5: Regulation of pol III transcription by the cell cycle in mammalian systems. At mitosis and G1, transcriptional activity is low compared to S and G2. At mitosis, transcription is repressed by phosphorylation-mediated inactivation of TFIIIB. In early G1, although TFIIIB phosphorylation is relieved, transcription still remains low due to RB repression of TFIIIB. After the G1/S transition, transcription increases 2- to 3-fold and remains elevated until the end of G2. Active TFIIIB is represented in green while inactive TFIIIB in red.

tion after mitosis, its activity remains low in early G1 phase and increases as cells move into S phase [147]. At S and G2 phases pol III transcription reaches its maximum (Fig. 1.5). During these phases, the availability of TFIIIB has increased so that is not the limiting factor anymore. At this stage, TFIIIC becomes the limiting factor for pol III transcription [147]. In G1, pol III transcriptional activity is mainly regulated by the retinoblastoma tumour suppressor protein (Fig. 1.5).

The Retinoblastoma Protein

The retinoblastoma protein (RB) is a 105 kDa nuclear phosphoprotein that is ubiquitously expressed in normal mammalian cells [3]. It regulates cell cycle progression by restraining the passage of G1 to S phase if growth factors are limiting [178]. In early G1 or when growth factors are limiting, RB is in a hypophosphorylated form which is able to bind and repress transcription factors like E2F. However, if growth factors are plentiful, at mid to late G1 RB becomes hyperphosphorylated at multiple sites by cyclin D- and E-dependent kinases (cyclin D/cdk4/6 and cyclin E/cdk2) [178]. This hyperphosphorylated form of RB cannot bind to E2F; therefore E2F becomes active and promotes transcription of a variety of class II genes involved in cell cycle progression, allowing transition to S phase [178]. RB remains hyperphosphorylated during S, G2 and M phases, being only active in early G1 and

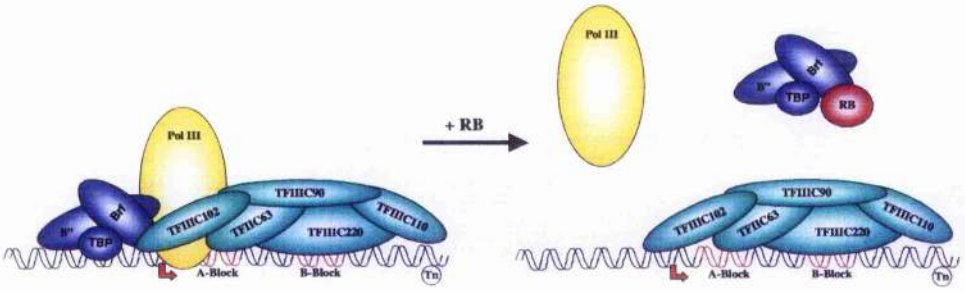

Figure 1.6: Mechanims of pol III inactivation by RB. Hypophosphorylated form of RB binds and sequesters TFIIIB from the initiation complex, disrupting interactions between TFIIIB and TFIIIC, and TFIIIB and pol III. Adapted from ref. [182].

G0.

The fact that TFIIIB's activity is low during the early part of G1 despite having been released from mitotic repression from, raises the possibility that another factor (or factors) are involved in its regulation at this stage. As mentioned above, TFIIIB activity increases at late G1 which coincides with RB inactivation, targeting RB as a good candidate for pol III repression in G1. There is a substantial weight of evidence showing this to be the case. RB can repress pol III transcription in vivo, when it is overexpressed in cells [3], and in vitro using purified pol III factors [179]. Primary fibroblasts from RB-knockout mice display four to fivefold increased pol III activity compared to wild-type cells [3]. In addition, pull-down and coimmunoprecipitation experiments demonstrate that RB interacts with TFIIIB, and SNAPc [179–181]. An interaction between TFIIIC2 and RB has also been reported, although confirmation has not been achieved [181]. Although RB interacts with several pol III TFs, TFIIIB is the principal target of RB-mediated repression [179, 180]. As with E2F, only the hypophosphorylated form of RB can bind to these proteins [4]. TBP is also involved in RB regulation, since its addition can restore efficient U6 transcription from RB-treated extracts [181]. One mechanism that RB uses to inhibit pol III transcription is by sequestering TFIIIB and disrupting the interactions between TFIIIB and TFIIIC2, and TFIIIB and pol III (Fig. 1.6) [182]. In conclusion, RB can be seen as a general regulator of pol III transcription, since it can repress transcription from every pol III template tested (tRNA, 5S rRNA, U6 snRNA, and Alu) [3, 162, 180].

RB contains a motif known as the pocket. There are two other RB-related proteins, p107 and p130, which also contain the pocket region and are referred to as

pocket proteins [178]. The pocket consists of two domains, A and B, separated by a nonconserved spacer. This region is essential for the interaction of RB, p107 and p130 with cellular proteins like E2F or with several viral oncoproteins [183]. p107 and p130 play an important role in cell cycle regulation, like RB [183]. Since they share a number of functions with RB, they might also regulate pol III transcription. Sutcliffe et. al. demonstrated in vivo and in vitro that indeed this is the case [184]. Overexpression of p107 and p130 in transfected cells results in a decrease of pol III transcription [184]. Furthermore, they associate stably with TFIIIB [184]. The RB family has also been shown to regulate pol III transcription in growth-arrested fibroblasts. In low serum conditions, fibroblasts undergo growth-arrest (G0), exiting the cell cycle . The levels of pol III transcription in these cells decrease significantly as they switch to G0 [4, 185]. This downregulation is due to specific reduction of TFIIIB activity, being inactivated by p130 during G0 and early G1 phase and RB during G1 [4].

Since RB controls cells progress into S phase, lost of RB's function results in uncontrolled proliferation. In many human cancers, if not all, RB's function appears to be compromised. This is achieved by mutations that inactivate the RB gene, e.g. in retinoblastoma, small-cell lung carcinomas and other sarcomas and carcinomas; or by the disruption of the regulatory pathway of RB [19]. A common control point that is disrupted in a number of cancers is the cyclin D-dependent kinases, specifically showing an increase in cyclin D1 levels [19]. Therefore, the mechanisms by which RB controls the cell cycle are very important to understand. All the oncoproteins studied in this doctoral thesis are able to deregulate RB activity. It will be interesting to study if RB inactivation is involved in their regulation of pol III transcription.

p53

Another tumour suppressor involved in the regulation of the cell cycle is p53. p53 inactivation is considered to be an important step in carcinogenesis, since more than half of all human tumours have lost p53 protein or have mutations in the gene [9, 19]. Normal cells express low levels of p53. When a cell suffers DNA damage there is an increase in the amount of p53. Two types of events can be triggered by the activation of p53: growth arrest and apoptosis, depending on various conditions. If it is in early G1, p53 can block further progression through the cell cycle, allowing DNA damage to be repaired. However, if it is committed to division, p53 may trigger apoptosis. Moreover, the decision between arrest and apoptosis is also determined by the cell type and the nature of stress, not only on the cell cycle's phase [9]. p53

has been shown to regulate pol III transcription. Overexpression of p53 in vitro or in transfected cells represses pol III activity [186]. Furthermore, using knockout technology it has been demonstrated that pol III transcription is increased 4-6-fold in p53 knockout fibroblasts from mice [187]. In addition, tumour-derived mutants of p53 lose their ability to repress pol III transcription. Primary fibloblasts from individuals with Li-Fraumeni syndrome, who inherit mutant forms of p53, show highly elevated pol III transcriptional activity [188]. When p53 is neutralized by oncoproteins like human papillomavirus E6 or cellular hdm2, pol III transcription gets elevated [188]. p53's repression of transcription has been shown to be through the inhibition of TFIIIB [186, 187]. Recombinant p53 interacts with TBP and Brf subunits of TFIIIB [186], and coimmunoprecipitates with TFIIIB at physiological ratios [187]. TFIIIB stability can also be affected by p53 in a human fibroblast cell line [189].

At the moment, cell cycle regulation of pol III transcription has been shown to be through regulation by the tumour suppressors p53 and RB family. There are other cell cycle regulators that have not been investigated yet, leaving an open window for their study. c-Myc is an important cell cycle regulator, making it a very interesting candidate.

1.4.3 Cell Growth and RNA Polymerase III

For a cell to double its DNA and divide, a critical growth rate has to be attained in order to conserve cellular resources for daughter cell survival. If DNA is manufactured faster than proper growth is achieved, the cell becomes unstable and dies [15]. Therefore, a tight equilibrium between cell growth and cell cycle must be accomplished for successful proliferation. A cell grows by increasing its proteins and macromolecules. Since proteins constitute the bulk of a cell's dry mass, the growth rate is directly proportional to the rate of accumulation of protein [11]. In fact, if protein synthesis is reduced by 50%, cells withdraw from cycle and quiesce [11]. Thus, an increase in the protein synthetic machinery will be required for successful growth. Ribosomes and tRNAs, as well as translation initiation factors, are constituents of the translational machinery. Thus, their availability is going to be an important determinant of the rate of translation. Since 5S rRNA and tRNAs are pol III products, a high level of pol III transcription is essential for cell growth.

Mitogenic stimulation triggers a coordinate induction of rRNA, tRNA, ribosomal proteins and translation factors, causing an increase in the rate of translation [1]. It would be interesting to study how this coordination of transcription from different

genes is accomplished. c-Myc has been shown to regulate transcription of ribosomal genes as well as that of translation initiation factors, however it is not known if it can regulate 5S rRNA or tRNA expression [15]. Since it would be a waste of energy to regulate only one set of genes and not the others, it is quite plausible that c-Myc also regulates transcription of these pol III products.

1.5 Objectives

As presented in this introduction, pol III transcription can be regulated by several proteins and conditions. Conditions like cell cycle progresion, growth, differentiation and transformation are regulated by a wide variety of proteins, from plasma membrane receptors to transcription factors. In particular, three proteins have received a lot of attention due to their oncogenic potential: the plasma membrane receptor ErbB2/neu, and the transcription factors c-Myc and Id2.

The plasma membrane receptor ErbB2/neu induces cell proliferation by activating several signalling cascades which will alter the expression of genes and ultimately induce this process. Some of the signalling cascades it activates include the Ras and PI3K signalling cascades. This receptor can also block the inhibitory function of RB by affecting its phosphorylation status. Since pol III transcription can be activated by the signalling pathways that ErbB2/neu activates, the first aim of this thesis was to investigate whether the activation of ErbB2/neu can induce pol III activity, and if so, what mechanism or mechanisms are involved in this process.

In addition to the effects already mentioned, ErbB2/neu can also stimulate the expression of the transcription factor c-Myc. This protein has been studied thoroughly because of its role in tumour formation. Furthermore, it has been shown to be a regulator of cell growth. As stated in this introduction, the products synthesized by pol III have a direct effect upon cell growth, and their availability is an important determinant of growth rate. Since c-Myc is a positive regulator of growth, another aim of this thesis is to investigate the participation of this transcription factor in the possible upregulation of pol III transcription in ErbB2/neu overexpressing cells, as well as when it is overexpressed by itself.

A possible mechanism by which c-Myc could regulate pol III transcription is through the inactivation of the RB family. In the literature there is a bulk of evidence showing that c-Myc is responsible for the induction of proteins that will ultimately phosphorylate RB, thus preventing its repressive influence. Since pol III transcription is tightly regulated by the RB family, it will be interesting to study if this is the mechanim by which c-Myc could be inducing pol III transcription.

Finally, the regulation of pol III transcription by a third oncoprotein, Id2, will also be studied. Id2 can bind and inactivate RB, and has been shown to prevent cells from differentiating. Furthermore, recent reports have shown that the induction of this transcription factor responds to N-Myc. This suggests that the ability of Id2 to overcome the inhibitory function of RB may provide an additional route that enables the Myc family to ensure high levels of pol III activity. Because pol III transcription reacts to RB activity, as well as being inhibited by differentiation, Id2 strikes as another possible regulator of its activity. Therefore, the last aim of this thesis will be to investigate whether Id2 can exert any effect on pol III transcription.

2 Materials and Methods

2.1 Cell culture

The ROSE cell lines were obtained from Bruce A. J. Ponder [190], which were produced by stably transfecting ecotropic retroviruses expressing the *β-galactosidase* gene or a mutated rat *neu/c-erbB2* oncogene. The ROSE cell lines, Balb/c 3T3 (A31), and mouse embryo fibroblasts (RB wild type and knockout) and primary fibroblasts from wild-type or RB, p107 and 130 triple knockout cells (TKO) were routinely cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal calf serum (FCS, Sigma), 2mM L-glutamine, 100U/ml penicillin and 100 μg/ml streptomycin and in a humidified atmosphere containing 5% CO2 at 37°C. The medium of the ROSE cell lines also contained 0.6 mg/ml G418. In the case of the RB mouse embryo fibroblasts (MEFs), the medium required addition of an insulin-transferrin-selenium supplement (5 ml in 500 ml of medium, Life Technologies). The primary and TKO cells medium was supplemented with 1 mM non-essential aminoacids, 1 mM sodium pyruvate and 0.1 mM β-mercaptoethanol. The Chinese hamster ovarian (CHO) cells expressing haemagglutinin (HA)-tagged Brf1 were grown in Glasgow's MEM containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 0.5 mg/ml G418. These cells were grown under the same criteria as the ones already mentioned.

Cell culture was performed in a class II hood, using aseptic technique and sterile equipment and reagents. Cells were passaged when subconfluent; approximately every 2 to 3 days. After media was aspirated from the flask, 2ml of buffered trypsin-EDTA (0.05% trypsin, 0.02% EDTA) was added to the cells, then aspirated immediately. A further 2ml was added and left for approximately 5 minutes at 37°C. Following addition of trypsin, fresh media was immediately added to the dissociated cells which were centrifuged at 1200g for 5 minutes. The media was removed and the pelleted cells were then resuspended in fresh 10% FCS DMEM solution at a ratio of 1:10, or as appropriate.

Cryo-freezing was used for storage of all cell lines. Cells were trypsinised as described and, following pelleting by centrifugation, were resuspended in a solution of DMEM, 20% FCS and 10% dimethylsulphoxide (DMSO). 1 ml aliquots of resuspended cells were placed into 1.5 ml cryo-tubes and frozen in stages by initially being placed at -80°C overnight and subsequently being transferred to liquid nitrogen storage. Thawing of cells was performed rapidly by placing cyro-tubes in a waterbath at 37°C until just thawed. Cells were then mixed with fresh media, centrifuged and the supernatant aspirated off to ensure removal of DMSO prior to resuspension in 10% FCS DMEM.

The samples from Chapters 5 and 6 came from Robert Eisenmann group in Seattle, prepared by Carla Grandori. Briefly, the $Myc^{+/+}$ and $Myc^{-/-}$ rat fibroblasts had been previously described ([191]), and correspond to the cell line TGR-1, an *hprt*$^-$subclone of the immortalized embryonic rat fibroblast cell line Rat-1 and *c-myc* knockouts produced by gene targeting of this cell line. The cells were grown in DMEM supplemented with 10% CS at 37°C in an atmosphere of 5% CO_2. The WI38 cells (ATCCcat ♯ 75-ccl) are human primary fibroblasts and were prepared as previously described [192]: cells were thawed and split once before infection. On day 1, subconfluent WI38 cells were infected with 5 ml of viral supernatant (pBabe-puro vectors expressing either c-Myc-ER^{TM} and Δ-Myc-ER^{TM}) in a 10 cm plate in the presence of 4 μg/ml polybrene. On day 2, infection was repeated again. On day 3 cells were split 1:4 or 1:6 in phenol-red free medium containing 10% fetal calf serum and selected in the presence of puromycin at 2.5 μg/ml. Selection was monitored by control cultures not exposed to the virus, that generally died in 2 or 3 days upon puromycin adition, while little or no cell death was detected in the infected cultures. Cells were grown to confluence, usually 1 week, without further media changes. Density-arrested cultures were induced with 200 nM 4-OHT or serum starve (0.1% FCS) for 36-48 h and then induced. Where specified, α-amanitin at 20 μg/ml was added prior to addition of 4-OHT.

2.2 Primers and Cycling Parameters

The primers and cycling parameters are shown in table 2.2 on the following page.

2.3 Plasmids

For the in vitro transcription assays, the plasmid templates used were as follows: pVA1 is a 221bp SalI-BalI fragment of adenovirus 2 DNA containing the VA1 gene subcloned into pUC18; PHu5S3.1 is a 638-bp *Bam*HI-SacI fragment of human ge-

Transcript	Primers	Product	No Cycles	Program (Denaturation; Cycling; Final Extension)
ARPP P0	5'-GCACTGGAAGTCCAACTACTTC-3' 5'-TGAGGTCCTCCATGGTCAACAC-3'	266 bp	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'-GGGGCTGGAGACATGGCT-3' 5'-CCATGTGGTTGCTGGGAT-3'	120 bp	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
BN51	5'-CCTACACAGTCTGTACCCTGG-3' 5'-GGCATATTTCGAGTGTCGTTCC-3'	150 bp	18-22	95°C for 150 s; 95°C for 30 s, 68°C for 30 s, 72°C for 30 s; 72°C for 5 min
Cyclin D2	5'-CTGCCCCCACCTAGATCATA-3' 5'-TCCCTTATGCTGTACTTCAAATAGG-3'	112 bp	20	94°C for 90 s; 94°C for 15 s, 62°C for 23 s, 72°C for 30 s; 72°C for 5 min
GAPDH	5'-TCCACCACCCTGTTGCTGTA-3' 5'-ACCACAGTCCATGCCATCAC-3'	452 bp	18-22	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
Id2	5'-TCCTCGCAGGCTTCTGAA-3' 5'-CCATTCAACTTGTCCTCC-3'	115 bp	18-22	94°C for 3 min; 95°C for 30 s, 66°C for 40 s, 72°C for 1 min; 72°C for 5 min
p21	5'-GCCTCCTTTCTGTGCCTGA-3' 5'-CCAGCCCTTCGATGGTTT-3'	254 bp	30-35	95°C for3 min; 95°C for 1 min, 52°C for 1 min, 72°C for 1 min; 72°C for 5 min
snRNA U6	5'-GCTCGCTTCGGCAGCACATATAC-3' 5'-TATCGAACGCTTCACGAATTTGCG-3	96 bp	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
TBP	5'-GCTCGCTTCGGCAGCACATATAC-3' 5'-TATCGAACGCTTCACGAATTTGCG-3'	660 bp	25	95°C for 2 min; 94°C for 1 min, 56°C for 1 min, 72°C for 3 min; 72°C for 10 min
TFIIIC 220	5'-TCCAGCGAGACCTTCACACC-3' 5'-GGATTCAGTCTTGCTCGGCT-3'	144 bp	20-25	95°C for 3 min; 94°C for 20 s, 62°C for 30 s, 72°C for 30 s; 72°C for 10 min
TFIIIC 110	5'-CCAGAAGGGGTCTCAAAAGTCC-3' 5'-CTTTCTTCAGAGAT-GTCAAAGG-3'	303 bp	20-25	94°C for 3 min; 6 cycles (95°C x1m, 66°C x40,72°C x40s 94°C for 20 s, 62°C for 30 s, 72°C for 30s; 72°C for 10 min
$tRNA^{Leu}$	5'-GAGGACAACGGCGACAGTAA-3' 5'-TCCACCAGAAAAACTCCAGC-3'	88 bp	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
$tRNA^{Tyr}$	5'-AGGACTTGGCTTCCTCCATT-3' 5'-GACCTAAGGATGTCCGCAAA-3'	84 bp	25-30	95°C for 3 min; 95°C for 1 min, 65°C for 30 s, 72°C for 15 s; 72°C for 5 min
5S rRNA	5'-GGCCATACCACCCTGAACGC-3' 5'-CAGCACCCGGTATTCCCAGG-3'	107 bp	15-18	94°C for 3 min; 95°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 10 min

Table 2.2: Primers and Cycling Parameters.

Protein	Antibody	Type	Company
TFIIIC220	4286	Serum	In house
TFIIB	C18	Polyclonal	Santa Cruz Biotechnology
pp44/42 MAPK	9102	Polyclonal	Cell Signalling Tecn.
phospho-p70^{S6K}	92055	Polyclonal	Cell Signalling Tech.
Cyclin D1	72-13G	Monoclonal	Santa Cruz Biotechnology
Cyclin E	C19	Polyclonal	Santa Cruz Biotechnology
RB	G3-245	Monoclonal	Pharmingen
phospho-RB	9300	Polyclonal	Cell Signalling Tech.
RB	C15	Polyclonal	Santa Cruz Biotechnology
TAF_I48	M19	Polyclonal	Santa Cruz Biotechnology
Brf	128	Serum	In house
TBP	SL-1	Polyclonal	Santa Cruz Biotechnology
TBP	mTBP-6	Monoclonal	In house
c-Myc	9E10	Monoclonal	Santa Cruz Biotechnology
Cyclin A	BF683	Monoclonal	Santa Cruz Biotechnology
BN51	113	Serum	Gift from M. Ittmann
Max	H-2	Monoclonal	Santa Cruz Biotechnology
Id2	C-20	Polyclonal	Santa Cruz Biotechnology
E7	TVG7 10Y	Monoclonal	Santa Cruz Biotechnology
E47	N-469	Polyclonal	Santa Cruz Biotechnology
TBP	58C9	Monoclonal	Santa Cruz Biotechnology
Cyclin D1	R124	Monoclonal	Santa Cruz Biotechnology

Table 2.3: List of antibodies.

nomic DNA containing a 5S rRNA gene, subcloned into pBR322 pBluescript SK+; $tRNA^{Leu}$ is a 240 bp EcoRI-Hind III fragment carrying a $tRNA^{Leu}$ gene subcloned into pAT153; $tRNA^{Arg}$ is a 500 bp Hind III fragment carrying a $tRNA^{Arg}$ gene subcloned into pAT153.

For the transfections, the plasmid templates used were as follows: pCAT (Promega) contains the *chloramphenicol acetyltransferase* (CAT) gene driven by the simian virus 40 promoter and enhancer; pRc-CMV-Myc is a 2.7 Kb (1.8 Kb *c-myc* cDNA + ~0.9 Kb HBV terminator and T3 excised from psp271-Myc) HindIII/XbaI fragment containing full length *c-myc* gene subcloned into pRc-CMV; pRc-CMV is a Stratagene vector; pcDNA3-Id2 contains the *Id2* gene subcloned into pcDNA3.

2.4 Antibodies

The list of antibodies is shown in table 2.3.

2.5 RNA Extraction

For the extraction of total cellular RNA, cells were grown to 80% confluence and then TRI reagent (Sigma) was utilised in accordance with the manufacturer's instructions. TRI reagent is a solution of guanidine thiocyanate and phenol. When cells were 80% confluent, media was aspirated off, and each dish was harvested by scraping in 1ml of TRI reagent per dish and transferred to a sterile eppendorf tube where they were left to stand for 5 min at room temperature allowing complete dissociation of nucleoprotein complexes. After this period of time, 0.2ml of chloroform was added to each tube and the samples vortexed for 15 seconds and left to stand for another 15 min at room temperature. Then the samples were centrifuged at 13000g for 15 minutes at 4°C, separating the contents 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 containing the RNA. 400 μl of the upper phase was carefully removed, avoiding contamination from the remaining phases, and transferred to fresh eppendorf tubes. The aqueous RNA phase was then mixed with isopropanol (500 μl) and thoroughly mixed by repeated inverting. This was left for 5 - 10 min at room temperature for the maximal precipitation to occur and then the samples were centrifuged again at 13000g for 10 min at 4°C. The supernatant was discarded and the remaining RNA pellet was washed with 1ml of 75% ethanol made up with diethypyrocarbonate (DEPC)-treated dH_2O (0.1% DEPC), thoroughly mixed into solution, left overnight at room temperature and then autoclaved to inactivate the remaining DEPC. After vortexing each sample, they were spun at 7500g for 5 min at 4°C. Then supernatant was thoroughly removed and appropriate volumes of DEPC-dH_2O (from 10 to 50 μl, depending on the size of the pellet) were added to the RNA pellets. The samples were heated in a 65°C waterbath for 10 - 15 min, shaking them throughout this period, allowing resuspension of the RNA. Once the RNA was resuspended, the samples were stored at -70°C. To determine the RNA concentration of each sample, UV spectrophotometry was used with the following formula: RNA concentration (μg/ml) = λ^{260} x 40 x dilution factor. To determine the purity of the RNA, the readings obtained at 260nm and at 280nm were divided and if the ratio was in the range of 1.8 - 2, it indicated that the RNA samples were relatively free from contamination with DNA or protein.

2.6 Northern Blot Analysis

10-20 μg of total RNA samples were usually used in analysis, made up to a total volume of 10 μl with DEPC-dH_20. Each RNA sample was mixed with 10 μl of 2 x RNA sample buffer composed of 1 x MOPS comprised of solutions made up with DEPC-dH_20 (20mM MOPS pH 7.0, 8mM sodium acetate, 1mM EDTA pH 8.0) plus 4.4M formaldehyde and 54% formamide and heated for 15 min at 65°C to denature the RNA secondary structure. After the incubation, the samples were immediately transferred to ice to prevent any renaturation and were briefly microcentrifuged to bring down evaporated solution. Then, 2 μl of 1mg/ml ethidium bromide and 2 μl of 10 x RNA loading buffer (50% glycerol, 1mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF) were added to the reaction. The samples were then loaded in a denaturing gel (1% agarose, 2.2M formaldehyde, 1 x MOPS) in 1 x MOPS, which had been previously pre-run at 40V for 20 min, and run for approximately 5 hours at 40V allowing separation of different sizes of RNA. After electrophoresis, the gel was visualised under a UV transilluminator in order to confirm separation and photographed. It was then washed for 20 minutes in 20 x SSC buffer (3M NaCl, 0.3M sodium citrate pH 7.0) prior to capillary transfer as described by Maniatis et al.

To transfer the gel, it was placed on a bridge of Whatmann 3MM chromatography paper supported on a glass plate and suspended over a reservoir of 20 x SSC buffer into which the tip ends of Whatmann 3MM paper were soaked. An appropriate size of Hybond N nylon membrane optimised for nucleic acid transfer (Amersham) was pre-soaked in 20 x SSC and positioned over the gel, followed by a further two layers of pre-soaked Whatmann paper; and taking care of removing all air bubbles. This arrangement was surmounted with a stack of paper towels and an appropriate weight in order to allow efficient capillary action. During transfer, the migration of the RNA from the gel to the nylon membrane is facilitated by the passive movement of the transfer solution through the gel. This was left overnight to ensure a high-transfer efficiency. Following transfer, the RNA was fixed to the membrane by UV-crosslinking at 1200 μJ and was then washed for 5 minutes in DEPC-dH_20.

Radiolabelled DNA probes complementary in sequence to a particular RNA of interest were used to locate it on the membrane. The pol III B2 gene probe was a 240bp EcoRI-PstI fragment from pTB14 and the pol II acidic ribosomal phosphoprotein P0 (ARPP P0) probe, a 1kb EcoRI-HindIII fragment from the mouse cDNA. The probes were labelled using a Megaprime DNA labelling system (Amersham) according to the random oligonucleotide priming method of Feinberg and

Vogelstein. Basicallly, 5 μl of random hexamer oligonucleotide sequences and the appropriate volume of DEPC-dH_20 were added to achieve a final volume of 50 μl to 25ng of purified DNA template. This mixture was denatured by heating at 95°C for 5 minutes. DNA synthesis is primed by the hexamer oligonucleotides which are able to anneal to the DNA during slow cooling to room temperature. After denaturation, 4 μl each of dATP, dGTP, dTTP (in Tris-HCl pH 8.0, 0.5mM EDTA), 50 μCi of [α-^{32}P] dCTP (10mCi/ml, 3000Ci/mmol) and 2U DNA polymerase I Klenow fragment (in 100mM potassium phosphate pH 6.5, 10mM 2-mercaptoethanol, 50% glycerol) were added and labelling was carried out at 37°C for 1hour in 1 x reaction buffer (containing Tris-HCl pH 7.5, 2-mercaptoethanol, MgCl2, Amersham). The reaction was stopped by heating at 80°C for 5 minutes and kept at 4°C until the nylon membrane with bound RNA had been pre-hybridised. This involved rotation in a hybridisation oven at 45°C for 20 minutes in 20ml of hybridisation buffer (0.2M sodium phosphate buffer pH 7.2, 1mM EDTA, 1% (w/v) BSA, 7% (w/v) sodium dodecyl sulphate (SDS), 45% (w/v) formamide in DEPC-dH_20). Following this, the radiolabelled probe was added to 20ml of fresh hybridisation buffer, in which the membrane was incubated with rotation at 45°C overnight.

The following day the nylon membrane was washed with rotation in wash buffer (40mM sodium phosphate buffer pH 7.2, 1mM EDTA, 1% (w/v) SDS in DEPC-dH2O) at room temperature for 2 minutes and then twice for 15 minutes at 65°C in order to remove non-specific radioactivity before being exposed to autoradiography film overnight at -80°C. Membranes were stripped by incubating in boiling water for 5 minutes and pre-hybridised again prior to being reprobed. Quantification of the RNA was achieved by scanning the autoradiograph and then analysed using the software NIH-Image.

2.7 Preparation of cDNAs

cDNAs were prepared from 3 μg of RNA. To each sample was added 1 μl of hexanucleotide diluted 1:10 (Roche) with DEPC-dH_2O and was brought to a final volume of 12 μl with DEPC-dH_2O. Primer annealing was carried out at 70°C and allowed to proceed for 10 minutes before transferral to ice. After quickly chilling on ice and microcentrifuging to bring down the evaporated material, 4μl of 5 x First Strand Buffer (Life Technologies), 2 μl of 0.1M DTT, 1 μl of 10mM dNTP mix (made up in DEPC-dH_20) were added and incubated for 2 min at 42°C. Following this incubation, 1 μl (200U) of Superscript II Reverse Transcriptase (Life Technologies) was added to initiate reverse transcription, which was performed for 50 min at 47°C

before the reaction was stopped by heating at 70°C for 15 min. An extra 20 μl of DEPC-dH_2O were added to give a final concentration of 60 μl.

2.8 Reverse transcriptase - Polymerase chain reaction (RT-PCR)

PCRs were carried out using a PTC-100 thermal controller (MJ Research, Cambridge, MA). 2 μl of cDNA was amplified with 20pmol of primers. Amplification reactions contained 0.5U of Taq DNA polymerase (Promega) in 20 μl of 1 x Taq DNA polymerase buffer (Promega) containing 1.5mM MgCl2, 0.2mM of each dNTP plus 1 μCi of [α^{32}P] dCTP (10mCi/ml, 3000Ci/mmol). After PCR, 20 μl of formamide loading buffer (98% formamide, 10mM EDTA pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol FF) were added to each sample and the reaction products were resolved on 7% polyacrylamide sequencing gels containing 7M urea and 1 x TBE (45mM Tris, 45mM boric acid, 0.625mM EDTA pH 8.0). Gels were pre-run for 30 minutes at 40W in 1 x TBE and 2 μl of each sample was loaded after being boiled at 95°C for 2 minutes and quenched on ice. Electrophoresis was carried out for a 1 hour at 40W 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. Quantification of results was achieved by scanning the autorads and analysed using the NIH-Image software.

2.9 Preparation of Whole Cell Extracts and Protein Fractions

Whole cell extracts were prepared from cells grown to approximately 80% confluence in 10cm tissue culture plates. Everything was performed on ice as rapidly as possible and all solutions and tubes were kept ice-cold to maintain cell activity and avoid protein degradation. Cells were washed twice with 5ml of ice-cold-PBS before being scraped with a plastic spatula into 5ml of fresh ice-cold PBS. They were collected in 50ml Falcon tubes and pelleted by slow centrifugation at 1200g for 8 minutes at 4°C. The supernatant was decanted and the cell pellet was resuspended in 1 ml of fresh ice-cold PBS and 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. 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 (450mM NaCl, 50mM NaF, 20mM Hepes pH 7.8, 25% glycerol, 1mM DTT, 0.5mM PMSF, 0.2mM EDTA, 40 μg/ml bestatin, 1 μg/ml trypsin inhibitor, 0.7 μg/ml pepstatin, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin) was added to the cells and, following resuspension, the cells were immediately snap-frozen on dry ice. Cells were then placed in a 30°C waterbath until just thawed, before being immediately returned to dry-ice. This freeze-thaw procedure was performed a total of 3 times to ensure optimal cell lysis. After the last thaw, the cells were microcentrifuged at 7000g for 7 minutes at 4°C. The supernatant was carefully recovered into a fresh tube, leaving behind the cell debris, and then promptly aliquotted and snap frozen. These whole cell extracts were then stored at -80°C.

Glutathione S-transferase (GST) fusion proteins GST-Myc-N262 and GST-Myc-C92, as well as GST-Max and GST, were kindly provided by Robert N. Eisenman. GST-Myc-N262 contains residues 1-262 of the N-terminal region of c-Myc, while GST-Myc-C92 contains the last 92 residues of the carboxyl-terminal region. GST-Max contains full size Max protein.

HeLa nuclear extracts were purchased from the Computer Cell Culture Center (Mons, Belgium). PC-B is the 0.1 to 0.35 M KCl step fraction from phosphocellulose step chromatography containing both TFIIIB and pol III. QS-PC-B contains TFIIIB that has been fractionated by gradient chromatography on Q-Sepharose followed by step chromatography on phosphocellulose. The TFIIIC fraction was oligo-purified (Phosphocellulose followed by S-Sepharose and then a B-block DNA-affinity column). The TFIIIB fractions were prepared by step fractionation on phosphocellulose followed by a MonoQ gradient. The pol III fraction was prepared by step fractionation on phosphocellulose and DEAE-Sephadex. All these fractions were kindly provided by Robert J. White.

In vitro translated pCITE-Brf was produced using the STP3 Kit (Novagen), according to manufacturer's procedures. Basically, 8μl of STP3 T7 Transcription Mix was mixed with 0.5 μg of DNA plasmid to a final volume of 10 μl. This reaction was stirred and incubated at 30°C for 15 min. After incubation, 30 μl of STP3 Translation Mix and 4 μl (40 μCi) ^{35}S-methionine were added and brought to a final volume of 50 μl with nuclease-free water. This reaction was gently stirred with the pipette tip and incubated at 30°C for 1 hour. The protein translation was confirmed by analysing with SDS/PAGE, followed by drying the gel and autoradiography at room temperature.

2.10 Determination of Protein Concentration

The protein concentration of samples was determined using Bradford's reagent (Bio-rad). Quantification of the colour reaction produced upon mixing 1ml of diluted reagent (1:4 in distilled water) was measured at an absorbance of 595nm in a UV spectrophotometer, which gave a reading proportional to increasing amounts of protein. At a range of 1-12 μg of protein, under these conditions, the absorbance is approximately linear. Absorbance readings obtained were then compared to a set of standards of known protein concentration using bovine serum albumin (BSA) measured at 595nm. A range of sample dilutions were measured and compared in this manner in order to obtain an average, which would provide a more accurate measurement of protein concentration. Where sample absorbance readings fell outside the linear range of the standard set, appropriate dilutions were made and the samples were re-measured.

2.11 Transformation of competent cells

E.coli XL-1 Blue supercompetent cells (Stratagene) were transformed for plasmid storage and propagation. Cells, which were stored at -80°C and highly temperature sensitive, were thawed on ice to prevent loss of transformation ability. Once thawed, 50 μl of cells per transformation reaction were exposed to 0.4 μl of β-mercaptoethanol (at a final concentration of 25mM), which enhances transformation efficiency by disrupting disulphide bonds of plasma membrane proteins. Typically 10-20ng of plasmid DNA was then gently mixed into the chilled cells. The reaction was left for a 30 minute incubation on ice, in which the contents were gently tapped occasionally. After the 30 minute incubation, the cells were 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 - 250rpm) following the addition of 450 μl of preheated (42°C) SOC medium (LB broth, 0.04% glucose, 10mM MgSO4, 10mM MgCl2). Typically, 150 μl of the transformation mixture was then plated on LB agar (2% LB, 2% 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.

2.12 Preparation of plasmid DNA

For large scale plasmid DNA preparation, a single isolated bacterial colony was selected from a freshly-streaked plate and used to inoculate 4 ml of LB medium con-

taining the selective antibiotic (50 μg/ml ampicillin). This was allowed to incubate with vigorous shaking at 37°C for ~6 hours and was then used to inoculate 250 ml of the same medium and left to grow overnight with vigorous shaking at 37°C on an orbital shaker (~300rpm). The following day, cells were harvested by centrifugation at 6000g for 15 minutes at 4°C and plasmid DNA was retrieved using the QIAGEN Plasmid Maxi Kit following the procedure described in it. The bacterial pellet was resuspended in 10 ml of Buffer P1 (50mM Tris pH 8.0, 10mM EDTA, 100 μg/ml RNase A) and then gently but thoroughly mixed with 10 ml of Buffer P2 (200mM NaOH, 1% SDS) to initiate an alkaline lysis reaction. This reaction was allowed to proceed for 5 minutes at room temperature before neutralising the lysate by the addition of 10 ml of chilled Buffer P3 (3M 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 decanted into a QIAGEN-tip 500, previously equilibrated with 10 ml of Buffer QBT (750mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100). The QIAGEN-tip 500 contains an anion-exchange resin to which the plasmid binds tightly while the the supernatant pass through by gravity flow. The resin was then washed twice with 30 ml of Buffer QC (1M NaCl, 50mM MOPS pH 7.0, 15% isopropanol) and the purified plasmid DNA was subsequently eluted with 15 ml of Buffer QF (1.25M NaCl, 50mM Tris pH 8.5, 15% isopropanol) and precipitated with 10.5 ml (0.7 volume) 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% ethanol, dried at room-temperature for 5 - 10 minutes and resuspended in an appropriate volume of sterile water or TE buffer, pH 8.0 (10mM Tris pH 8.0, 1mM EDTA).

2.13 RNA Pol III *In Vitro* Transcription Assay

In vitro transcription of class III genes was reconstituted using 15 μg of whole cell extracts or nuclear extracts to provide the basal pol III transcription components. The samples were kept on ice while the reactions were prepared. Each sample was supplemented with 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 12mM HEPES pH 7.9, 60mM KCl, 7.2mM MgCl2, 0.28mM EDTA, 1.2mM DTT, 10% (v/v)

glycerol, 1mM creatine phosphate, 0.5mM each of rATP, rCTP and rGTP and 10 μCi [α^{32}P] UTP (400mCi/mmol) (Amersham). The reaction was performed in a waterbath at 30°C for 1 hour. In the case of assays incorporating additional reagents, a 15 minute pre-incubation at 30°C was carried out prior to adding the nucleotides required to initiate transcription. Transcription was terminated by the addition of 250 μl of 1M ammonium acetate/0.1% SDS containing 20 μg of yeast tRNA, which acts as a stabiliser for the synthesised RNA. Phenol-choloroform extraction of the samples was performed to remove protein and DNA by adding 250 μl of a 25:24:1 ratio solution of PhOH/CHCl3/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 and were inverted several times to ensure that they were thoroughly mixed. In order to precipitate the RNA, the samples were 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 the pellet was washed in 750 μl of 70% ethanol and spun again. The supernatant was carefully removed to avoid dislodging the pellet and the samples were heated at 47°C for 5 - 10 minutes to dry. Once dried, 4 μl of formamide loading buffer (98% formamide, 10mM 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. 1.5 μl of each sample was loaded on a pre-run 7% polyacrylamide sequencing gel containing 7M urea and 1 x TBE (45mM Tris, 45mM boric acid, 0.625mM EDTA pH 8.0) after being boiled at 95°C for 2 minutes and quenched on ice. Electrophoresis was performed at 40 W for 1 hour in 1 x TBE, before being dried and exposed to autoradiography film in order to detect the radiolabelled transcripts.

2.14 Immunoprecipition and Immunodepletion

Antibodies for immunoprecipitation experiments were coupled to either protein-A Sepharose (polyclonal antibodies) or protein-G Sepharose beads (monoclonal antibodies). 25 μl of packed beads were used per sample and beads were washed twice with 1 x LDB (20 mM Hepes-Cl pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 17% glycerol) prior to incubation with the appropriate antibody on a shaker for 1 hour at 4°C. Equivalent amounts of antibodies were used for these experiments, to give a final concentration of 1 μg in a volume of 20 μl. Protease inhibitors were used throughout the experiment to reduce loss of activity. Following incubation, the beads were washed twice with LDB to ensure removal of unbound an-

tibody. For immunodepletion experiments, these beads, carrying equivalent amounts of prebound immunoglobulin (IgG) were incubated on ice with approximately 225 μg of cell extract (made up to a volume of 30 μl with LDB0 if they were whole cell extracts or with LDB in the case of nuclear extracts) for 2 hours, gently mixed by tapping every 5 minutes to avoid loss of activity. For co-immunoprecipitation reactions, the cell extract was incubated with the prepared protein-A Sepharose beads at 4°C for 3 hours on an orbital shaker. After the incubation, beads were pelleted by gentle pulse microcentrifugation and the supernatant was discarded in the case of immunoprecipitation. The supernatant of the immunodepletions was recovered (20 μl) and 2 μl (~15 μg) were analysed by in vitro transcription assay. The beads were then subjected to extensive washing before the bound material was released by the addition of an equal volume of 2 x protein sample buffer and analysed by SDS-PAGE and subsequent western blotting for the protein of interest.

2.15 Protein Analysis by Polyacrylamide Electrophoresis (SDS-PAGE) and Western Blot

Proteins were resolved on denaturing polyacrylamide gels according to molecular weight by electrophoresis. Polyacrylamide resolving minigels (375mM Tris pH 8.8, 0.1% SDS) were used with a stacking layer comprised of 4% polyacrylamide gel (125mM Tris pH 6.8, 0.1% SDS) based on the discontinuous buffer system described by Laemmli. Samples were boiled for 3 minutes in 1 x protein sample buffer (62.5mM 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, 76.8mM glycine, 10mM Tris, pH 8.3) at an initial voltage of 70 V while the bromophenol blue dye front moved through the stacking gel and a subsequent voltage of 140 V after it reached the resolving gel. Electrophoresis was allowed to proceed until the dye front had reached the bottom of the gel, approximately 1 - 1.5 hours. After gel separation, the proteins were transferred to a nitrocellulose membrane by electrophoretic transfer using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system. Transfer was carried out in 1 x transfer buffer (76.8mM glycine, 10mM Tris, pH 8.3, 16.5% methanol) at 40 V for 1 hour or overnight. Following transfer, the membrane was washed in TBS for 10 min and then blocked in milk buffer (32.5 mM Tris, 150 mM NaCl, 0.2% Tween-20, 4% skimmed milk powder (Marvel)) for 30 min at room temperature. Membranes were incubated with primary antibodies (typically a 1:1000 dilution in milk buffer) for one hour at room temperature or overnight at 4°C. Excess primary antibody was removed by wash-

ing the blot 3 times for 2 minutes in fresh milk buffer before incubating for one 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 each, followed by 2 washes for 15 minutes. After one further 5 minute wash using 1 x TBS (2.5mM Tris-HCl pH 7.6, 15mM NaCl), the blot was developed using the enhanced chemiluminescence method (ECL, Amersham), as directed by the manufacturers.

2.15 Pull-Down and Pull-Down Depletion

GST or GST-fusion proteins were coupled to glutathione-agarose beads (SIGMA) previously prepared according to manufacturers' instructions. GST, GST-Myc-N262, GST-Myc-C92 and GST-Max bound-beads, as well as BSA samples at different known concentrations were then analysed by SDS-PAGE followed by Coomasie staining of the gel to measure the concentration of each GST protein and equalize them to the same final concentration (5 $\mu g/\mu l$). Once they were at the same concentration, they were stored at -80°C or used for pull-down experiments. Typically, 250 μg of whole cell extracts or nuclear extracts were incubated with 25 μl of packed glutathione agarose-beads carrying GST alone, GST-Myc-N262, GST-Myc-C92 or GST-Max for 3 hrs on an orbital shaker at 4°C. After the incubation, beads were pelleted and the supernatant of the pull-downs discarded or transferred into a new fresh eppendorf tube. Equal amounts of depleted supernatants were used in transcription assays. In the case of the pull-downs, the beads were washed thoroughly to remove any unbound material, once with 1 ml of TBS (2.5mM Tris-HCl pH 7.6, 15mM NaCl), 0.25mM NaCl, 0.5% Triton X-100 and a further 4 washes in 1 ml of TBS. After extensive washing, the bound material was released by the addition of an equal volume of 2 x protein sample buffer and analysed by SDS-PAGE and subsequent western blotting for the protein of interest. Beads were probed by immunoblotting.

2.16 c-Myc Antisense Experiment

ROSE cells were plated in 6 cm dishes out at $1x10^7$ cells/per well and left for 3 hours until cells adhered to the surface. Once adhered, the medium was replaced by medium containing either c-Myc-Sense oligonucleotide (5'-ATGCCCCTCAACGTG-3') or c-Myc-Antisense oligonucleotide (5'-CACGTTGAGGGGCAT-3') at a final concentration of 20 μM. The cells were left for 48 hrs with medium containing the

oligos, after which time total RNA was extracted with TRI Reagent and analysed by RT-PCR.

2.17 Transient transfection

Expression vectors were kindly provided by Robert N. Eisenman (pRc-CMV-Myc and empty vector) and Antonio Iavarone (Id2 and pcDNA3) to transform E.coli XL-1 Blue supercompetent cells (Stratagene). Large scale plasmid preparation using a QIAGEN Plasmid Maxi kit was performed to have sufficient material for the transfections. Again, plasmid preparations were verified and DNA concentrations determined by UV spectrophotometry.

2.17.1 Transfection with Superfect

Balb/c 3T3 (A31) and RB wild-type and RB knockout cells were transfected using the Superfect reagent (Qiagen). The transient transfection with Superfect required cells to be plated out at 3 x 10^5 cells/well on 6-well plates 24 hours prior to transfection, resulting in a confluency of ~70-80% at the time of transfection. Two wells were transfected per treatment, with a total of 4 μg of plasmid DNA per well of cells; this was comprised of 0.5 μg of VA1, 0.5 μg of SV40 CAT (Promega) and 3 μg of "empty" pRc-CMV (for c-Myc) or pcDNA3 (for Id2) expression vector, pRc-CMV-Myc or pId2. Mastermixes for each set of wells were made up comprising the appropriate plasmid DNA and neat DMEM media to give a volume of 100 μl per well. Following addition of 8 μl of Superfect per well, the reactions were vortexed and incubated at room temperature for 5 minutes. After this incubation period, 600 μl of DMEM (10% fetal calf serum (Sigma), 2mM L-glutamine, 100U/ml penicillin and 100 μg/ml streptomycin) was used to dilute each Superfect mix. Media was removed from the cells prior to addition of the mix and washed once with sterile-PBS. The reaction mix was added to the plates and they were swirled to ensure the wells were evenly covered. Treatment was carried out for 3 hours at 37°C before removing the mix, washing the cells once with warm PBS and applying fresh media. Cells were incubated for a further 48 hours to allow expression of transfected DNA; with media being renewed again after 24 hours. Cells were harvested and total RNA extracted for analysis by primer extension.

2.17.2 Transfection with Lipofectamine

Primary mouse embryo fibroblasts and RB-p107-p130 knockout cells were transfected using the Lipofectamine reagent (Gibco). The transient transfection with Lipofectamine required cells to be plated out at 3 x 10^5 cells/well on 6-well plates

24 hours prior to transfection, resulting in a confluency of ~70-80% at the time of transfection. Two wells were transfected per treatment with a total of 4 μg of plasmid DNA per well of cells; this was comprised of 0.5 μg of VA1, 0.5 μg of SV40 CAT (Promega) and 3 μg of "empty" pRc-CMV (for c-Myc) or pcDNA3 (for Id2) expression vector, pRc-CMV-Myc or pId2. Mastermixes for each set of wells were made up comprising the appropriate plasmid DNA and 0.5 ml of OptiMEM (Gibco) per tube containing plasmid DNA. In a separate tube, 0.5 ml of OptiMEM was mixed with 25 μl of Lipofectamine and then this reaction was transferred into the tube containing the plasmid with OptiMEM and they were mixed by pipetting up and down. The tubes were incubated for 45 min in the dark at room temperature. During the incubation period, each 6-well plate to be transfected was washed with 1 ml of OptiMEM per well, and then 0.76 ml of OptiMEM were added per well. The plates were left to incubate at 37°C for the rest of the incubation time (~30 min). After the incubation, 1 ml of the Lipofectamine-DNA-OptiMEM mix was added to each well and the plates were left at 37°C for 3 hrs in the incubator. After the incubation, the Lipofectamine-DNA mix was removed from each well and replaced with 3 ml per well of fresh medium. Cells were incubated for a further 48 hours to allow expression of transfected DNA; with media being renewed again after 24 hours. Cells were harvested and total RNA extracted for analysis by primer extension.

2.18 Primer Extension

Expression levels of the transfected pol III template VA1 and the CAT gene, which was co-transfected as an internal control for transfection efficiency, were analysed by primer extension. VA1 (5'-CACGCGGGCGGTAACCGCATG-3') or CAT (5'-CGATGCCATTGGGATATATCA-3') oligonucleotides were ^{32}P end-labelled using T4 polynucleotide kinase (PNK). For each primer extension reaction, 10 μg of total RNA (made up to 10 μl with DEPC-dH_20) and incubated at 80°C for 10 minutes with 9 μl of 5 x First Strand Buffer (Life Technologies) and 1 μl (2.5ng) of the relevant probe to act as a primer. Samples were immediately transferred to a 50°C hotblock for a further 2 hours incubation. 30 μl of an elongation mix (23 μl DEPC-dH_20, 0.5 μl 1 M DTT, 5 μl 5 mM dNTP mix (5 mM in DEPC-dH_2O), 0.5 μl 4 mg/ml actinomycin D, 0.5 μl RNasin, 0.5 μl (100U) of Superscript II Reverse Transcriptase (Life Technologies)) was then added to the samples to initiate reverse transcription and the reaction was allowed to proceed for 1 hour at 42°C. Reaction products were precipitated overnight at -20°C by the addition of 5 μl of 3 M sodium acetate and 125 μl of ethanol and subsequently pelleted by microcentrifugation at 13,000g for 15 minutes. Pellets were

washed with 300 μl of 75% ethanol and dried at 47°C for 5 minutes before being resuspended by vortexing for 10 minutes in 4 μl of formamide loading buffer (98% formamide, 10mM EDTA pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol FF). Electrophoresis through a 7 M urea 7% polyacrylamide gel was used to resolve the samples. The reaction products were detected by overnight exposure to autoradiography film at -80°C and quantified by scanning the autorad and analysing the image with the software NIH-Image.

2.19 Chromatin Immunoprecipitation

Cells (roughly 2 x 10^7 per immunoprecipitation) were collected and washed with ice-cold PBS and then crosslinked for 10 min at 37°C in 10 ml of 0.5% NP-40/PBS containing 1% formaldehyde. Crosslinked cells were washed with ice-cold 0.5% NP-40/PBS and incubated for 30 min with 40 ml of high salt buffer (0.5% NP-40, PBS, 1 M NaCl). Further washing with 0.5% NP-40/PBS was followed by hypotonic disruption for 30 min in 40 ml of low salt buffer (0.1% NP-40, 10 mM TrisHCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl). After centrifugation at 478g for 10 min, nuclei were obtained with 10 strokes through a 23-gauge needle and re-centrifuged. Nuclei were resuspended in 2.7 ml of low salt buffer and lysed with 300 μl of 20% sarkosyl. Samples were transferred to a sucrose cushion and spun for 10 min at 4,000g. After resuspending the pellet in 3 ml of TE (10 mM Tris, pH8.0, 1 mM EDTA), the process was repeated. After resuspension in 2 ml of TE, genomic DNA was sheared by sonication (Branson sonifier 250, 10 x 10 s, duty cycle 30%). Sonicated material was adjusted with 1/10 volume of x11 NET (1.56 M NaCl, 5.5 mM EDTA, 5.5% NP-40, 550 mM TrisCl, pH7.4) and immunoprecipitated overnight at 4°C with 20 μl (4 μg) of antibody. Protein A or protein G Sepharose beads were added for a further 2 h incubation and then recovered on Polyprep columns (BioRad). After washing twice with 10 ml of RIPA buffer (50 mM TrisHCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40), twice with 10 ml of LiCl buffer (10 mM TrisHCl, 250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, pH 8.0) and twice with 10 ml TE, beads were transferred to 1.5-ml tubes and immunoprecipitated material was eluted twice with 200 μl of 1% SDS in TE. To isolate precipitated DNA, proteins and antibodies were degraded by proteinase K treatment overnight at 42°C. DNA was extracted twice using phenol/chloroform/isoamylalcohol and ethanol-precipitated. Immunoprecipitated DNA was quantified by PCR performed on the input and bound fractions.

3 Activation of RNA Polymerase III Transcription by the Tyrosine Kinase Receptor ErbB2/Neu

3.1 Introduction

The human proto-oncogene *erbB2* (also referred to as HER2) and its rat homologue *neu* encode a 185 kDa transmembrane glycoprotein that is ubiquitously expressed in different tissues. The erbB2/neu gene was identified as the oncogene associated with the development of neuroblastomas in rats exposed to ethylnitrosourea in utero [193,194]. It belongs to class I of the superfamily of receptor tyrosine kinases. This class is known as the ErbB family and includes three other members: the epidermal growth factor receptor (EGFR), also termed ErbB1/HER1, ErbB3/HER3 and ErbB4/HER4 [195]. All members contain an N-terminal extracellular ligand-binding domain, a single membrane-spanning region and a C-terminal cytoplasmic tyrosine kinase domain [195]. Activation occurs upon ligand binding to the extracellular domain, which leads to the formation of homo- and heterodimers. Dimerization activates the tyrosine kinase activity of the intracellular domain in each monomer and triggers autophosphorylation of specific tyrosine (Tyr) residues within the cytoplasmic regions of each other (Fig. 3.1) [9,195]. Consequently, the phosphorylated tyrosine residues serve as docking sites for signaling molecules which will transfer activation to intracellular signaling cascades (Fig. 3.1) [13,195]. Ultimately, receptor activation will affect gene expression.

In the embryonic rat and human fetus, ErbB2/neu is expressed in a variety of tissues of epithelial, mesenchymal and neuronal origin, excluding those of hematopoietic origin . However, in adult animals immunoreactive ErbB2 is localized to epithelia. Cultured cell lines of epithelial or mesenchymal origin express moderate levels

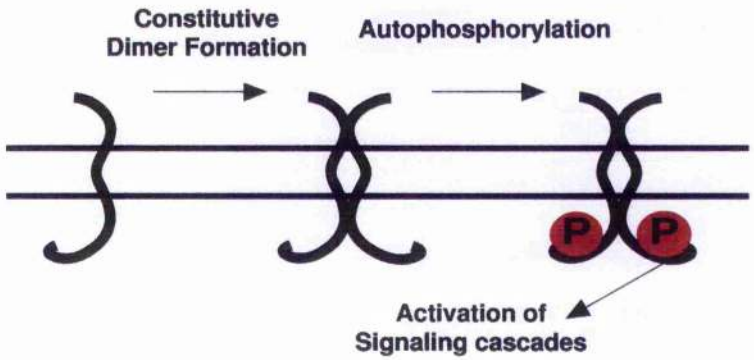

Figure 3.1: Activation of the ErbB2/neu receptor. Upon ligand binding to the extracellular domain or through overexpression, ErbB2 protein binds to ErbB2 molecule or to other members of the Erb family, creating homo- or heterodimers respectively. The formation of dimers produces autophosphorylation of Tyr residues which will transduce the activation to signaling cascades.

of ErbB2 (approximately 10^4 molecules/cell) [13]. This receptor is fundamental in development, since ErbB2 null mice die at midgestation (E10.5) due to trabeculae malformation of the heart [196]. It is also required for proliferation and differentiation. In particular, this has been studied in the mammary gland. Most of the proliferation and differentiation of this organ happens postnatally. Expression of a dominant-negative mutant of ErBb2 in the mammary gland of mice results in defective lobuloalveoli and reduced milk protein secretion, although ductal growth is normal [197].

3.1.1 Ligand

Although there are several ligands that can bind and activate the ErbB receptors, no direct ligand has been identified for ErbB2 yet. The first candidate ligands to be studied were the neuregulin (NRG) peptides (also referred to as heregulins, neu differentiation factors, acetylcholine receptor-inducing activity and glial growth factor) [13]. NRGs contain epidermal growth factor-related domains which were shown to activate Tyr phosphorylation and substrate binding of ErBb2. However, this effect was dependent upon the presence of ErbB4 or ErbB3, since in lymphoblastoid T cell lines and Sf9 insect cells, as well as fibroblasts that were engineered to express ErbB2 solely, NRG couldn't stimulate dimerization nor Tyr phosphorylation [198–200]. Therefore, NRGs activate ErbB2 by binding to other ErbB members and stimulating heterodimerization with ErbB2. In fact, ErbB2 is the

preferred heterodimerization partner of ErbB family members, mediating lateral signal transduction from them [201]. Other ErbB2 agonists that have been proposed include the neu activating factor (NAF), and the mucin MUC4/sialomucin complex (SMC). NAF was found in medium from a transformed human T cell line ATL-2. It specifically binds to the ErbB2 extracellular domain stimulating dimerization and tyrosine kinase activity [202]. In addition, Samanta et. al. found that NAF binds to purified ErbB2 expressed in baculovirus and promotes dimerization in vitro [203]. However, no further studies have been published on this ligand since 1994, and the scientific community hasn't completely accepted this protein to be a true ErbB2 ligand. MUC4/SMC (also referred to as ascites sialoglycoprotein-2 or ASGP2) is a major cell surface protein in rat ascites 13762 cells [204]. Its transmembrane subunit contains two EGF-like domains, one of which binds directly to and specifically activates ErbB2 [204, 205]. However, MUC4/SMC rather than a ligand, is a modulator of ErbB2's activity, potentiating its phosphorylation in the presence of other ligands like NRG1 [205].

3.1.2 ErbB2/neu and Cancer

There is a considerable amount of data demonstrating the participation of ErbB2/neu in cancer development and progression. Overexpression of ErbB2/neu is implicated in the genesis of carcinomas affecting different organs, like breast, ovary, stomach, colon, kidney, bladder and salivary gland [13]. In human breast and epithelial ovarian cancer, ErbB2 is overexpressed in 20-30% of the cases and is usually associated with poor prognosis [206–210]. The overexpression is generally caused through gene amplification, and tumours from patients containing multiple copies of the gene respond poorly to chemotherapy and have an increased propensity to metastasize [206 210]. Furthermore, their tumours have shorter time to relapse and a shorter overall survival, and the presence of *erbB2* gene amplification can be used as a predictor of clinical outcome [206–210]. ErbB2 overexpression is also accomplished through transcriptional upregulation of the *erbB2* gene, localized on chromosome 17q21 [206,207]. When ErbB2 is overexpressed, constitutive homodimerization and kinase activation is achieved in the absence of ligand (Fig. 3.1) [211].

The rodent models of neuroblastomas induced by exposure to N-nitrosoethylurea present a different mechanism of ErbB2/neu activation [212, 213]. In these cases, a single point mutation in the membrane-spanning region, from valine at position 664 to glutamic acid yields constitutive homodimerization as well as kinase activation and elevated Tyr phosphorylation of cellular substrates [212]. This activating

mutation alone is sufficient to induce Schwann cell immortalization and transformation, without requiring activation of another oncogene [214]. Analysis of a panel of neu proteins with second-site mutations has shown that not only the Glu664 substitution is responsible for dimerization and transformation but also the aminoacids surrounding it, forming the domain Val 663-Glu-664-Gly665 (VEG) [215]. If this domain is localized elsewhere within the transmembrane region, it can still promote weak dimerization but its transforming activity is lost, suggesting that in some cases receptor dimerization is not sufficient for transformation [216]. In fact, the enhanced receptor dimerization seen in the neu Glu664 substitution appears not to be a direct effect of Glu contacts, but of changes produced in the relative orientation of the dimerization interface [217]. Moreover, a recent report has demonstrated that a sequence (VVI at position 966-968) in the intracellular domain is responsible for homomeric association of ErbB2 in the absence of ligand [218]. When these amino acids are mutated to alanines, no self-association is observed with wild type or mutant ErbB2, indicating that the Glu664 substitution only causes stabilization of the homodimer, but does not initiate ErbB2 self-association [218]. It is interesting to note that neither the Glu664 substitution nor any other mutation have been identified in human malignancies. Nevertheless, an ErbB2 gene polymorphism at codon 655[1] (isoleucine to valine) in the coding region corresponding to the transmembrane domain has shown an increased risk of breast cancer [219]. The same polymorphism was studied for the susceptibility to develop bladder cancer [220]. Although no association with the Ile/Ile genotype was found, an increased risk of disease progression was observed in tumours containing this genotype [220].

As mentioned before, ErbB2 is involved in the pathogenesis of different types of human cancers. Breast cancer and ovary carcinoma account for one quarter of cancer-related female deaths in the United States [206]. In the case of epithelial ovarian cancer, it is usually detected when patients are in advanced stages of the malignancy, with poor chance of survival. Therefore, it is paramount to understand the changes involved in the neoplastic transformation of the ovarian surface epithelium which might also be entailed in other malignancies. Since ErbB2 overexpression is one of the changes observed in this malignancy, it is important to study what other cellular changes this overexpression leads to. Cancer cells proliferate at increased rates, requiring to double their DNA and cellular contents faster than normal cells do. Indeed, elevated growth rates are observed in transformed cells. Since

[1]This human codon (655) does not correspond to the rat 644 codon which is frequently mutated from valine to glutamate inducing constitutive activation. The corresponding human codon is 659, which also encodes a valine [218].

pol III transcripts are important determinants of growth rate (as they are involved in protein synthesis), ErbB2 overexpression might trigger activation of pol III transcription. Winter et. al. have shown that epithelial ovarian tumours present high levels of pol III transcripts compared to adjacent healthy tissue, although ErbB2 expression was not analysed [12]. Furthermore, overexpression of pol III transcripts has been observed in breast, lung and tongue carcinomas, the same types of carcinomas where ErbB2 overexpression is involved [171,172,206]. Therefore, the regulation of pol III transcription by ErbB2/neu was investigated.

To study ErbB2/neu regulation of pol III transcription, a cell line was used instead of tumour samples, since tumour cells have the disadvantage of presenting modifications other than ErbB2/neu overexpression. Furthermore, a cell line allows one to analyse the mechanisms involved in ErbB2 regulation of pol III transcription. The ROSE 199 cell line was chosen for this research because it is a spontaneously immortalized rat ovarian surface epithelial cell line that is normal and nontumourigenic[2] [221]. This cell line was stably transfected by Ponder's group with ecotropic retroviruses containing the mutated rat *neu* oncogene (with the activating Val to Glu664 substitution) or the *β-galactosidase* (β-gal) gene, under the control of the β-actin promoter [190]. The untransfected cells express low levels of endogenous neu; after *neu* oncogene is transfected, an eightfold induction in its expression is observed [190]. The increase in neu expression is not due to the process of transfection *per se*, since the cells transfected with β-gal do not show any change in neu protein levels [190]. For this reason, ROSE-βgal transfected cells were utilised as a control for the experiments reported in this and in chapter four.

[2]It is interesting to note that spontaneous transformation of the ovarian surface epithelium of animals is very rare, so animal transformed cell lines from this tissue are quite unusual [190].

3.2 Results

3.2.1 Pol III Transcription is Increased in Neu Overexpressing Cells

The ROSE-neu transfected cells (ROSE-neu) exhibit an increase in proliferation, induction of anchorage independence, reduced intercellular and cell/substrate adhesion and increased invasive potential with the creation of tumours relative to the parental ROSE 199 or ROSE-βgal transfected cells (ROSE-βgal) [190]. One of the changes induced by neu overexpression is a reduction in the doubling time of almost one third, compared to that of ROSE-βgal (ROSE-neu take 12.5 hrs while ROSE-βgal take 17.2 hrs to double), so it can be concluded that ROSE-neu cells are proliferating faster, thus might present faster growing rates. Since pol III transcripts are involved in cellular growth, their expression was compared in these two cell types. First, expression of the B2 gene was tested. Induction of the B2 transcript in malignant cells and in cells infected with viruses like SV40 and papillomavirus is well documented [154, 222, 223]. Furthermore, Crone et. al. have shown that downregulation of the cytoplasmic B2 transcript causes a direct reduction in proliferative rate of up to 80% [224]. Therefore, if neu overexpression is accelerating the growth rate of ROSE cells, an induction of the B2 gene might be expected. Indeed, primer extension analysis revealed that the RNA levels of the B2 transcript are induced by neu overexpression compared to ROSE-βgal transfected cells (Fig. 3.2).

The upregulation seen in the B2 transcript could be a specific effect of neu on the expression of this gene and not a general activation of pol III transcription. To assess if neu upregulation of the B2 transcript reflects a general effect upon pol III transcription, the expression of different class III genes was tested. RT-PCR was performed to assay the levels of primary transcripts from $tRNA^{Tyr}$, $tRNA^{Leu}$, and U6 snRNA genes by using as template cDNAs derived from RNA extracted from each cell group (ROSE-βgal or ROSE-neu transfected cells). As shown in Fig. 3.2, the levels of all the pol III transcripts tested are increased compared to the control. This effect upon pol III transcription by neu overexpression is specific, since the mRNA levels of the pol II transcribed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) did not change (Fig. 3.2, *Bottom left*). The tRNA levels reflect pol III transcriptional activity at the time of extraction of RNA and not the build up of these products, because the primers used for the tRNAs were designed to the intron sequences of short-lived precursor tRNAs, which are rapidly degraded. These results demonstrate that neu overexpression activates pol III transcription

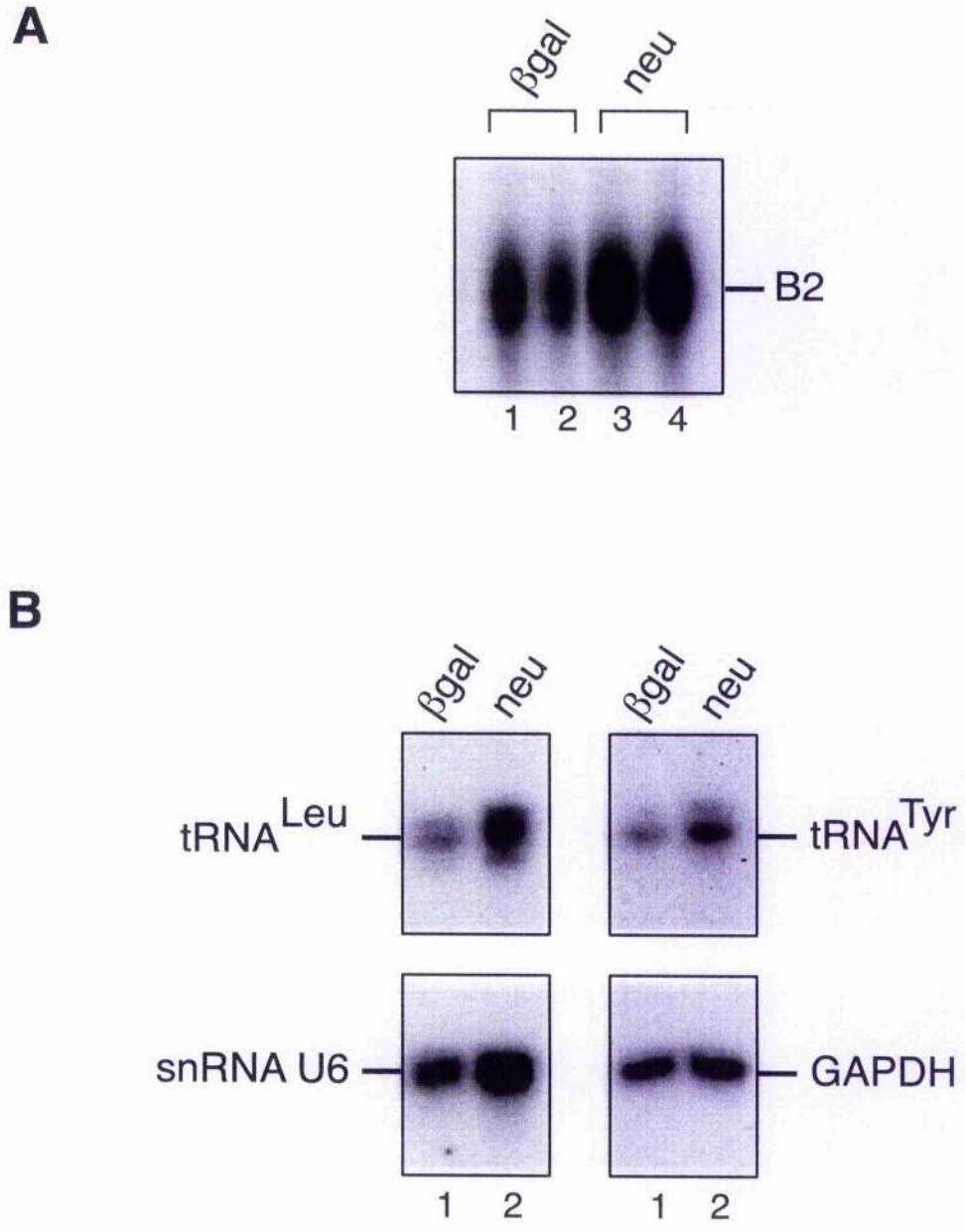

Figure 3.2: Induction of pol III products by neu overexpression. (A) Primer extension analysis of total RNA (10μg) extracted from ROSE-βgal (lanes 1 and 2) or ROSE-neu transfected cells (lanes 3 and 4). The probe used was against the B2 product. (B) RT-PCR from the the same total RNA samples used in panel A. cDNA was prepared from 3μg of RNA and amplified by PCR using primers specific for $tRNA^{Leu}$ (*top right*), $tRNA^{Tyr}$(*top left*), U6 snRNA (*bottom right*) and GAPDH (*bottom left*).

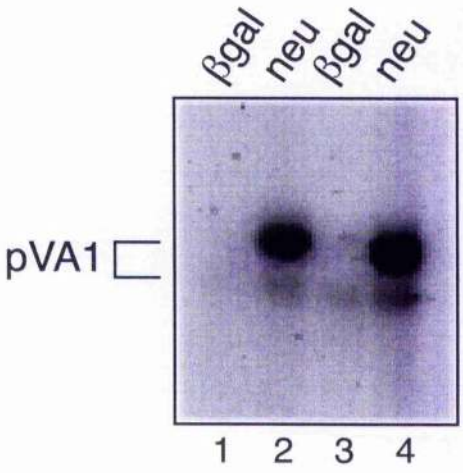

Figure 3.3: Extracts from neu overexpressing cells contain higher pol III transcriptional activity. The class III gene, pVA1, was utilised as template for an in vitro transcription assay to assess the activity of pol III transcription. 15μg of whole cell extracts prepared from ROSE-βgal or ROSE neu cells were used in each reaction. Lanes 1 and 3 are from two different extractions of ROSE-βgal cells, while lanes 2 and 4 are from two different extractions of ROSE-neu cells.

from different class III genes, and therefore can be regarded as a general activator of pol III transcription.

To further address the transcriptional activity of pol III in the neu overexpressing cells, an in vitro transcription assay was performed. Whole cell extracts prepared from ROSE-βgal or ROSE-neu cells were incubated with the adenovirus VA1 gene for 50 min at 30°C. Then the RNA produced was extracted and analysed in a 7% polyacrylamide gel. As shown in Fig. 3.3, extracts from ROSE-neu transfected cells are significantly more active than those from ROSE-βgal transfected cells. From these experiments it can be concluded that pol III transcription is increased in neu overexpressing cells.

3.2.2 TFIIIC2 Expression Is Not Affected by Neu Overexpression

There are several possible mechanisms that could mediate the induction of pol III transcription by the overexpression of neu. The first mechanism investigated was the induction of TFIIIC2. Increased activity of TFIIIC2 has been observed in ovarian tumours, as well as in adenovirus-infected and SV40-transformed cells [12, 67, 160, 162, 225]. In human ovarian tumours with induced pol III activity, the deregulation

in TFIIIC2 appears to reflect the overexpression of the TFIIIC2 subunits, at the mRNA and protein levels [12]. Therefore, TFIIIC2 overexpression was investigated in the ROSE model. Again, RT-PCR was performed with cDNAs produced from mRNA of ROSE-βgal and ROSE-neu cells to assay the levels of different TFIIIC2 subunits. In contrast to expected, the mRNA levels of all TFIIIC subunits were reduced in ROSE-neu cells compared to the control cells (Fig. 3.4A and data not shown). Since the mRNA levels of the TFIIIC2 subunits might not reflect the actual amount of protein present in the cells, the protein levels of the subunit TFIIICα was examined by immunoblot analysis of whole cell extracts. As shown in Fig. 3.4B, the levels of this subunit are the same in ROSE-βgal and ROSE-neu cells, suggesting an increase in protein stability of the subunits in neu-overexpressing cells. Furthermore, TFIIIC2 activity is slightly elevated in extracts from ROSE-neu cells compared with that of ROSE-βgal cells [226]. The overexpression of the U6 transcript induced by neu overexpression could not have been explained through an induction of TFIIIC2 activity, since this TF is not required for U6 transcription [42]. Therefore, these results implicate a different mechanism used by neu for pol III transcription upregulation.

3.2.3 Signal Transduction from Neu

Neu's activation induces its carboxyl-terminal kinase which phosphorylates Tyr residues within a 200-300 amino acid stretch at the carboxyl-terminus [227]. The phosphorylated Tyr become docking sites for downstream cytoplasmic or plasma membrane associated proteins involved in the transduction of signals to the nucleus [228]. Downstream proteins bind stably to these sites through SH2 (Src homology) domains and trigger different signaling cascades, depending on the protein bound [13]. Proteins that have been seen to interact specifically with ErbB2/neu phosphorylated Tyr residues include phospholipase Cγ1 (PLCγ1) [229, 230], c-Src [231], and Grb7 (growth factor bound 7) [232, 233] as well as proteins involved in the modulation of the Ras/MAPK (mitogen-activated protein kinase) pathway by either promoting the active Ras-GTP complex formation through Sos GTP exchange proteins like Shc [234, 235], Grb2 [232, 234, 235] and Nck [236] or by accelerating the hydrolysis of Ras-GTP to its inactive Ras-GDP state like Ras-GAP (GTPase-activating protein) [229, 230, 236] (Fig. 3.5). The phosphatidylinositol 3 kinase (PI3K) pathway, implicated in cell survival, has also been observed to be active in ErbB2/neu overexpressing cells, although this activation is likely to be through ErbB3 interactions with the PI3K regulatory subunit p85 [237]. The contribution

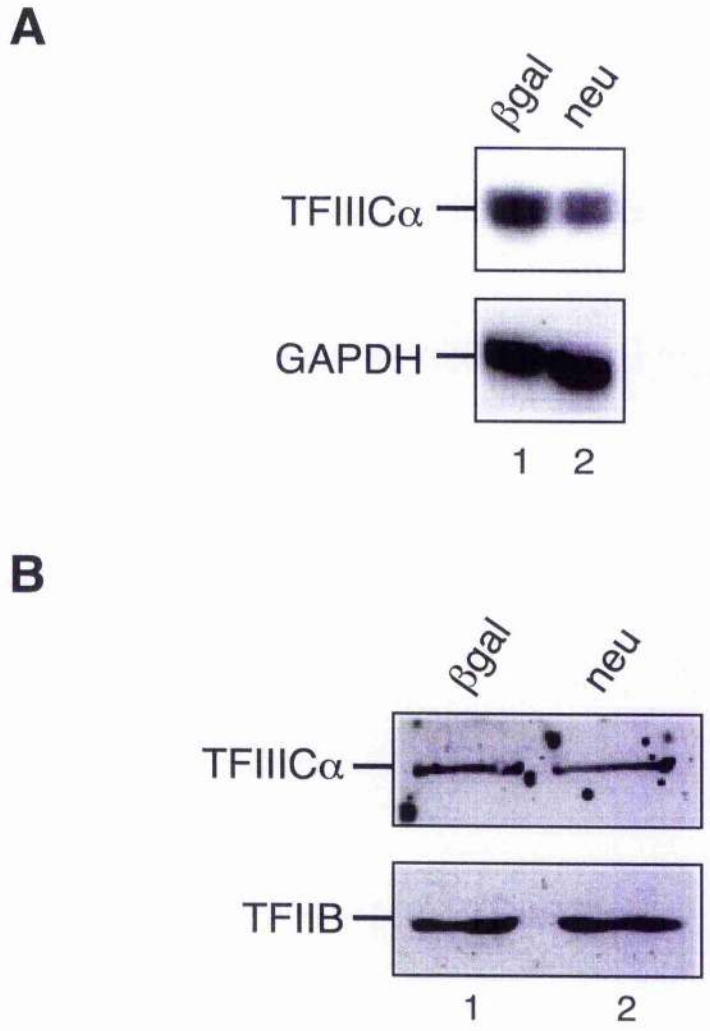

Figure 3.4: Expression of the TFIIIC subunit, TFIIICα, in ROSE-βgal and ROSE-neu cells. (A) cDNAs prepared from total RNA (3μg) extracted from ROSE-βgal (lane 1) or ROSE-neu cells (lane 2) were used as template for RT-PCR analysis. The primers used were designed against TFIIICα (*upper panel*) or GAPDH (*lower panel*). (B) Proteins (100μg) extracted from ROSE-βgal (lanes 1) or ROSE-neu cells (lanes 2) were resolved in a 7.8% polyacrylamide gel and then analysed by Western blotting with antiserum Ab2 against TFIIICα (*upper panel*) or the rabbit antibody C-18 against TFIIB (*lower panel*).

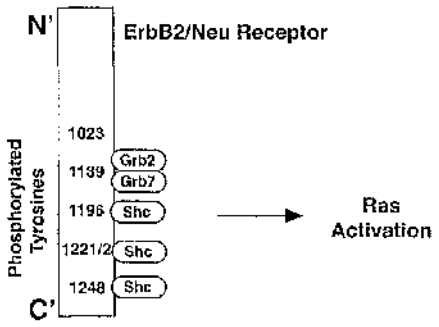

Figure 3.5: Binding of signaling molecules to specific phosphotyrosine residues from the ErbB2/neu receptor. The tyrosine residues that have been found to be phosphorylated in the ErbB2/neu receptor are indicated [227]. The protein Shc has been found to bind to Tyr1196 and Tyr1248 via its PTB domain, and to Tyr1221/2 and Tyr1248 via its SH2 domain [234, 235]; Grb2 binds to Tyr1139 through an SH2 domain [228, 234]; and Grb7 interacts with Tyr1139 through its SH2 domain [232].

towards tumourigenesis of the signaling pathways activated by ErbB2/neu still remains to be elucidated.

Activation of these pathways induces changes in gene expression which will affect the cell's proliferation state. The activation of Ras is of particular importance, since it plays a pivotal role in a wide variety of cellular processes, as well as being frequently deregulated in carcinogenesis. It can activate multiple effector-mediated signaling pathways like the Raf-MEK-MAPK or PI3K cascades (Fig. 3.6A) [238]. The mammary tissue of transgenic mice overexpressing v-Ha-ras present mammary carcinomas [239]. In addition, Ras effector pathways are found activated in many breast cancer samples and derived cell lines, suggesting Ras activation in primary tumours [228]. The activation observed in transformed cells is probably due to the action of receptors like ErbB2/neu on its function, although Ras mutations are extremely common (~30% of human tumours present mutated Ras). Infection with HBV, which is implicated in hepatocarcinogenesis, activates the Ras/MAPK pathway [240]. This activation is essential for successfull transformation of hepatocytes by the HBV X protein [241]. As mentioned in the introduction, HBV X protein induces pol III transcription through activation of the Ras/MAPK pathway [170]. Therefore, the activity of this pathway was investigated in ROSE-βgal and ROSE-neu transfected cells. To assess this, western blot analysis of whole cell extracts was performed. The membrane was probed with an antibody that recognises the active phosphorylated forms of MAPKs/extracellular signal-regulated kinase-1/2 (Erk1/2).

As shown in Fig. 3.6B, upper panel, ROSE-neu transfected cells present higher levels of ERK1/2 phosphorylation than control cells. This result suggests that neu overexpression is causing an increased activation of the Ras/MAPK pathway which might have an effect on pol III transcriptional upregulation.

Since neu overexpressing cells proliferate faster than control cells, it would be interesting to study if other pathways involved in cell growth regulation, in particular that of ribosome synthesis are being activated. The ubiquitous mitogen-activated Ser/Thr kinase, p70 ribosomal protein S6 kinase ($p70^{S6K}$) phosphorylates the 40S ribosomal protein S6, which drives translation of 5' TOP (terminal oligopyrimidine tract) mRNA [242]. This family of mRNA generally encode ribosomal proteins and components of the translation apparatus like elongation factors; thus $p70^{S6K}$ activation is a prerequisite for protein synthesis [242]. As neu activation has been shown to stimulate the $p70^{S6K}$ pathway [243], kinase activity was tested in the ROSE system. Immunoblot analysis of whole cell extracts using an antibody against phosphorylated $p70^{S6K}$ showed that neu overexpression causes increased phosphorylation of the kinase (Fig. 3.6B, middle panel). The antibody used was raised against phosphorylated Thr-389, which is essential for kinase activity [244]. The phosphorylation of Thr-389 is mediated mainly through the mammalian target of rapamycin (mTOR) [244], although recently it has been reported that the Raf/MAPK pathway can also phosphorylate this residue [245]. As shown in Fig. 3.6A, activation of mTOR can be through the PI3K pathway which responds to Ras activation, although it can also be activated by amino acids [246]. TOR has been ascribed as a central controller of cell growth, which regulates a diverse set of growth-related readouts including pol III transcription [246].

The data presented so far demonstrate that the ROSE-neu transfected cells behave in a similar fashion to ErbB2/neu overexpressing cells studied before, activating the Ras/MAPK and mTOR signaling cascades. These pathways have been shown to induce pol III transcription, thus it is very probable that they are involved in neu's regulation of this process.

3.2.4 Cyclin Levels in ROSE Transfected Cells

ROSE-neu cells present faster rates of growth than control cells. This could be explained through the activation of the Ras/MAPK pathways, since it contributes to cell cycle progression from G1 to S phase [247, 248]. Overexpression of mutationally activated Ras leads to shortening of the G1 phase of the cell cycle [249]. The Ras/MAPK pathway achieves this by stimulating the activity of TFs which will in-

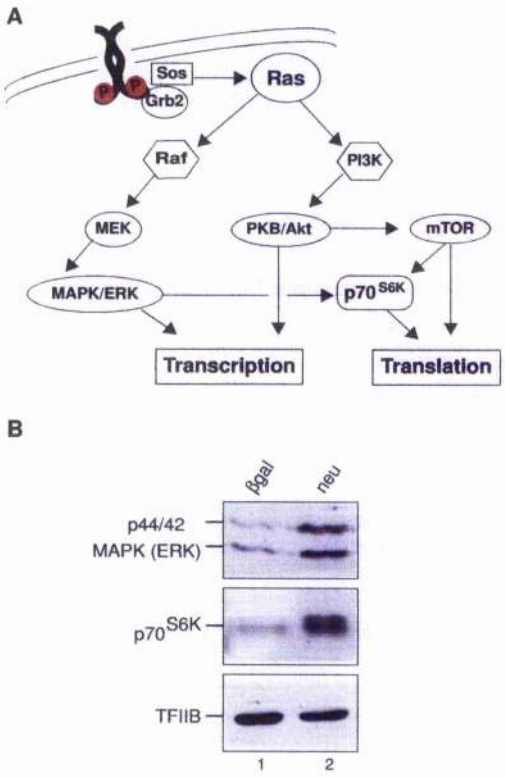

Figure 3.6: Neu overexpression causes activation of the Ras/MAPK and $p70^{S6K}$ pathways. (A) A suggested cascade triggered by neu activation for the induction of transcription and translation. Neu activation produces phosphorylation of its carboxy-terminal tyrosine residues which will serve as docking sites for Grb2. Grb2 will transduce the activating signal to Ras through Sos. Activated Ras will then induce the MAPK pathway and the mTOR pathway, maybe through PI3K, which will activate transcription and translation of genes involved in cell growth and cell proliferation.(B) Western blot analysis of proteins ($50\mu g$) extracted from ROSE-βgal (lane 1) or ROSE-neu cells (lane 2) resolved in a 7.8% polyacrylamide gel. Immunoblotting was performed with antibodies against the phosphorylated forms of ERK1/2 (p42/44) (*upper panel*), phosphorylated $p70^{S6K}$ (*middle panel*) or TFIIB (*lower panel*). TFIIB was used as a loading control.

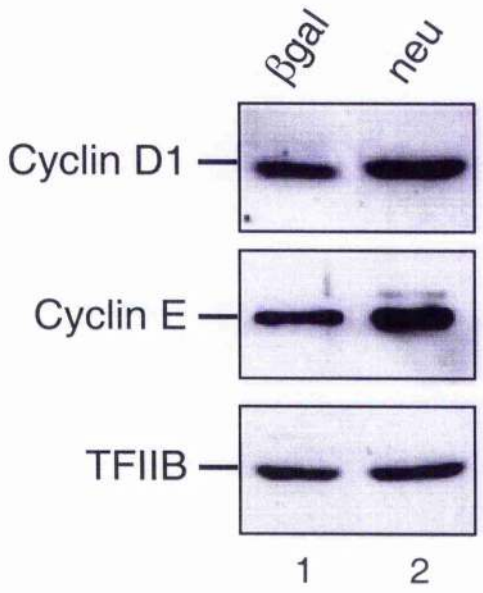

Figure 3.7: Neu overexpressing cells contain higher protein levels of cyclin D1 and cyclin E. Proteins (50μg) extracted from ROSE-βgal (lanes 1) or ROSE-neu cells (lanes 2) were analysed by Western immunoblotting with antibodies from Santa Cruz Biotechonology against cyclin D1 (antibody 72-13G, *upper panel*), cyclin E (antibody C19, *middle panel*), or TFIIB (antibody C-18, *lower panel*).

duce transcription from cyclin D genes [247,249]. The TFs involved in the induction of cyclin D1 expression have not yet been elucidated, but there is evidence to suggest that the AP1 complex of transcription factors (consisting of c-Fos, Fos B, Fra-1, Fra-2, c-Jun, JunB and JunD), c-Ets-2, Sp1 and E2F-1 might be involved [250,251]. Activation of mTOR also plays a role in cyclin D1 protein availability. mTOR controls cyclin D1 translation by activating the translation initiation factor eIF4E, which regulates translation of mRNAs containing a highly structured 5' untranslated region, like that of the cyclin D1 transcript [252]. Since ROSE-neu transfected cells present both Ras/MAPK and mTOR activation, the cyclin D1 levels are most probably induced.

Aberrant expression of cyclin D1 has been observed in breast tumor cell lines and the majority of breast cancers and in epithelial ovarian tumours [249,253]. Mammary tumours produced by overexpression of wild-type neu contain increased cyclin D1 protein levels [250]. Lee et. al. also demonstrated that neu-induced transformation of Rat-1 cells requires cyclin D1 induction, and that this induction involves activation of the Ras/MAPK pathway, as well as Rac, Rho, c-Jun N-terminal kinase and p38

[250]. Therefore, protein levels of cyclin D1 were analysed in the ROSE transfected cells by western blot. Figure 3.7, upper panel, shows that indeed cyclin D1 levels are induced in the neu overexpressing cells.

Cyclin E induction is also required for G1/S transition [254]. Its expression is regulated by the TFs E2F and c-Myc [251,255]. Overexpression of cyclin E has been observed in breast cancer and in epithelial ovarian tumours and it is significantly associated with malignancy in ovarian tumours [256–258]. When cyclin D1 function is lost, activated mutant neu can no longer induce oncogenesis of the mammary gland [259]. However, 35% of the cyclin D1 null mice regain mammary tumour potential through overexpression of cyclin E [259]. These data suggest that neu's induction of both cyclin D1 and cyclin E expression are important events for the modulation of neu-induced tumourigenesis. Since ROSE cells transfected with neu are transformed cells that produce tumours when injected into mice peritoneal cavities, the protein levels of cyclin E were investigated. As expected, cyclin E levels are also increased by neu overexpression (Fig. 3.7). Both cyclin D1 and E induction is a specific event, since TFIIB protein levels remain constant in normal and transformed cells (Fig. 3.7, lower panel). Thus, ROSE-neu transfected cells induce cyclin D1 and cyclin E, which are required for transformation. As these cyclins have been shown to regulate pol III function through inactivation of the RB protein, this mechanism was explored.

3.2.5 RB Activity

Induction of cyclin levels are required for G1/S progression. Cyclin D/cdk4/cdk6 complexes partially phosphorylate RB [260,261]. This process is completed by cyclin E/cdk2 [260, 261], which is activated shortly after cyclin D/cdk4/cdk6 activation, producing complete RB phosphorylation and abrogating its growth inhibitory activity upon TFs like E2F and TFIIIB, which will then induce transcription of different genes involved in cell cycle progression and growth (Fig. 3.8) [4,178]. As mentioned in the thesis introduction, RB phosphorylation status plays an important role in controlling pol III transcriptional activity. When RB is in the hypophosphorylated form, it binds to TFIIIB and blocks its activity, inhibiting pol III transcription; however when it is phosphorylated, it can no longer bind to TFIIIB and pol III transcription increases [3, 4, 179, 182]. Since ROSE-neu cells overexpress cyclin D and E proteins, RB might be hyperphosphorylated with the concomitant activation of pol III transcription. For this reason, the phosphorylation state of RB was investigated in the ROSE system.

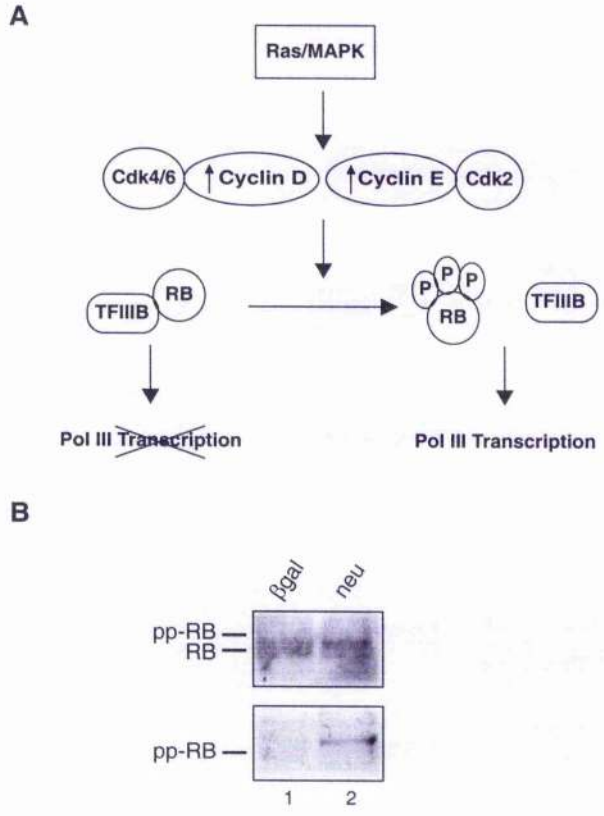

Figure 3.8: RB is hyperphosphorylated in ROSE-neu cells compared to control. (A) Mechanism of activation of pol III transcription by inactivation of RB through cyclinD,E/cdk complexes. An increase in the protein levels of cyclin D and cyclin E produce activation of the cyclin D/cdk4/6 and cyclin E/cdk2 complexes which are involved in the phosphorylation of RB. Hypophosphorylated RB binds to TFIIIB and inhibits pol III transcription; however, when it is phosphorylated by the cyclin/cdk complexes, it can no longer bind to RB and repression of pol III transcription is released [11]. (B) Western blot from whole cell extracts (50μg) prepared from ROSE-βgal (lane 1) or ROSE-neu cells (lane 2). Upper panel shows that RB from ROSE-neu extracts is primarily in the hyperphosphorylated form compared to that of ROSE-βgal (*upper panel*). The antibody used was G3-245. *Lower panel* shows again that RB from the neu overexpressing cells is highly phosphorylated. The antibody used was specific for phosphorylated serine 780.

First, western blotting with an antibody that reacts with both the hypo- and hyperphosphorylated forms of the RB protein was tested in ROSE whole cell extracts. The two forms of RB can be distinguished by their electrophoretic mobility, since the hyperphosphorylated form has retarded mobility compared to the hypophosphorylated protein in SDS-polyacrylamide gels [262,263]. As expected, a bigger fraction of the hyperphosphorylated form of RB is present in ROSE-neu cells compared to RB present in ROSE-βgal cells (Fig. 3.8). This demonstrates that phosphorylation of RB is increased in neu overexpressing cells, eventhough both groups are cycling. To test this further, an antibody that exclusively recognizes cyclin D/cdks phosphorylation of RB at serine 780 was utilised [264,265]. Extract from neu overexpressing cells gave a stronger signal compared to that of control cells (Fig. 3.8). Taken together, these results demonstrate that RB is being phosphorylated as a result of neu overexpression.

3.2.6 TFIIIB Association with RB

Once it had been demonstrated that RB phosphorylation is increased in the neu-overexpressing cells, association of RB with TFIIIB in the two ROSE-transfected cell types was investigated. Since only the underphosphorylated form of RB can bind to TFIIIB [4], it would be expected that less TFIIIB from neu overexpressing cells is associated with RB than in control cells. Evidence of this can be seen in figure 3.9A, where coimmunoprecipitation experiments show that the interaction between RB and the TFIIIB subunit Brf in ROSE-neu extracts is diminished compared to that in ROSE-βgal extracts. For this experiment, an antibody raised against RB was used to immunoprecipitate proteins from ROSE-transfected cells and the precipitated material was then probed by Western blotting using an antiserum for the Brf subunit of TFIIIB. Precipitation with an antibody against TAF_I48 was used as a negative control (Fig. 3.9). The same samples were also analysed for RB concentration in each cell type (data not shown), demonstrating that RB is present at similar levels in both. From this, it can be concluded that TFIIIB from ROSE-neu cells is less tightly associated with the inhibitor RB, and this suggests that the increase in pol III transcription observed in these cells might be through activation of TFIIIB.

3.2.7 TFIIIB Regulation by Neu

The reduction observed in the association between RB and Brf might be one mechanism by which neu induces pol III transcription. In addition to this effect, neu might be inducing pol III transcription by mechanisms independent of RB. Activa-

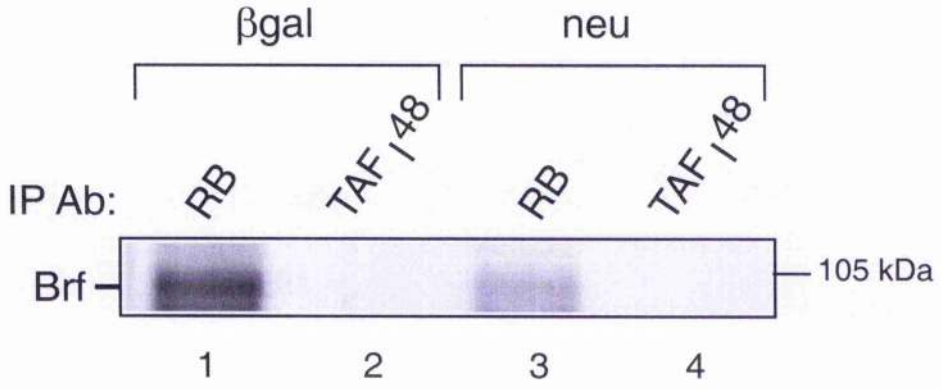

Figure 3.9: Decreased association between TFIIIB subunit Brf and RB in ROSE-neu extracts. (A) 150μg of ROSE-βgal (lanes 1 and 2) or ROSE-neu extracts (lanes 3 and 4) were immunoprecipitated with anti-RB antibody C-15 (lanes 1 and 3) or anti-TAF_I48 antibody (lanes 2 and 4). Precipitated material was resolved on a 7.8% SDS-polyacylamide gel and then analysed by Western immunoblotting with antiserum against Brf.

tion of the Ras/MAPK pathway has been shown to stimulate pol III transcription not only by inducing RB phosphorylation, but also by producing an elevation in the protein levels of the TFIIIB subunit TBP [170]. To address this possibility, Western blot analysis was carried out to determine the amount of TBP and Brf present in whole cell extracts from ROSE-neu cells compared to ROSE-βgal cells. The signals of these proteins from ROSE-neu extracts appeared as a doublet for both Brf and TBP (Fig. 3.10A). Nevertheless, it can be observed that the levels of TBP are higher in the ROSE-neu cells compared with the ROSE-βgal (Fig. 3.10A, lower panel).

The retardation in electrophoretic mobility of these proteins suggests that a modification is occurring. There are several modifications that could affect the electrophoretic mobility of a protein, including phosphorylation, acetylation, and ubiquitination. Recently, it has been demonstrated that casein kinase 2 (CK2) is able to phosphorylate TBP and Brf *in vivo*, stimulating pol III transcription in both mammals and yeast [266–268]. The data presented in these reports suggested that phosphorylation of Brf and TBP might facilitate recruitment of TFIIIB by TFIIIC to the promoter [266, 268]. Furthermore, activated ERK phosphorylation of Brf has been detected in mouse fibroblasts after addition of serum, and this phosphorylation is required for pol III transcriptional activation [269]. Thus, phosphorylation was tested as the possible modification observed in the neu overexpressing cells. Whole cell extracts were treated with phosphatase and then analysed by Western blot.

Although the experiment was performed several times and with different conditions, no clear result could be obtained for Brf. In the case of TBP, phosphatase treatment did not have any effect on the doublet (data not shown). From this result it could be concluded that the modification observed in TBP is not phosphorylation; however, it has to be considered whether the experiment did not work, maybe because it was not properly designed or because different reagents had to be used, since no clear result could be seen for Brf.

To investigate this further, the induction of TBP mRNA was studied. RT-PCR was performed using cDNAs prepared from the mRNA of ROSE-βgal and ROSE-neu cells. The primers utilised were designed to recognise TBP mRNA. Neu overexpression produces an increase in the mRNA levels of TBP compared to control (Fig. 3.10B). This result suggests that activation of pol III transcription by neu overexpression not only involves RB inactivation, but also an increase in TBP mRNA. Overexpression of TBP has been shown to induce transcription of tRNA and U6 snRNA genes in *Drosophila* cells [270]. This is quite interesting since the same genes are induced by neu overexpression (Fig. 3.2). Furthermore, it has also been observed that TBP mRNA is overexpressed in human breast carcinomas compared to normal tissue [271]. Since neu is overexpressed in 20 to 30% of human breast tumours, the induction observed in TBP mRNA might be regulated through ErbB2/neu. The result obtained with the ROSE-neu overexpressing cells indicate that this might be the case.

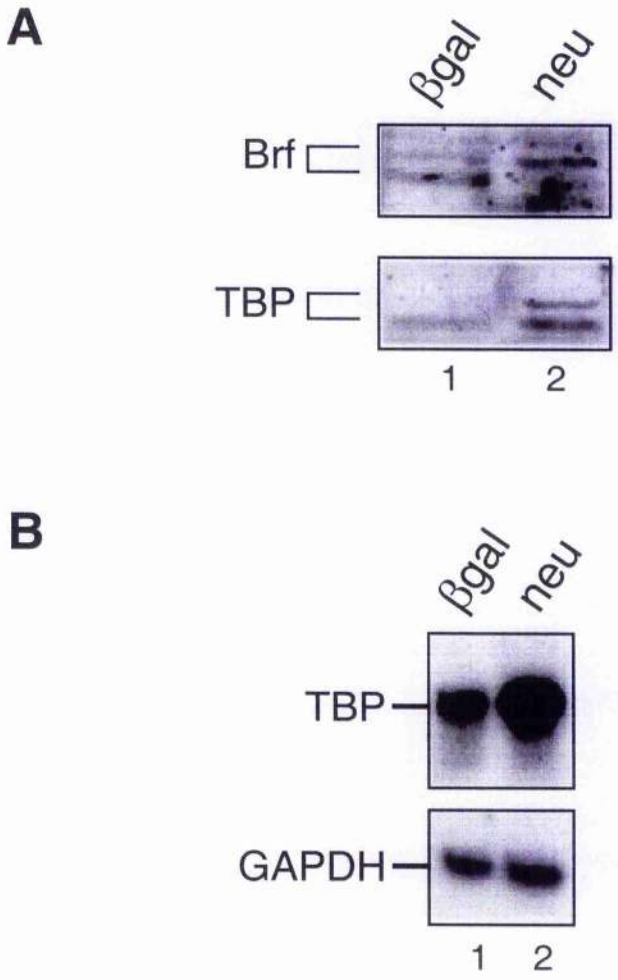

Figure 3.10: Expression of the TFIIIB subunits TBP and Brf. (A) Proteins (100μg) extracted from ROSE-βgal (lanes 1) or ROSE-neu cells (lanes 2) were resolved in a 7.8% polyacrylamide gel and then analysed by Western blotting with antiserum128 against Brf (*upper panel*), or antibody SI-1 against TBP (*lower panel*). (B) cDNAs prepared from total RNA (3μg) extracted from ROSE-βgal (lane 1) or ROSE-neu cells (lane 2) were used as template for RT-PCR analysis. The primers used were designed against TBP (*upper panel*) or GAPDH (*lower panel*).

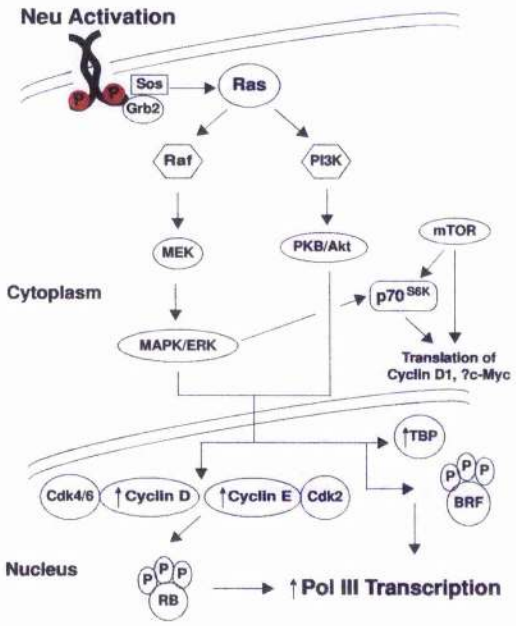

Figure 3.11: Possible mechanisms of pol III transcriptional induction by the activation of the ErbB2/neu receptor. Neu activation triggers induction of different signaling cascades, like Ras/MAPK and PI3K which will then increase the expression of different genes, including cyclins and transcription factors like TBP. Cyclin induction will inactivate RB, allowing pol III transcription to occur. Furthermore, an increase in the TFIIIB subunit TBP will also increase the activity of pol III transcription; plus phosphorylation of Brf may also promote pol III activity.

3.3 Discussion

Over the past 20 years since the discovery of ErbB2/neu, intense research has been performed to study its normal cellular functions and the mechanisms involved in its tumourigenicity. Its gene is among the most frequently altered in human cancer, which makes it an attractive candidate to study towards the understanding of this process. Cancer cells often proliferate at a much faster rate than normal cells; for this reason, they require to increase their growth rate to achieve a sufficient size before they can divide. As explained in the introduction chapter, high levels of pol III transcription are essential to mantain rapid growth. Indeed, pol III transcription is deregulated in rapidly growing cells like human cancers and transformed cell lines. Thus, activation of pol III transcription might be a necessary event leading to cell transformation and tumourigenesis by neu overexpression.

The results presented in this chapter show that neu overexpressing cells, which are transformed cells that proliferate at a faster rate than control non-transformed cells, have increased pol III transcription. It has been shown in ovarian epithelial cancer that pol III transcripts and TFIIIC2 are overexpressed [12]. Although pol III transcription is increased in the ovarian epithelial neu-transformed cell line, these cells do not overexpress the TFIIIC2 subunits (Fig. 1.2). In fact, a reduction in TFIIIC2 mRNA levels was observed in ROSE-neu compared to ROSE-βgal; however, the protein levels were similar in both transfected cell lines and TFIIIC2 activity is very slightly higher in ROSE-neu cells compared to control cells (Fig. 1.2 and [226]).

There are other possible mechanisms involved in neu's regulation of pol III transcription. Pol III transcription can be stimulated by factors that have been shown to be induced by neu overexpression. These factors include Ras activation, increase in cyclin levels and RB inactivation. As demonstrated in this chapter, the ROSE-neu transfected cells present all these alterations, so the activation of pol III transcription in the ROSE-neu cells might be through these mechanisms. Ras activation induces RB inactivation by increasing the levels of cyclin/cdk complexes which will phosphorylate this protein (Fig. 3.11). Since inactivated RB can no longer bind and inhibit TFIIIB, neu might be using this mechanism to stimulate pol III transcription. The coimmunoprecipitation experiments presented here demonstrate that TFIIIB subunit Brf from ROSE-neu cells is largely dissociated from this inhibitor. Furthermore, the Ras/MAPK pathway can also increase the cellular levels of the TFIIIB subunit TBP [170]. TBP has been shown to be a limiting factor for pol III transcription and its induction can mediate transactivation of class III genes [168, 270]. ROSE-neu cells also express elevated levels of TBP mRNA. Therefore TFIIIB activity is expected to be increased in these cells compared to that of control, and this activation might be responsible for the induction of pol III transcription (Fig. 3.11).

Induction of cyclins and RB phosphorylation by activated Ras has been shown to be regulated also through the TF c-Myc. Neu stimulation of Ras produces activation of the MAPK and PI3K pathways (Fig. 3.6) [5, 13, 228]. These pathways have been implicated in the control of c-Myc cellular levels. In addition, the mTOR pathway, observed to be induced in the ROSE-neu cells through the phosphorylation of $p70^{S6K}$, also regulates c-Myc expression. This TF is frequently deregulated in human cancers, and it not only regulates transcription of genes involved in cell cycle progression, but also genes involved in cell growth, like ribosomal proteins and translation initiation factors [15]. It has been proposed that c-Myc is a primary effector of ErbB2/neu oncogenicity [5]. c-Myc expression is induced upon activation

of ErbB2/neu through the Ras/MAPK and PI3K pathways in breast tumour cells and can partially rescue the neu phenotype from cells that had lost functional neu [5]. Hynes group showed that expression of c-Myc lead to an increase in cyclin D levels and phosphorylation of RB [5]. It would be interesting to study if this oncoprotein is exerting any role upon neu's upregulation of pol III transcription. The following chapter will address this question.

4 c-Myc's Participation in Neu's Induction of RNA Polymerase III Transcription

4.1 Introduction

Cancer probably occurs due to the accumulation of mutations within growth regulatory genes, which will produce the activation and/or suppression of several different pathways. The different resulting combinations will affect the characteristics of the tumour, like its agressiveness or its response to treatment. In breast and epithelial ovarian tumourigenesis, abnormal expression of the *crbB2/neu*, *c-myc* and *ras* genes has been implicated, although other genes are also involved [206, 207, 210, 272–275]. In addition, combinations of abnormal coexpression between these genes may act synergistically, endowing tumour cells with a highly aggressive phenotype. Studies from patients with epithelial ovarian cancer have shown that tumours containing both *c-myc* amplification and high ErbB2 coexpression have significantly worse survival than those of patients with normal expression of ErbB2 [273, 275]. Moreover, simultaneous overexpression of ErbB2 and Ras have been also observed in epithelial cancer, and this is associated with shorter disease free and overall survival than tumours presenting overexpression in only one oncoprotein [273, 275]. Furthermore, when these three oncoproteins are overexpressed in the same cancer, the survival of patients is significantly worse that that of patiens with tumours that do not overexpress these proteins [273]. In the case of human breast carcinoma, a correlation between c-Myc and ErbB2 overexpression has also been observed [274]. As well, a role for Ras activation and ErbB2 overexpression in mammary tumourigenesis has also been documented, and alterations in both oncoproteins also lead to a more aggressive tumour phenotype [235]. In addition to the ovarian and breast cancers,

simultaneous abnormal expression of these oncoproteins have also been found in other types of cancers (e.g. lung carcinomas [276]). Taken together, these findings suggest that the abnormal expression of the oncoproteins ErbB2, Ras and c-Myc are a common feature of different cancers. Therefore their study provides a good system to investigate the process of carcinogenesis.

4.1.1 ErbB2 Regulation of c-Myc Expression

Overexpression of ErbB2, Ras and c-Myc are usually observed in late stage cancers, and happen through a succession of genetic alterations; thus, the modification of one protein might have an effect upon the regulation of the other two. There is increasing data demonstrating that the activation of ErbB2 triggers the activation of the Ras/MAPK pathway; therefore, this receptor might be contributing to the increased activity of this oncoprotein found in tumours [5, 228, 234, Chapter 3]. On the other hand, c-Myc expression can be affected by ErbB ligands as well. Li et. al. demonstrated that neuregulins, which bind and activate ErbB2 in association with ErbB3 or ErbB4, can upregulate the expression of c-Myc mRNA and protein [277]. This experiment showed that this transcription factor can be induced by the ErbB family; however, it did not address the possible regulation of c-Myc expression upon activation of ErbB2 solely. Neve et. al., using a strategy for efficient down-regulation of the ErbB2 receptor through intracellular expression of an ErbB2-specific single chain antibody, demonstrated that the activation of the ErbB2 receptor does indeed stimulate c-Myc expression [5]. This report also showed that the induction of both c-Myc's mRNA and protein levels by ErbB2/ErbB3 dimers is through the activation of the signaling cascades Ras/MAPK and PI3K [5]. In a different report, it was shown that ErbB2 overexpression produces both Ras/MAPK activation and upregulation of c-Myc expression following epidermal growth factor stimulation (EGF). However, in this study the activation of the PI3K was lower in the ErbB2 overexpressing cells than in the control. From this study, it can be concluded that ErbB2 overexpression can induce c-Myc expression through the activation of the Ras/MAPK pathway solely [278]. Taken together, these findings show that there is a link between c-Myc expression and ErbB2 activation, and that the induction of c-Myc expression by ErbB2 can be achieved through the activation of the Ras/MAPK pathway, as well as in conjunction with the PI3K signaling pathway. Hence, the signaling pathways act as intermediaries transferring the growth-promoting signal from ErbB2 at the plasma membrane to c-Myc at the nucleus. c-Myc is a transcription factor whose increased expression has been associated with increased cell survival, stimulation

of cell growth, and cell cycle progression; furthermore, its overexpression can also cause apoptosis [279, 280]. Its induction will affect the expression of its different target genes (discussed in detail in the following chapter).

4.1.2 Regulation of c-Myc Expression by Signalling Pathways

The Ras/MAPK and the PI3K pathways utilize different strategies to upregulate c-Myc expression. These signaling pathways increase c-Myc expression at both the transcriptional and translational levels; in addition, the Ras/MAPK pathway can also enhance c-Myc protein accumulation through post-translational modifications [245, 281–284]. Evidence for the regulation of c-Myc expression at the transcriptional level by these pathways comes from experiments using erythroid cells stimulated by the hematopoietic growth factor erythropoietin. In these cells, erythropoietin, when interacting with its cognate receptor (EpoR), initiates the activation of a cascade of signaling pathways, including the PKC, the MAPK and the PI3K pathways, which produce an increase in *c-myc* gene expression [281]. In this system, if the MAPK is blocked after Epo stimulation, a reduction in c-myc elongation at the 3'-end of exon 1 is observed, whereas inhibition of PI3K blocks transcription initiation at exon 1; therefore, PI3K is required for *c-myc* gene transcriptional initiation, while MAPK acts on elongation [281].

Upregulation of c-Myc expression at the translational level by these signaling pathways is mediated through their activation of $p70^{S6K}$ (the 70-kDa isoform of S6 protein of 40S small ribosomal subunit protein kinase) [245, 284]. This kinase is important for G1 cell cycle progression and is responsible for the phosphorylation and activation of the S6 protein of the ribosomal translational complex, causing accelerated protein synthesis [242]. Following activation, the S6 protein induces protein synthesis biased toward mRNA transcripts that contain a polypyrimidine-rich 5' untranslated region, like that of the mRNA of c-Myc, as well as early growth response genes, cyclins and ribosomal elongation factors [242]. Thus, activation of $p70^{S6K}$ diverts the translational machinery to translate c-Myc mRNAs, among others, increasing the levels of this protein. It is interesting that ErbB2 activation has been shown to stimulate the phosphorylation of $p70^{S6K}$ at Thr^{389} (Chapter 3). The phosphorylation of this residue has been shown to regulate c-Myc levels, since when its dephosphorylation is induced, a dramatic down-regulation of c-Myc levels is observed [285]. Therefore, ErbB2 induction of Thr^{389}phosphorylation of $p70^{S6K}$ might provide another positive control for c-Myc's upregulation.

In addition to positive effects exerted by the Ras/MAPK pathway upon *c-myc* transcription and translation already mentioned, this signaling cascade is also able to enhance the accumulation of the c-Myc protein through post-translational modifications by increasing its stability. c-Myc has a very short half life of approximately 30 min, being rapidly degraded by the 26S proteasome [283]. However, this rapid degradation is slowed dramatically by the activation of the Ras/MAPK pathway, resulting in an increase of its half life to approximately 50-65 min [283]. This increase is achieved by the phosphorylation of c-Myc Ser 62 through the action of MAPK (ERK) [282].

In the previous chapter, it was demonstrated that overexpression of ErbB2 in a spontaneously immortalized rat ovarian surface epithelial cell line (ROSE 199) produced an increase in pol III transcription compared to control cells. The ErbB2/neu overexpressing cells present higher levels of the Ras/MAPK and the $p70^{S6K}$ pathway activation than the control cells, which might account for the induction of pol III transcription. As mentioned in the previous chapter, it is known that the Ras/MAPK pathway can upregulate pol III activity by affecting TFIIIB activity through different mechanisms, like by increasing the protein levels of the TBP subunit as well as by relieving TFIIIB from RB repression [170, Chapter 3]. These alterations are indeed present in the ErbB2/neu overexpressing cells; thus, they might account for the activation of pol III transcription. However, other factors might also be involved. Since these cells show higher activation of the Ras/MAPK and $p70^{S6K}$ pathways than the normal cells, it is quite probable that they will express higher levels of the c-Myc protein as well. Recently, it has been proposed that c-Myc is a primary effector of ErBb2/neu oncogenicity and its expression can partially rescue the ErbB2 phenotype from cells that had lost functional ErbB2/neu [5]. Furthermore, c-Myc is an important regulator of cell growth; and since the transcripts produced by pol III are essential in this process, it might be quite probable that c-Myc is participating in the upregulation of pol III transcription by ErbB2 overexpression. This chapter will address if the oncoprotein c-Myc is involved in the ErbB2/neu activation of pol III transcription. To assess this, the same cell line as the one studied in the previous chapter (a spontaneously immortalized rat ovarian surface epithelial cell line (ROSE 199) overexpressing ErbB2/neu or β-galactosidase) is going to be used.

4.2 Results

4.2.1 c-Myc Expression Is Increased in Neu Overexpressing Cells

The first experiment performed was to test whether overexpression of neu could affect the levels of the c-Myc protein in the ROSE system. As mentioned in the introduction, c-Myc protein levels are known to be controlled by the activation of different signaling pathways, including the Ras/MAPK cascade and $p70^{S6K}$. The Ras/MAPK pathway, through ERK activation, increases the stability of the c-Myc protein, enhancing its accumulation. As shown in chapter 3, the ROSE cells that overexpress the receptor neu present higher levels of ERK and $p70^{S6K}$ activity than that of the cells overexpressing β-galactosidase (ROSE-βgal, Fig. 3.6). These findings suggest that there might be an induction in the expression of the c-Myc protein in the ROSE-neu cells compared to that of the control cells. To assess whether this is indeed happening, whole cell extracts from both ROSE-βgal and ROSE-neu cells were analysed by Western immunoblotting, with antibodies specific for c-Myc or TFIIB, the latter used as a loading control. As shown in figure 4.1, c-Myc protein levels are increased in ROSE overexpressing neu compared to the expression in control cells, whereas the TFIIB protein levels remain constant. The increase in the levels of c-Myc is highly reproducible and not due to unequal protein loading because equal amounts of total protein were loaded into each lane based on protein assays, and based on immunoblotting with a TFIIB antibody as a loading control (Fig. 4.1, lower panel). From this result it can be concluded that constitutive activation of neu upregulates c-Myc expression in ROSE cells.

4.2.2 Regulation of Pol III Transcription by c-Myc in ROSE cells

Having established that the ROSE-neu cells overexpress c-Myc, the participation of this protein in the upregulation of pol III transcription was investigated. Since pol III transcriptional activity can be assessed by in vitro transcription assays, an experiment was designed to test whether the removal of the c-Myc protein from whole cell extracts could have any effect on pol III transcription from ROSE-neu extracts compared to ROSE-βgal extracts. Whole cell extracts were immunodepleted using an antibody against c-Myc or an antibody against the pol II-specific factor TFIIB and then were used for in vitro transcription of the class III gene, VA1. Mock-depletion or immunodepletion of c-Myc or TFIIB from ROSE-βgal extracts

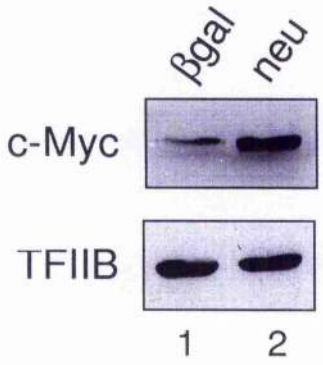

Figure 4.1: Neu overexpression induces c-Myc protein levels. Proteins (150μg) extracted from ROSE-βgal (lane 1) or ROSE-neu cells (lane 2) were resolved in a 7.8% polyactylamide gel and then analysed by Western blotting with an antibody against c-Myc (*upper panel*), or an antibody against TFIIB (*lower panel*).

has little effect on pol III transcription (Fig. 4.2, lanes 1-3); however, when ROSE-neu extracts are depleted of c-Myc with an anti-Myc antibody, they display reduced VA1 transcription (Fig. 4.2, lane 6). This effect is specific, since mock-depletion or immunodepletion of the pol II-specific factor TFIIB has no effect (Fig. 4.2, lanes 4 and 5, respectively). These results demonstrate that c-Myc overexpression plays a role in the increase of pol III transcriptional activity observed in the ROSE cells overexpressing neu, while the low levels of pol III transcription observed in ROSE-βgal cells are not dependent on c-Myc expression. They suggest that the high pol III activity caused by neu overexpression is via the induction of this transcription factor. Thus, neu upregulation of c-Myc might be responsible for the activation of pol III transcription.

4.2.3 Inhibition of c-Myc Expression and Pol III Transcription in ROSE Cells

In order to further investigate whether c-Myc induction is required for the activation of pol III transcription in the neu overexpressing cells, the following experiment was designed to inhibit c-Myc expression at the mRNA level. Previously, Heikkila et. al. have shown that c-Myc protein expression can be inhibited by using a pentadecadeoxyribonucleotide complementary to the initiation codon and four downstream codons of human *c-myc* mRNA [286]. Therefore, the same approach was utilised to analyse c-Myc participation in pol III transcriptional upregulation by neu overexpression in the ROSE system. Cell cultures from ROSE-βgal and

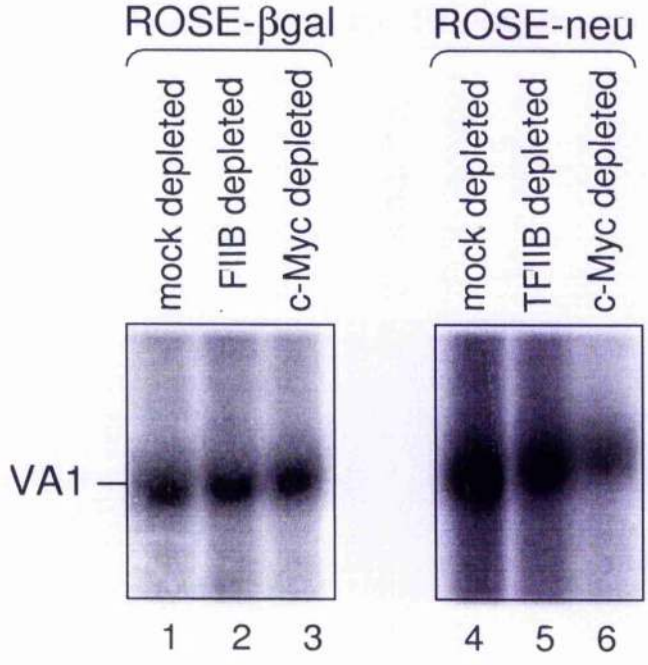

Figure 4.2: Immunodepletion of c-Myc reduces pol III activity in ROSE-neu overexpressing cells. Transcription of the VA1 gene was assayed using whole cell extracts prepared from ROSE-βgal or ROSE-neu cells, which were mock depleted (lane 1 for ROSE-βgal and lane 4 for ROSE-neu) or previously immunodepleted for 2 hours on ice with antibodies raised against TFIIB (C-18 Santa Cruz, lane 2 for ROSE-βgal and 5 for ROSE-neu), or c-Myc (9E10 Santa Cruz, lane 3 for ROSE-βgal and 6 for ROSE-neu).

ROSE-neu cells were exposed to antisense oligodeoxyribonucleotides (complementary to the first five codons of rat *c-myc* mRNA) or sense oligodeoxyribonucleotides (complementary to the antisense construct) at a final concentration of 20μM for 48 hours. Total RNA was extracted from these cells and used for cDNA preparation. This cDNA was used as template for RT-PCR reactions to assay levels of tRNA precursors and 5S rRNA, as well as GAPDH mRNA. An intron-specific primer was employed to study levels of tRNA precursors, which are processed very rapidly and so provide an assay of ongoing transcriptional activity [12]. When ROSE cells overexpressing the oncoprotein neu were exposed to *c-myc* antisense oligonucleotides, both tRNA and 5S rRNA levels decreased considerably (Fig. 4.3, lane 6), while the levels of the pol III products in the control cells did not suffer any change (Fig. 4.3, lane 3). This effect is specific since exposure to *c-myc* sense oligonucleotides did not have any effect on pol III transcription in both ROSE-βgal or ROSE-neu cells. Furthermore, the expression of GAPDH mRNA did not change in response to either sense or antisense c-myc oligonucleotides. Taking together the results obtained here with those of the immunodepletion experiment, it can be concluded that neu overexpression requires the induction of the transcription factor c-Myc in order to activate pol III transcription. Furthermore, they are the first results to demonstrate that this trancription factor is capable of regulating pol III activity.

4.2.4 Induction of the pol III subunit BN51 by Neu Overexpression

Having established that expression of the oncoprotein c-Myc does affect pol III activity in the neu overexpressing cells, it was investigated whether other proteins might also be induced in this system. In the literature, there is evidence showing that the c-Myc-ER fusion protein in an immortalized Rat1 cell line, can upregulate expression of the pol III-specific subunit BN51 [287]. Since the ROSE-neu cells are also a transformed cell line that overexpress the oncoprotein c-Myc, through constitutive activation of the receptor neu, the protein expression levels of the BN51 subunit were analysed in the ROSE system. Equal amounts of whole cell extracts prepared from either ROSE-βgal or ROSE-neu cells were analysed by Western blotting using an antiserum raised against BN51. Elevated levels of the BN51 protein were observed in the neu overexpressing cells compared to the cells overexpressing β-gal (Fig. 4.4, upper panel). Induction of BN51 in the ROSE-neu cells is specific, since the levels of the pol II-specific subunit TFIIB remained constant in both cell types (Fig. 4.4, lower panel). These data show that not only the c-Myc oncoprotein

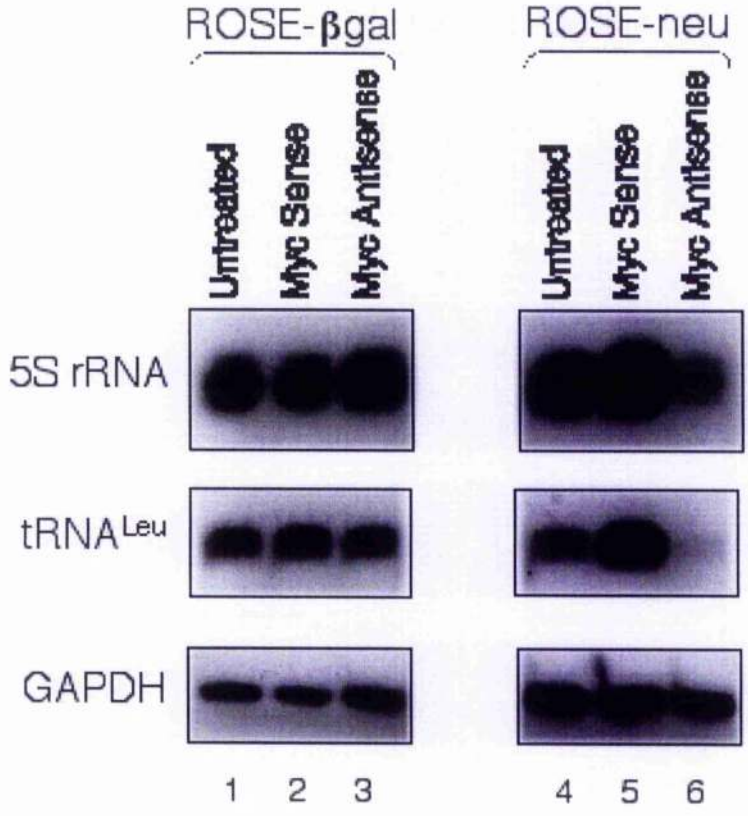

Figure 4.3: Depletion of c-Myc by antisense oligonucleotides selectively reduces 5S rRNA and $tRNA^{Leu}$ gene expression in neu-overexpressing cells. Growing ROSE-βgal or ROSE-neu cells were grown in the presence of 20 μM sense (lanes 2 and 5, respectively) or antisense (lanes 3 and 6, respectively) c-Myc oligonucleotides. RNA was harvested 48 hrs after transfection and analysed by RT-PCR, for the expression of 5S rRNA, $tRNA^{Leu}$ and mRNA encoding GAPDH.

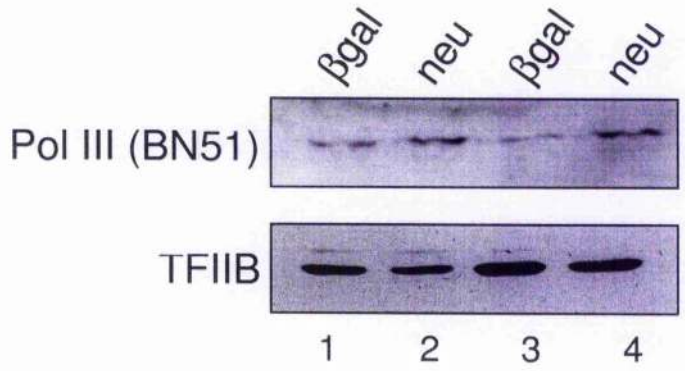

Figure 4.4: Expression of the pol III subunit, BN51, in ROSE-βgal and ROSE-neu cells. Proteins (100μg) extracted from ROSE-βgal (lanes 1 and 3) or ROSE-neu cells (lanes 2 and 4) were resolved in a 7.8% polyacrylamide gel and then analysed by Western blotting with antiserum 113 against BN51 (*upper panel*) or the rabbit antibody C-18 against TFIIB (*lower panel*).

is upregulated when the neu receptor is constitutively activated, but also the pol III-specific subunit, BN51 is induced. The fact that the transcription factor c-Myc has been shown to positively regulate transcription of the BN51 gene suggests that the induction observed of this protein in the ROSE-neu cells might be through a c-Myc-dependent mechanism; however, this still has to be examined.

4.3 Discussion

In the literature, there is compelling evidence showing that ErbB2/neu receptor activation leads to the activation of several signaling cascades that will consequently affect gene expression. In the previous chapter, it was demonstrated that constitutive expression of neu in an immortalized ovarian epithelial rat cell line produces activation of different kinases, shown by the phosphorylation of MAPK (ERK1/ERK2) as well as $p70^{S6K}$, which are downstream kinases of the Ras and PI3K pathways (see Fig. 3.6). These cascades are well known to be induced by activation of ErbB2/neu (see introduction); thus the results obtained were expected. Activation of the signaling cascades will affect gene transcription and, ultimately, the rate of proliferation of a cell. ErbB2/neu activation in a breast luminal cell line and in a breast tumour cell line has been shown to induce c-Myc oncoprotein expression through activation of the MAPK and the PI3K cascades [5, 278]. In this chapter, it is shown that overexpression of neu also upregulates the expression of c-Myc in an ovarian epithelial cell line. This cell line does display activation of the MAPK cascade, as well as $p70^{S6K}$; however, it was not determined if this is responsible for the observed upregulation of c-Myc levels. Nevertheless, it is quite probable that these pathways are involved in this process, but it would be interesting to address the mechanisms of this induction.

The c-Myc oncoprotein is known to be a positive regulator of both cell growth and proliferation [279, 280, 288]. In the previous chapter, it was demonstrated that constitutive activation of neu in the epithelial cells produces an activation of pol III transcription (see Chapter 3). As previously mentioned in the introduction of this thesis, the genes transcribed by pol III are essential factors of cell growth, since they function in mRNA maturation and protein synthesis required to achieve an increase in cell mass. The fact that a transcription factor like c-Myc, which induces cell growth, is upregulated in the same cells where pol III transcription activity is observed to be higher, suggested a possible participation of this transcription factor in pol III regulation. As shown in this chapter, this appears to be the case. Depletion of c-Myc either *in vitro* (by immunodepleting whole cell extracts of c-Myc, see 4.2) or *in vivo* (by antisense oligos specific to c-Myc mRNA, see 4.3) in the ROSE-neu cells reduced pol III transcription to the levels obtained in the control cells (ROSE-βgal), or even less for some transcripts (see 4.3, for $tRNA^{Leu}$). These findings show that indeed pol III transcription is sensitive to the presence of the transcription factor c-Myc, and suggest a role for this protein in its regulation. To date, this is the first evidence demonstrating that pol III transcription responds to c-Myc oncoprotein levels. Although the antisense experiments show that c-Myc

downregulation reduces transcription from class III genes to the levels observed in the control cells and suggests upregulation of c-Myc to be the mechanism by which oncogenic neu impinges on pol III transcription, it has also to be considered that constitutive expression of neu produces other alterations that will positively regulate pol III transcription (like RB phosphorylation and TFIIIB derepression, see Chapter 3) and might be involved in the induction of pol III activity in the ROSE-neu cells. To address this, different experiments have to be performed to investigate the contribution of each alteration. c-Myc is known to affect RB phosphorylation, so it might also account for the hyperphosphorylation observed in the ROSE-neu cells. Moreover, the pol III-subunit BN51 was observed to be upregulated in the neu-overexpressing cells, and there is evidence in the literature showing that is a c-Myc target gene [287]. Nevertheless, the data presented here present a new mechanism of inducing pol III transcription, through the oncoprotein c-Myc, and it will be interesting to investigate whether c-Myc regulation of pol III transcription is a characteristic particular to this type of cell line, or if it is a general effect in multiple types of cells.

In summary, it can be concluded that constitutive activation of the oncoprotein ErbB2/neu positively regulates pol III transcription. It might be doing so through alterations at multiple points, including TFIIIB release from RB repression, upregulation of the TFIIIB subunit TBP and the pol III-subunit BN51, and induction of the transcription factor c-Myc. Regulation of pol III transcription by RB and induction of TBP through Ras has been studied in numerous publications; however, c-Myc regulation of pol III activity has not been addressed yet and presents an interesting area of research. For this reason, the following chapters will investigate c-Myc participation in pol III transcription. To study this, the ROSE cell line will no longer be used since it presents so many different changes; instead, primary cells will be used to investigate if c-Myc is a general regulator of pol III transcription.

5 Activation of RNA Polymerase III Transcription by the Oncoprotein c-Myc

5.1 Introduction

The oncogene *myc* was discovered around 20 years ago as the transforming sequence of neoplastic avian retroviruses (e.g. the myelocytomatosis virus and the avian leukosis virus), referred to as *v-myc*, (for review see [288]). Soon after, *v-myc* cellular homologues (*c-myc*) were identified, including those of chicken, mice and humans [288–291]. Later on, two different genes were also identified containing strong homology to c-myc in human neuroblastomas and small cell lung carcinomas, and were dubbed the N-*myc* and L-*myc* genes, respectively [288, 292, 293]. Other cellular sequences with high homology to some portions of *c-myc* have been found, including B-*myc* and S-*myc* [294, 295]. The *c-myc* allele is localized to chromosome 8 (8q24) [296] and its translocation to different chromosomes has been shown to be involved in the generation of cancer [272, 280, 288, 296]. Homozygous deletion of *c-myc* is lethal at embryonic day 9.5-10.5; *myc* null embryos are smaller, retarded in development and show abnormalities of the heart, pericardium, neural tube, and a delay or failure in embryonic turning [280, 297].

The myc members are considered to be proto-oncogenes, since alterations in their expression play an important if not essential role in the development of a wide variety of human and animal cancers [272, 280, 288, 290, 293]. A good example of this are the translocations of the *c-myc* locus, on chromosome 8, to immunoglobulin loci of B lymphocytes, on chromosomes 2, 14, or 22, observed in Burkitt's lymphomas in humans and plasmacytomas in mice [272, 288, 298, 299]; as well as amplification of the N-*myc* and L-*myc* genes in neuroblastomas or small cell lung carcinomas,

respectively [292, 293]. Furthermore, overexpression of the Myc proteins with no genetic alterations can be seen in a wide variety of human cancers (like in breast and epithelial ovarian cancer) [272, 290]. In fact, c-Myc deregulation contributes to one seventh of all cancer deaths in the USA [272, 280, 288]. However, the myc genes can only partially transform primary cells in culture [288]. To elicit a tumourigenic phenotype, cooperation between Myc plus the activation of another oncogene (like *ras*), or the loss of a tumour suppressor gene, is required [300, 301]. This is also confirmed by its ability to transform when overexpressed in established cell lines that already contain deregulation in other genes [288].

Overexpression of c-Myc in transgenic animals has also confirmed and extended the results mentioned above. Transgenic mice with c-Myc overexpression under the control of the glucocorticoid-responsive murine mammary tumour virus long terminal repeat, MMTV LTR, develop tumours in breast, testicles, or B and T lymphocytes [302, 303]. Although c-Myc was widely expressed in different tissues, it only produced malignancies in certain organs, which implies the requirement for a secondary event for *c-myc* tumour formation [288, 302]. Other strategies have been developed to target c-Myc overexpression in specific tissues. When a *c-myc* transgene containing the Eμ immunoglobulin enhancer is expressed in mice, they develop B cell malignancies which vary according to the susceptibility of each of the inbred mouse strains used [272, 288, 304]. Taken together, these results establish the *c-myc* gene as an important factor in the etiology of cancer.

5.1.1 c-Myc Protein Structure

c-Myc protein is mainly localized in the cell nucleus and contains a transactivation domain (TAD) in the N-terminal region and a basic helix-loop-helix leucine zipper (bHLH-LZ) domain in the C-terminal region (Fig. 5.1) [305–308]. The TAD comprises the first 143 amino acids (aas) and is responsible for gene activation [306]. It contains two regions, Myc box I (MBI; from approximately aas 45-63) and Myc box II (MBII; from approximately aas 128-143) (Fig. 5.1), which are highly conserved among the Myc family [306, 309]. MBII is absolutely required for c-Myc transforming activity, while a deletion in MBI only attenuates its transforming activity [310, 311]. The C-terminal bHLH-LZ domain is also required for c-Myc transcriptional activities, biological activities and transformation [308, 312–314]. Proteins containing bHLH-LZ domains can form homo- and heterodimers, bind specific DNA sequences and usually act as transcription factors [19]. However, c-Myc cannot form homodimers nor bind DNA by itself. It heterodimerizes with the ubiquitously

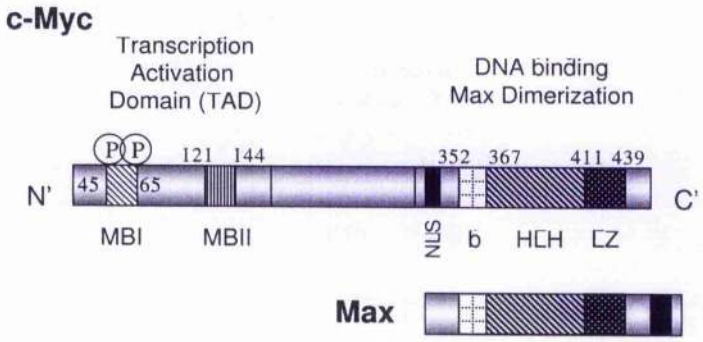

Figure 5.1: Schematic model of the c-Myc and Max proteins. The structural elements in c-Myc and Max are indicated. In the amino-terminal domain of c-Myc: transactivation domain (TAD), including Myc Box 1 (MB1) from aas 45 to 65 and Myc Box 2 (MB2) from aas 121 to 144. In the carboxy-terminal domain of c-Myc and Max: the nuclear localization signal (NLS); the basic region (b) from aas 352 to 357; the helix-loop-helix region from aas 357 to 411; and the leucine zipper from aas 411 to 439. P indicates sites of *in vivo* phosphorylation.

expressed bHLH-LZ protein Max (myc associated X protein) [280, 315, 316]. Myc-Max heterodimers bind DNA, recognizing specifically the hexameric DNA sequence CACGTG (which belongs to the sequences known as E-boxes, with the CANNTG consensus that is recognized by most bHLH proteins) and activate transcription of genes containing it [308, 317]. Usually, the E-boxes recognised by Myc-Max complexes are localized in the first introns of its target genes [308]. Max can homodimerize and bind to DNA, but Max homodimers appear to be transcriptionally inert [260,280]. Activation of transcription by Myc-Max heterodimers of certain genes, like cyclin D2, appears to be mediated by the recruitment of histone acetyltransferases and chromatin remodelling, though activation by other mechanisms might also be involved (discussed further in the next chapter) [318–321].

5.1.2 Funtions of c-Myc in Cell Proliferation

c-Myc participates in a wide variety of cellular processes including cell cycle progression, cell growth, differentiation, and apoptosis, which explains its involvement in the process of tumourigenesis [15, 272, 279, 280, 314, 318, 322–325]. No expression of c-Myc can be detected in quiescent cells; entry to the cell cycle by mitogen or serum stimulation, produces a rapid and transient burst in c-Myc mRNA and protein levels, which peaks 3-4 h after growth factor stimulation, followed by a gradual decline to low levels in proliferating cells [326, 327]. Because its expression is increased by

mitogenic stimuli during the G0 to G1 transition, it belongs to the immediate early response genes, which are involved in the cell's progression through the cell cycle. In cycling cells (in the presence of growth factors), c-Myc expression remains low and even throughout the cell cycle [325, 327, 328]. However, when there is growth factor withdrawal, c-Myc levels decline to undetectable values [329]. Differentiation also produces a decline in c-Myc levels, which is mantained in differentiated cells [288, 314, 330]. c-Myc is required for G1 to S phase progression and its overexpression in fibroblasts decreases the cell requirement for serum growth factors, as well as shortening the G1 phase [280, 286, 325, 330, 331]. Furthermore, activation of a conditional c-Myc is sufficient to drive quiescent cells into the cell cycle [6, 332]. In neoplastic cells, deregulated c-Myc expression drives cells to uncontrolled proliferation [280, 288, 325]. However, in primary fibroblasts, c-Myc overexpression alone cannot sustain a complete round of cell cycling, allowing progression of the cell cycle followed by arrest in the G2 phase. The tumour suppressor proteins p53 and p21 appear to play a role in this G2 checkpoint, since the arrest is compromised in the absence of these proteins [330]. Overexpression of c-Myc in primary cells induces apoptosis in low serum conditions, through p53-dependent and independent mechanisms [324].

5.1.3 c-Myc Target Genes

Induction as well as repression of a number of potential c-Myc target genes have been implicated in its regulation of the cell cycle [279, 280, 323, 330]. A list of these genes is presented in table 5.1. It is important to mention that validation of these putative genes as c-Myc target genes has been difficult because c-Myc only produces a slight increase (on the order of 2-5 fold) in their induction. Nevertheless, it can be noted from table 5.1 that c-Myc potential targets are required for RB phosphorylation and progression of G1. Since RB affects pol III transcriptional activity, it is quite probable that c-Myc is inducing pol III transcription.

5.1.4 c-Myc and Growth Control

c-Myc also influences cell growth. For a cell to divide, it needs to gain a certain size before it can separate into two daughter cells. As mentioned in this thesis introduction, the increase in size depends primarily on protein synthesis. Induction of c-Myc produces an increase in protein synthesis that precedes DNA synthesis [340]. More evidence of c-Myc regulation of cell growth comes from experiments in which myc alleles have been deleted in RAT1 fibroblasts. Loss of a single myc allele produces a 3 hr delay in S phase entry and a similar increase in doubling time [342]; when both al-

Table 5.1: c-Myc target genes.

	Myc target gene	Effect	Cellular Function	Reference
Cell Cycle	cdc25A	A	Cell cycle phosphatase	[333]
	cdk4	A	Cell cycle kinase	[334]
	cyclin D1	A	Cell cycle regulator	[335]
	cyclin D2	A	Cell cycle regulator	[323, 336]
	Id2	A	Inactivation of RB	[16]
	p21	R	Cell cycle inhibitor	[323]
	p27	R	Cell cycle inhibitor	[335]
Cell Growth	ODC	A	Polyamine biosynthesis	[323, 337]
	cad	A	Pyrimidine biosynthesis	[338]
	LDH-A	A	Metabolic enzyme	[339]
	GAPDH	A	Metabolic enzyme	[287]
	Nucleolin	A	rRNA processing	[287, 323]
	Fibrillarin	A	rRNA processing	[323]
	BN51	A	Pol III subunit	[287]
	RPS11	A	Ribosomal protein	[323]
	eIF-2α	A	Tranlation initiation factor	[340]
	eIF-4E	A	Translation initiation factor	[340]
	IARS	A	Isoleucine-tRNA synthetase	[323]
	DDX18	A	RNA helicase	[341]

Abbreviations: cdk4, cyclin dependent kinase 4; ODC, ornithine decarboxylase; cad, carbamoyl-phosphate synthase carbamoyl transferase/dihyroorotase; LDH-A, lactate dehydrogenase-A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RPS11, ribosomal protein S11; DDX18, Dead box helicase. A stands for activated; R stands for repressed.

leles are deleted, a 2.5 fold increase in doubling time with a delay in both G1 and G2 phases is observed [191]. These cells present a reduction of 2.5 fold in net protein synthesis as well as decreased rates of rRNA synthesis, resulting in reduced growth [191]. Moreover, its direct role on cell growth was demonstrated in Drosophila, where mutations that diminished *dmyc* expression (Drosophila ortholog of vertebrate *c-myc*) resulted in smaller but developmentally normal flies [343]. When imaginal disc cells were analysed, dmyc-mutant cells were smaller, but the cell cycle distribution stayed the same as in normal cells. However, if *dmyc* is overexpressed, the cells become larger than normal without significant change in cell cycle length (although G1 became shorter while G2 increased) [343]. These findings demonstrate that dmyc is affecting only cell size, leaving the cell cycle normal, and suggest that it may do so by directly regulating the cell growth machinery. In mammals, overexpression of c-Myc in B-cells both *in vivo* and *in vitro*, in the absence of growth factors, also produces larger cells without cell cycle progression or RB hyperphosphorylation [344]. Furthermore, in hepatocytes and the adult myocardium *in vivo*, overexpression of c-Myc provokes hypertrophy in the absence of proliferation [167,345]. In the hepatocytes, prominent nuclei and nucleoli were observed, associated with an upregulation of large- and small-subunit ribosomal protein genes and nucleolar genes [346]. In fact, several candidate c-Myc genes are involved in cell growth, including ribosomal proteins and translation initiation factors as well as genes involved in cell metabolism (Table 5.1). Because pol III is tightly linked to growth, c-Myc might also regulate its activity by a direct mechanism. This chapter will address c-Myc regulation of pol III transcription and study the possible mechanisms responsible for this induction.

5.2 Results

5.2.1 Pol III Transcription in c-Myc Knockout Cells

Due to the fact that the *c-myc* null RAT1 fibroblasts present a general reduction in cell growth, this system was chosen to study whether pol III transcription could be affected in the absence of *c-myc*. The group of Sedivy generated this cell line by gene targeting from a previous rat fibroblast cell line that contained a genetic disruption of one copy of the *c-myc* gene. A second disruption was generated, resulting in homozygous deletion of *c-myc* [191]. The parental cell line used for the targeted deletion was TGR-1, an *hprt-* subclone of the immortalized embryonic rat fibroblast cell line Rat-1, and was utilised as control. The *c-myc-/-* cells do not express c-Myc protein. In addition, no detectable expression of N-myc or L-myc is observed either in the parental cell line or in the *c-myc* null cells [191]. Northern blot analysis of total RNA extracted from quiesced parental and c-Myc knockout cells, as well as at different time points after serum stimulation, was performed. When c-myc null cells and matched wild-type fibroblasts are compared, the former show ~6 fold lower expression of pol III transcripts from the B2 repetitive gene family (Fig. 5.2). Transcription of the B2 gene provides a reliable indicator of the rate of pol III transcription since its transcripts are rapidly degraded after being transcribed [10], reflecting pol III transcriptional activity at the time of extraction of RNA and not build up of products. In all the samples, the B2 RNA levels are lower in the c-Myc knockout cells than in the parental cell line. The decrease observed is specific, since mRNA encoding acidic ribosomal phosphoprotein (ARPP) P0 is unaffected (Fig. 5.2). ARPP P0 was chosen as a control due to the fact that it is not modulated by c-Myc, estradiol, OHT, mitogens or serum growth factors, the RB pathway or upon cell cycle entry [287]. The results presented demonstrate that pol III activity is sensitive to the presence of endogenous c-Myc.

5.2.2 Rescue of Pol III Activity by Myc-ER in c-Myc Null Cells

To test whether the decrease in pol III activity in the *c-myc* null cells is due to the lack of c-Myc expression and not because of possible secondary mutations produced during the gene targeting process of the *c-myc* gene deletion, *c-myc* null cells infected with a conditional Myc were used to study if c-Myc can recover pol III activity. Retroviral transduction of a modified inducible c-Myc-estrogen receptor (Myc-ER) system into c-Myc knockout cells has been shown to rescue the null phenotype, with

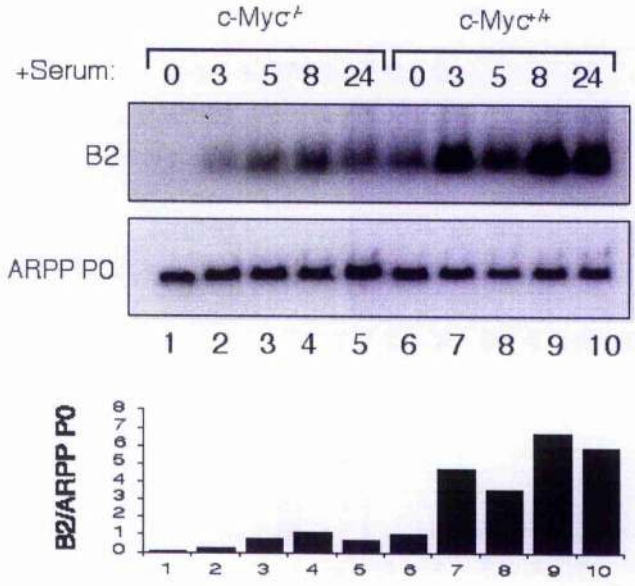

Figure 5.2: c-Myc expression affects pol III activity *in vivo*. Total RNA extracted from Myc-null fibroblasts ($Myc^{-/-}$) or wild type ($Myc^{+/+}$) was analysed by Northern blot using a DNA probe against the pol III transcript B2 (*upper panel*) and ARPP P0 (*middle panel*). The *lower panel* shows the quantified values of B2 RNA normalized to the levels of ARPP P0 mRNA, which were obtained from a scanned image analysed with the NIH-Image software.

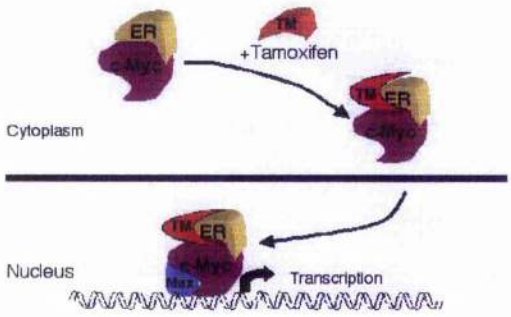

Figure 5.3: Schematic representation of c-Myc-ER fusion protein activation. An inactivated c-Myc-ER fusion protein, composed of c-Myc and the ligand binding domain of the estrogen receptor protein which only binds to 4-hydroxytamoxifen (OHT; TM in the figure) and not to estrogen, is expressed in the cells. The fusion protein remains in the cytoplasm in the absence of tamoxifen. Addition of tamoxifen promotes dissociation of the inhibitory complex and triggers translocation of the c-Myc-ER protein to the nucleus. Once in the nucleus, c-Myc-ER transactivates transcription from different target genes [347].

the cells regaining normal growth [191, 192, 348]. The Myc-ER system allows the expression of an inactive c-Myc-ER fusion protein that is composed of a modified estrogen receptor ligand-binding domain that binds to hydroxytamoxifen (OHT), fused to the c-Myc protein (Fig. 5.3) [347]. The Myc-ER fusion protein stays in the cytoplasm; addition of (OHT) causes translocation of the Myc-ER protein into the nucleus, activating c-Myc functions (Fig. 5.3) [347, 348]. Northern blot analysis was carried out with total RNA extracted from TGR-1 following serum stimulation or from Myc knockout cells infected with Myc-ER or Myc-S-ER following OHT addition in the absence of serum. Reintroduction of Myc-ER into the c-Myc knockout cells stimulated B2 expression (Fig. 5.4). Some increase occurred in the absence of OHT, which reflects leakiness of the conditional protein that has been previously reported. However, OHT induced a further increase in pol III transcript levels. Again, this response is specific, since the mRNA of ARPP P0 was not induced (Fig. 5.4).

Previously, it has been reported that the reintroduction of the c-Myc naturally occurring variant, MycS can also rescue the slow growth phenotype of *c-myc* null cells, albeit at lower efficiency than wild-type Myc-ER [192,348]. The MycS protein arises from translation initiation at two downstream AUG codons of wild-type c-Myc, yielding proteins that lack the first ~100 aas from the amino-terminus. Thus,

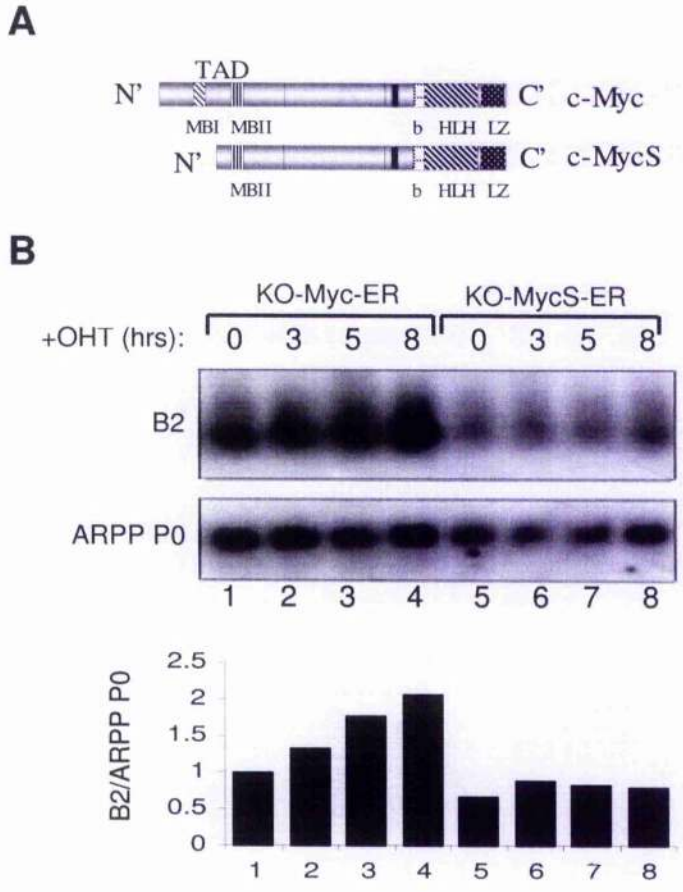

Figure 5.4: Expression of c-Myc rescues pol III transcription in c-Myc null cells. (A) Representation of c-Myc protein and its naturally occurring mutant c-MycS, lacking the first 100 aas of the N-terminal domain which includes MB1. (B) RNA extracted at the indicated times after addition of OHT, from c-Myc null fibroblasts transduced with retroviral vectors carrying Myc-ER (KO-Myc-ER) or MycS-ER (KO-MycS-ER) [192], was analysed for B2 (*upper panel*) and ARPP P0 (*middle panel*) expression by northern blotting. The *lower panel* shows the values of B2 RNA normalized to the levels of ARPP P0 mRNA, which were produced by scanning the image and quantitating the values of each sample using the NIH-Image software.

MycS lacks much of the TAD, including MBI, but preserves MBII [295]. In immortalized rodent cell lines, MycS promotes cell proliferation and apoptosis; however, in primary cells it cannot mimic c-Myc activities [192,348]. It has also been shown to act as a weak transactivator of endogenous target genes [192]. Figure 5.4 shows that the reintroduction of an inducible MycS-ER fusion protein into c-myc null cells does not rescue pol III activity; however, it would have been interesting to study MycS effect upon pol III transcription at longer time points than those chosen.

5.2.3 Activation of Myc-ER is Sufficient to Induce Pol III Transcription in Quiescent Primary Cells

Having demonstrated that pol III transcription responds to c-Myc expression in established cell lines that are immortal, it was investigated if it could have the same effect in mortal cells. Mortal cells offer the opportunity to study c-Myc effects exerted on pol III transcription in the absence of possible mutations that commonly occur during the immortalization process. Early passage human primary fibroblasts referred to as WI38 were chosen for these experiments. The extracted RNA from cells previously prepared by Carla Grandori from Bob Eisenman's group were used for RT-PCR. Early passage WI38 were infected with a retroviral vector expressing the Myc-ER fusion protein or with empty vector, then selected with puromycin and grown to confluence with no further passages. Once confluent, the cells were quiesced by leaving in the same medium for 7 to 8 days after plating [192]. It has been previously reported that under these conditions the levels of endogenous c-myc RNA are very low [341]. Once quiesced, OHT was added to the cells and total RNA was extracted at different time points.

cDNA was prepared from total RNA and used as template for RT-PCR. An intron-specific primer was employed to study levels of tRNA precursors, which are processed very rapidly and so provide an assay of ongoing transcriptional activity [12]. Pol III activity was induced by Myc-ER in these cells (Fig. 5.5, *upper panel*). Precursor $tRNA^{Leu}$ levels increase ~7-fold in quiescent cells infected with Myc-ER relative to vector-infected controls; a further increase occurs within three hours of adding OHT, whereas OHT has no effect on cells carrying empty vector (Fig. 5.5, *upper panel*). Similar results were obtained with $tRNA^{Tyr}$ (data not shown). Activation of tRNA genes reached 12-fold in the presence of Myc-ER and OHT, while the established Myc target, cyclin D2, had a response of only ~3.6 fold induction in these conditions (Fig. 5.5, *middle panel*). The effects observed by Myc-ER and OHT are specific, since ARPP P0 mRNA is unchanged (Fig. 5.6, *bottom panel*). Further

evidence of specificity came from the mutant ΔMyc-ER, which lacks a region within the TAD, from aa 106 to 143, that includes MBII. ΔMyc-ER failed to activate both tRNA and cyclin D2 transcription upon addition of OHT. Therefore, activation of pol III transcription is induced by Myc-ER and this upregulation requires the TAD of c-Myc. It can also be observed that pol III transcription can be activated in the absence of growth factors by the sole presence of c-Myc.

5.2.4 Activation of Pol III Transcription by c-Myc and Induction of Pol III Machinery

Several plausible mechanisms might explain the c-Myc responsiveness of pol III-transcribed genes. The first one considered was if c-Myc might be inducing genes that encode proteins from the pol III transcriptional machinery. It has been reported that in Rat1 cells c-Myc upregulates the BN51 gene, which encodes a subunit of pol III [287]. c-Myc activates transcription from the *BN51* gene via E-boxes present in its first intron. RT-PCR analysis from cDNAs prepared from total RNA of human primary fibroblasts containing Myc-ER before and after addition of OHT was performed using primers designed for the BN51 mRNA. As shown in figure 5.6, no induction of BN51 by c-Myc was found in the primary human fibroblasts.

Another pol III-specific factor that has been shown to be overexpressed in transformed cells that present high pol III activity is TFIIIC2 [12]. Therefore, the same analysis as the one mentioned above was performed to study the expresssion levels of different mRNAs encoding the subunits of TFIIIC2. Again, no increase was observed in TFIIIC2 subunits TFIIICα/220 and TFIIICβ/110 (Fig. 5.6). Therefore, c-Myc activation of pol III transcription does not require induction of these proteins from the pol III apparatus.

5.2.5 RB Family Participation in the Pol III Response to c-Myc

Activation of c-Myc can provoke hyperphosphorylation of RB [6]. c-Myc induces a number of genes that are involved in this process at different levels, to ensure RB phosphorylation. It induces the expression of cyclin D1 and cyclin D2 genes, that form complexes with ckd4 and cdk2 [323,335,336]. These complexes are responsible for phosphorylating RB. Cyclin D1 and D2 are also responsible for sequestrating the cyclin dependent kinase inhibitors p21^{Ink}, p27^{Kip} and p21^{Cip}, which bind to cdk4/2 and inhibit their activation (Fig. 5.7) [335,336]. Furthermore, another c-Myc target gene encodes the phosphatase cdc25A, which removes inhibitory phosphates from

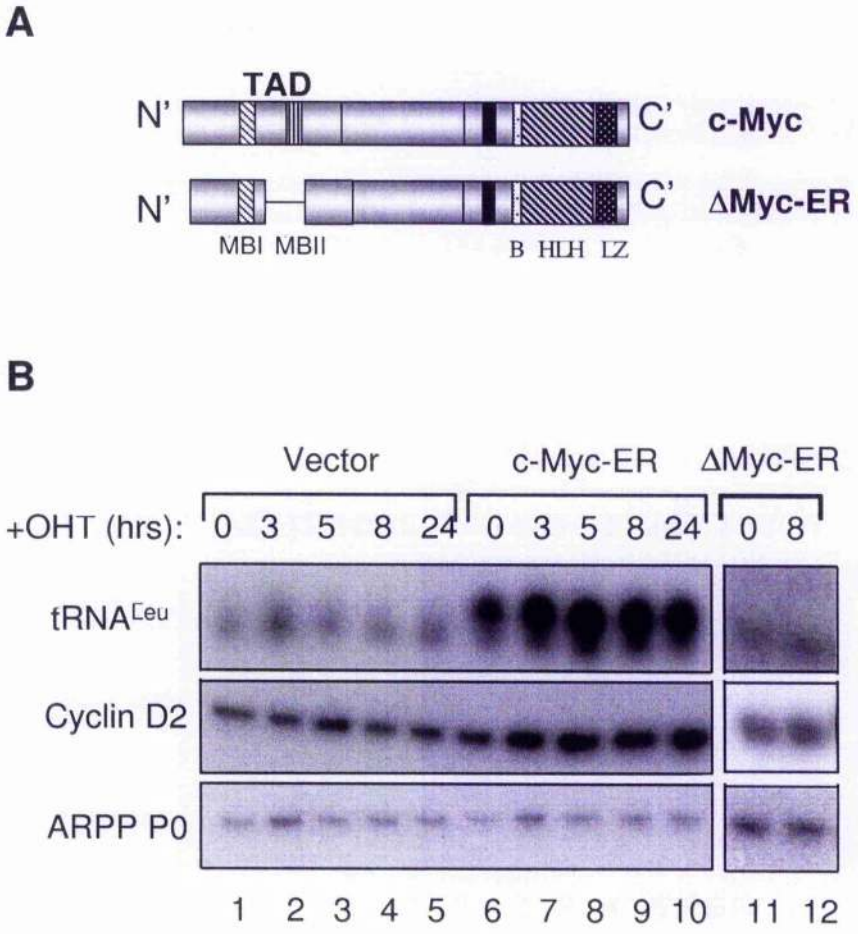

Figure 5.5: Pol III transcribed genes respond to c-Myc *in vivo* in quiesced human fibroblasts. (A) Representation of c-Myc protein and the mutant ΔMyc-ER, in which residues 106-145 (including MB2) within the TAD have been deleted. (B) Gene expression was analysed in WI38 cells transduced with empty retroviral vector (Vector) or vector carrying c-Myc-ER. Cells were serum-starved for 48 hrs to downregulate endogenous c-Myc expression and then treated with 200 nM tamoxifen (OHT) ([192]). RNA was extracted at the indicated times after induction and used for cDNA preparation (3μg). RT-PCR analysis of $tRNA^{Leu}$ (*upper panel*), cyclin D2 (*middle panel*) and ARPP P0 (*lower panel*) was performed.

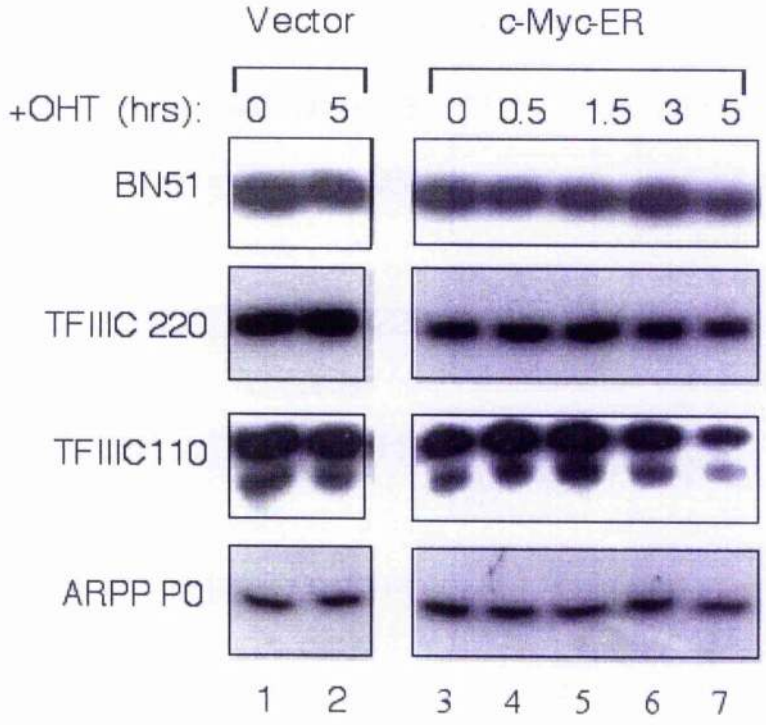

Figure 5.6: Activation of pol III transcription by c-Myc does not depend on induction of TFIIIC2 or BN51. WI38 cells were transduced with empty retroviral vector (Vector) or vector carrying c-Myc-ER (c-Myc-ER), as indicated. Cells were serum starved and then treated with 200 nM OHT. RNA was extracted at the indicated times after induction and analysed by RT-PCR for expression of TFIIIC220, TFIIIC110, BN51 and ARPP P0.

cdk2/4 proteins (Fig. 5.7) [333]. In addition, it has been recently demonstrated that c-Myc activates transcription from the Id2 gene, whose product binds and sequestrates RB, inactivating this tumour suppressor [7, 16]. The induction of all these proteins ensures that RB gets phosphorylated and inactivated. Activation of Myc-ER in the quiescent primary human fibroblasts produces hyperphosphorylated RB by 8 to 13 hrs after OHT treatment, promoting cell-cycle progression [192].

Since RB represses tRNA synthesis in a phosphorylation-dependent manner [3,4], it was tested whether the pol III-response to c-Myc is RB-dependent. To achieve this, immortalized fibroblasts from wild-type or RB knockout mice were transiently transfected with a c-Myc expression vector or empty vector, together with the class III gene adenovirus VA1 and the SV40-CAT vector as reporter. Total RNA was extracted and analysed by primer extension. Transfection of c-Myc expression vector stimulated pol III transcription of the adenovirus VA1 gene in fibroblasts from either wild-type or RB knockout cells, whereas the cotransfected SV40-CAT reporter was unaffected (Fig. 7.6). Although the RB knockout cells have higher pol III activity, the overexpression of c-Myc produced a $\sim$3 fold increase of VA1 in both the wild-type and the RB null cells (Fig. 7.6).

The RB-related proteins p107 and p130 also repress pol III transcription [184]; since c-Myc can bind and neutralise p107 [301], it was tested whether c-Myc overexpression continues to activate VA1 in the absence of the entire RB family. The same experiment as the one mentioned above was performed in primary fibroblasts from wild-type or RB-p107-p130 triple knockout mice [349]. A robust and specific VA1 induction was obtained even in triple knockout fibroblasts lacking all three pocket proteins (Fig. 7.8). Taken together, from these findings it can be concluded that pol III regulation by c-Myc does not require the RB family.

5.2.6 Involvement of Pol II Transcription in c-Myc Activation of Pol III Transcription

So far, there is a bulk of evidence showing that c-Myc can transactivate transcription from a vast amount of genes involved in different processes [280, 323]. c-Myc induction of pol III activity might require the transactivation of class II genes that have not been investigated yet in our system. Since it would be time-consuming to study all the class II genes with a potential role in pol III induction, instead the effect of blocking pol II transcription was investigated. From the experiments with the human primary fibroblasts it can be observed that the tRNA induction following activation of Myc-ER parallels that of the cyclin D2 gene, which is known

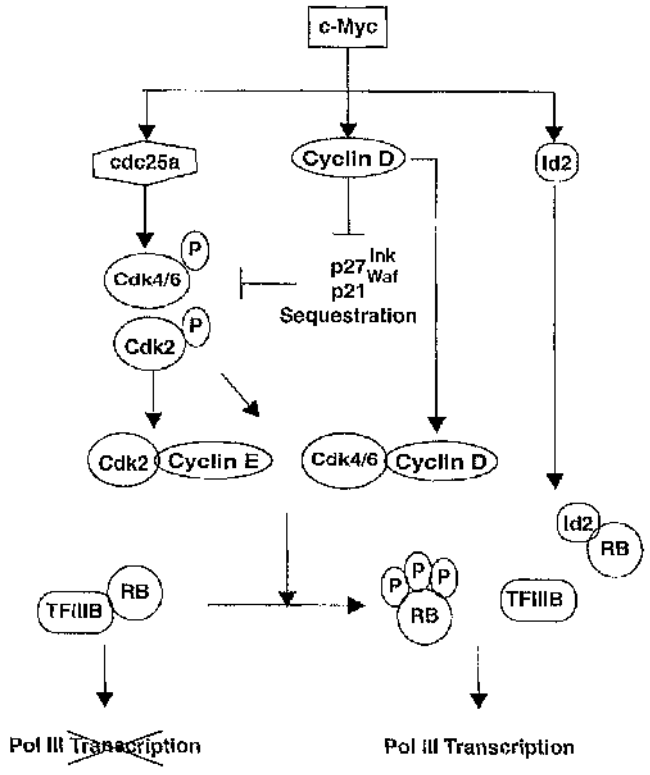

Figure 5.7: Schematic model of c-Myc inactivation of RB leading to a probable activation of pol III. c-Myc induces expression of different genes, like cdc25a, cyclin D and Id2, which are involved in the process of RB phosphorylation. The phosphatase cdc25A removes inhibiting phosphate groups from cdk, allowing them to associate with their respective cyclin partners. Induction of cyclin D produces sequestration of cki which block cdks from binding to their cyclin partners; as well as increasing cyclin D/cdk4,6 complexes. The cyclin/cdk complexes phosphorylate RB, which will no longer be able to bind and inactivate TFIIIB, inducing pol III transcription. On the other hand, Id2 binds and sequestrates hypophosphorylated RB, releasing TFIIIB from RB repression.

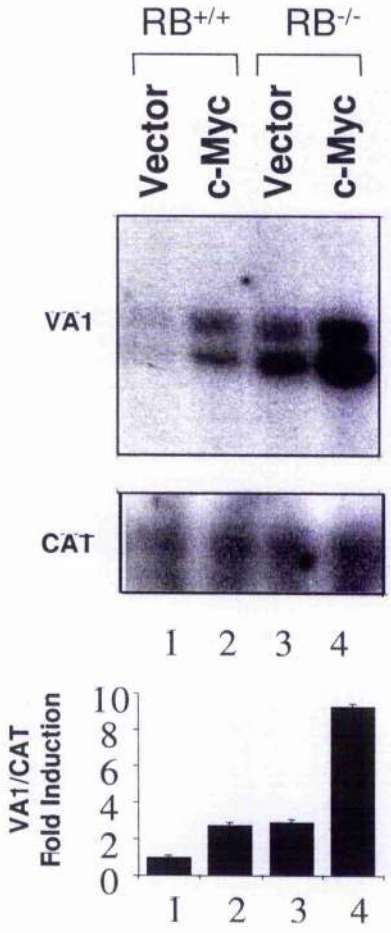

Figure 5.8: Activation of pol III transcription by c-Myc does not require neutralisation of RB. Immortalized fibroblasts from RB null ($RB^{-/-}$, lanes 3 and 4) or wild type ($RB^{+/+}$, lanes 1 and 2) mice were transiently transfected with pVA1 (0.5μg), pCAT (0.5μg) or plasmid (2μg), encoding wild type c-Myc (lanes 2 and 4) or empty vector (lanes 1 and 3). RNA was extracted after 48 hrs of transfection. VA1 (*upper panel*) and CAT (*middle panel*) RNA levels were assayed by primer extension analysis and quantified using the NIH/Image software. Values shown in the *lower panel* represent the signal for VA1 normalized to the levels of CAT expression to correct for transfection efficiency.

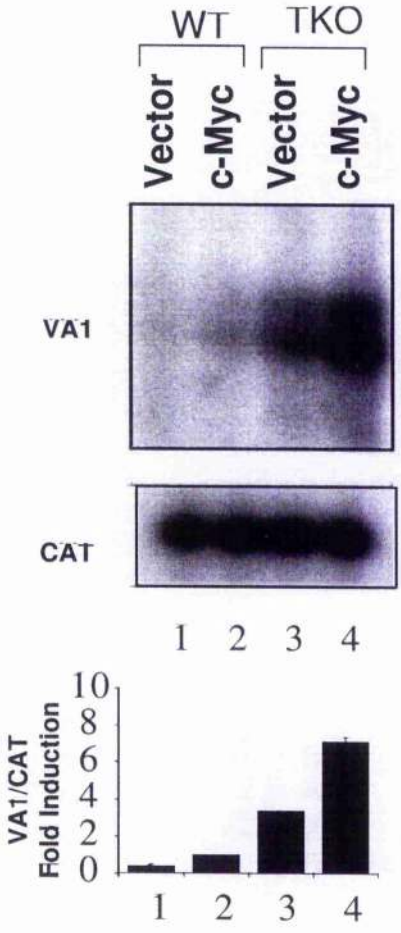

Figure 5.9: Activation of pol III transcription by c-Myc does not require neutralisation of the pocket proteins. Primary fibroblasts from p107-p130-RB triple knockout mice (TKO, lanes 3 and 4) or wild type (WT, lanes 1 and 2) were transiently transfected with pVA1 (0.5μg), pCAT (0.5μg) or pMyc (2μg), encoding wild type c-Myc (lanes 2 and 4) or empty vector (lanes 1 and 3). RNA was extracted after 48 hrs of transfection. VA1 (*upper panel*) and CAT (*middle panel*) RNA levels were assayed by primer extension analysis and quantified using the NIH/Image software. Values shown in the *lower panel* represent the signal for VA1 normalized to the levels of CAT expression to correct for transfection efficiency.

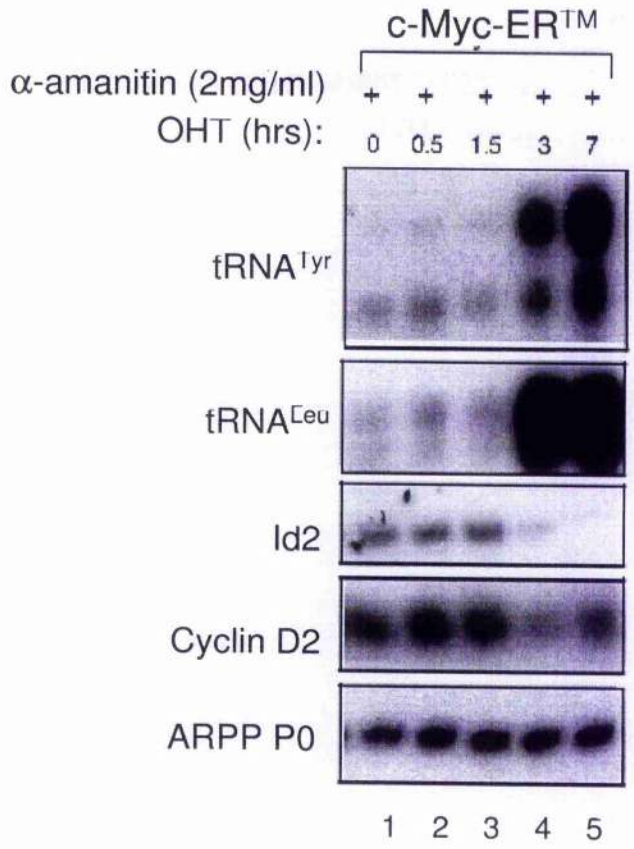

Figure 5.10: Activation of pol III transcription by c-Myc does not depend on induction of pol II transcription. WI38 cells were transduced with retroviral vector carrying c-Myc-ER, serum-starved and then treated with 2μg/ml α-amanitin, 2.5 hrs before induction with 200 nM OHT. RNA was extracted at the indicated times after induction (Grandori). The RNA was analysed by RT-PCR for $tRNA^{Tyr}$, $tRNA^{Leu}$, Id2, cyclin D2 and ARPP P0 expression.

to be a direct target of c-Myc [323,335,336]. If pol III induction by c-Myc needs the action of another c-Myc target, such a quick induction would be unlikely. Indirect c-Myc targets generally take more than 9 hr to respond in this system and S phase entry takes ~17 hr [323]. Therefore, because of the rapidity of pol III activation, it is quite possible that c-Myc activates pol III transcription directly. So, if pol III transcription is still being induced upon activation of Myc-ER by OHT when pol II transcription is inhibited, a direct effect can be inferred.

To inhibit transcription only from pol II, while leaving pol I and III active, the different sensitivities to the drug α-amanitin of the pols were exploited. α-amanitin is a bicylic octapeptide derived from the poisonous mushroom *Amanita phalloides*

[9]. Pol II has the highest sensitivity to it, being affected at low concentations; pol I, has the lowest, requiring high concentrations to inhibit its function, and pol III has intermediate sensitivity [19, 56]. A concentration that would only inhibit pol II transcription was chosen to study the requirement for pol II transcription in c-Myc activation of pol III. Tamoxifen treatment was carried out in the presence of $2\mu g/ml$ α-amanitin, specifically blocking pol II. Whereas cyclin D2 and Id2 mRNA levels decline after α-amanitin treatment (Fig. 5.10, lanes 4 and 5), the more stable ARPP P0 transcript remains steady throughout the experiment (Fig. 5.10). By contrast, $tRNA^{Leu}$ and $tRNA^{Tyr}$ were induced robustly even in the presence of α-amanitin (Fig. 5.10). This suggests that c-Myc regulation of pol III templates does not require stimulation of protein-encoding genes and may therefore be direct.

5.2.7 c-Myc Interaction with Pol III Templates

Since no participation of class II c-Myc target genes appears to be required for pol III activation, the presence of this oncoprotein on pol III templates might be involved in their transactivation. To test if endogenous c-Myc is actually associating with class III genes in vivo, chromatin immunoprecipitation was performed on either mice fibroblasts (Balb/c 3T3) or rat ovarian epithelial cells (ROSE-neu). Antibodies against c-Myc, TBP, Brf, the pol III subunit BN51, TFIIIC220 and heamaglutinin (HA) were used to precipitate their respective proteins that had been previously cross-linked to chromatin. As shown in figure 5.11, the TFIIIB subunits TBP and Brf are detected at $tRNA^{Leu}$, $tRNA^{Tyr}$ and 5S rRNA genes in both cycling fibroblasts and epithelial cells, but not at the ARPP P0 gene or at the $p21^{Waf1}$ pol II promoter. This interaction is specific, since no signal was observed from TAF_I48 or HA controls. These results demonstrate that c-Myc is located at pol III templates in vivo.

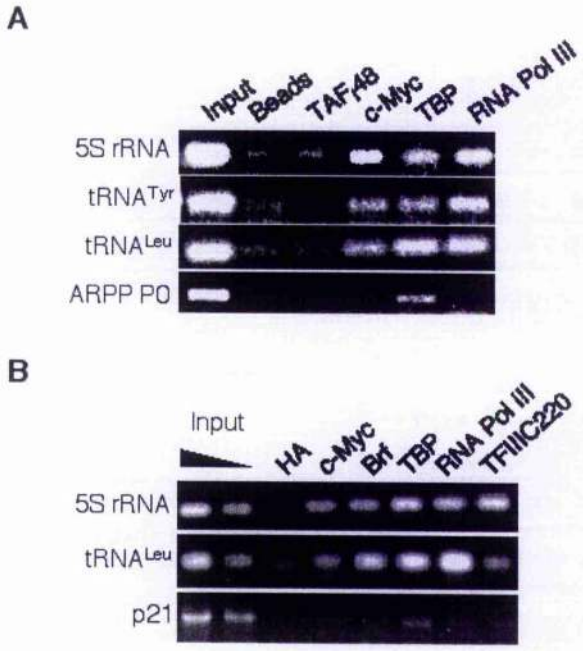

Figure 5.11: c-Myc interacts with pol III templates *in vivo*. (A) Chromatin immunoprecipitation of Balb/c 3T3 cells ($2x10^7$ per immunoprecipitation) was performed with $20\mu l$ of the respective antibodies (HA, c-Myc 9E10 , serum against Brf, serum against TBP, serum against RNA Pol III subunit BN51 and serum against TFIIIC220). Immunoprecipitated DNA was quantified by PCR performed on the input (diluted 1:200) and bound fractions, using primers against 5S rRNA, $tRNA^{Tyr}$, $tRNA^{Leu}$, and ARPP P0. (B) The same experiment as the one described above was performed on ROSE-neu cells; for these cells, PCR against 5S rRNA, $tRNA^{Leu}$ and p21 were performed.

5.3 Discussion

Over the past few years, it has been shown that c-Myc can stimulate cellular growth and that this effect can be distinguished from its effects on cell proliferation. Several lines of evidence support this hypothesis. In mammalian cells, it has been shown in quiesced B-cells, and hepatocytes, as well as in cells that have lost the ability to proliferate like cardiomyocytes, that overexpression of c-Myc can produce hypertrophy without inducing cell proliferation [344–346]. Furthermore, a mutation in *Drosophila melanogaster* of c-Myc leads to diminutive flies that present a slow growth phenotype, while overexpression of c-Myc produces bigger flies without any apparent change in cell division [343]. These findings suggest that the upregulation of c-Myc can increase cell mass in the absence of cell proliferation and might be an essential function of this oncoprotein. Since pol III products are required to achieve a coordinated increase in cell mass, it was investigated whether c-Myc could induce pol III transcription. The results shown in this chapter demonstrate that indeed pol III transcription is regulated by c-Myc expression.

From the experiments using the c-Myc knockout mice, it can be observed that in the absence of c-Myc expression pol III transcription is quite diminished compared to the wild-type cells (Fig. 5.2). The diminished pol III activity observed in the *c-myc* null cells can be rescued by the introduction of a c-Myc-ER fusion protein upon activation with OHT, which demonstrates that the observed reduction in pol III transcription is due to the loss of *c-myc* expression. c-Myc-ER has been reported to be quite leaky; in fact, c-myc null cells harbouring c-Myc-ER can be phenotypically rescued in the absence of hormone, which explains the increased B2 transcript observed in quiesced cells before addition of OHT (time point 0) (Fig. 5.4) [322]. In these experiments, introduction of the naturally occurring variant c-MycS-ER could not recover pol III transcription. c-MycS is still able to produce proliferation and apoptosis of immortalized cells and can still repress some genes while giving weak transcriptional activation of certain genes (like ODC) [192, 348]. It has also been documented that it cannot induce S phase progression in quiescent primary human fibroblasts or induce transcription of the known c-Myc target gene cyclin D2 [192, Grandori pers. communication]. Its expression in c-Myc knockout cells can rescue the slow growth phenotype; however, this rescue was observed starting from day two, being more clear at day three, while c-Myc-ER rescued the slow growth phenotype from day 1, expressing the same growth rate at day two as the wild-type cells [192]. The last time point chosen to study the pol III transcript B2 was at 8 hrs after addition of OHT, which is quite early compared to the time

at which Myc-S-ER starts to rescue the slow growth phenotype. Thus it might be interesting to study the effect of this mutant after longer periods of time.

The results presented here also demonstrate that c-Myc induction of pol III activity is by direct action on pol III transcription rather than by inducing class II genes which will then affect pol III transcriptional activity. Several known and unknown c-Myc target genes transcribed by pol II could be affecting pol III transcription (like BN51 or induction of pocket proteins phosphorylation). However, the fact that pol III transcription is still responsive to c-Myc expression while pol II transcription is inhibited demonstrates that c-Myc activates pol III transcription directly (Figs. 5.10, 7.6, and 7.8). Furthermore, no induction of BN51 or TFIIIC subunits expression could be observed in these experiments (Fig. 5.6). Previously, BN51 has been shown to be a c-Myc target gene [287]. In that study, they utilised immortalized rat cells, while in the experiments presented here, primary human fibroblasts were used. In immortalized rat cells, the induction observed for cyclin D2 was ~8 fold while that of BN51 was ~2 fold [287]. The primary fibroblasts used here only presented an increase of ~3.6 fold in cyclin D2 expression (Fig. 5.5). These findings suggest that primary cells may be less responsive than immortalized cells, which might explain the lack of response of the BN51 gene to c-Myc expression. Nevertheless, pol III transcription showed a clear induction upon activation of c-Myc-ER, which reflects a direct effect on it. Moreover, it is quite interesting to note that c-Myc can activate transcription from most class III genes tested; since the expression of these genes may be expected to impact strongly on cell growth, the effect exerted upon pol III activity by this oncoprotein might explain the slow growth phenotype observed in the *c-myc* null cells, although other factors like pol I transcription are also involved [191]. Furthermore, induction of tRNA levels by Myc-ER exceeds the weak transactivation often observed for established c-Myc targets, such as cyclin D2 (Fig. 5.5). In addition, a direct interaction of endogenous c-Myc could be observed at pol III templates *in vivo* (Fig. 5.11).

In this chapter it has been demonstrated that c-Myc induces pol III activity through a direct mechanism, although it has not addressed how this is achieved. Thus, it would be interesting to study the mechanism or mechanisms involved in this activation. The following chapter will identify the way this regulation is being achieved.

6 c-Myc's Association with the RNA Polymerase III Transcription Machinery

6.1 Introduction

The transcription factor c-Myc is able to activate transcription of different target genes, including those transcribed by pol II and III [279, Chapter 5]. It is still not well understood how c-Myc exerts its transactivation properties. Two regions of c-Myc protein have been shown to be required for the activation of transcription from class II target genes, its C-terminal DNA binding/dimerization domain and its N-terminal transactivation domain (TAD). The C-terminal DNA binding/dimerization bHLH-LZ domain recognizes and binds to E-Box DNA sequences with the core motif 5'-CACGTG through its basic region, while it heterodimerizes with Max through its HLH-LZ region (although the HLH-LZ region has also been shown to make contacts with DNA, [308]) [315, 316]. c-Myc target genes usually contain E-boxes downstream of the promoter regions, in introns, 5' untranslated, or coding regions. However, class III genes, which have been shown to be c-Myc target genes [Chapter 5], do not contain matches to the E-box DNA sequence; thus c-Myc transactivation of these genes must be through protein-protein interactions in the absence of a DNA recognition site.

6.1.1 Myc Binding Partners other than Max

Different proteins, in addition to Max, have been identified to interact with c-Myc, mainly with the TAD, although there are some that interact with the C-terminal domain. The C-terminal domain has been shown to interact with the transcription factors YingYang-1 (YY1), AP-2, TFII-I, the breast cancer susceptibility gene (BRCA1), and Miz-1 [350]. In the case of YY1 and TFII-I, c-Myc binding modulates

their activity by sequestering them or preventing their interaction with other factors like TBP [351]. It is interesting to note that c-Myc interacts with the factor that is responsible for transactivating ribosomal protein genes, YY1; however, until now, c-Myc's binding to this transcription factor appears to repress rather than activate transcription from these genes, although the biological relevance has to be studied further. This result strikes as surprising, since there are several studies showing that c-Myc transactivates ribosomal protein genes rather than inhibiting them [346]; therefore, the YY1-c-Myc binding should be analysed in detail to understand what is the physiological significance of this association. These findings suggest that the C-terminal domain might also be involved in the regulation of transcription, although the main regulator of this activity in c-Myc is the TAD. It would be interesting to study whether the activation of pol III transcription by c-Myc is through an interaction between a protein from the pol III machinery and the C-terminal domain of c-Myc.

The N-terminal TAD has been shown to interact with different proteins involved in various processes, like cell cycle regulation, histone acetylation and apoptosis; it is responsible for both activation and repression of transcription. As mentioned previously, the cell cycle regulator p107 when it is hypophosphorylated binds to c-Myc through the TAD [301]. This interaction inhibits c-Myc transactivation properties. It has been shown that it might have this effect by inhibiting cell cycle phosphorylation of MBI *in vitro*. However, p107 does not appear to be required for c-Myc's regulation of pol III transcription, since in its absence c-Myc can still induce transcription of class III genes [Chapter 5].

Another protein that has been observed to interact with c-Myc's TAD is TRRAP (TRansformation/tRanscription-domain-Associated Protein) [352]. TRRAP is a protein of >400 kD (3830 aa's) with homology to the PI3K/ATM family, lacking the kinase function [352]. It works as a transcriptional cofactor, mediating the recruitment of histone acetyltransferase (HAT) complexes like GCN5/PCAF and Tip60/NuA4 to sequence-specific activators, like c-Myc [353]. It binds to c-Myc's MBII, which is essential for c-Myc oncogenic transformation. In fact, mutations in MBII which disrupt TRRAP interaction render c-Myc unable to transform cells, thus c-Myc recruitment of TRRAP might be critical for its oncogenic activity, although the interaction with some other molecules is also MBII-dependent. Histone acetylation has been shown to activate gene expression, therefore TRRAP binding to c-Myc might mediate HAT recruitment to c-Myc target genes with their consequent induction. Recently, it has been demonstrated that indeed activation of *cyclin D2*

gene expression by c-Myc is dependent on TRRAP recruitment and histone acetylation at the *cyclin D2* promoter [318]. Since c-Myc can bind to class III genes and induce their transcription, it would be interesting to study whether the recruitment of TRRAP is necessary for this induction. However, this would not show how c-Myc is recognizing and binding to class III genes, which it remains to be determined.

An interesting candidate for c-Myc recruitment to class III genes is TBP. TBP binds to the c-Myc N-terminal TAD [354]. This interaction appears to require the full TAD, since shorter fragments of it cannot bind to TBP [355]. Furthermore, complex formation between TBP and c-Myc's TAD produces a change in protein conformation in the TAD, with the induction of a more structured transactivation domain conformation. This rearrangement in structure is common for transactivation domains which remain unstructured until they interact with specific target factors, and suggests the physiological importance of the c-Myc-TBP interaction. Since TBP is a component of TFIIIB [82], it is very probable that it is involved in c-Myc's recruitment to class III genes.

This chapter will address which protein or proteins are participating in the recruitment of c-Myc to class III genes. It will also investigate which regions of this transcription factor are required for the interaction with the pol III transcriptional machinery.

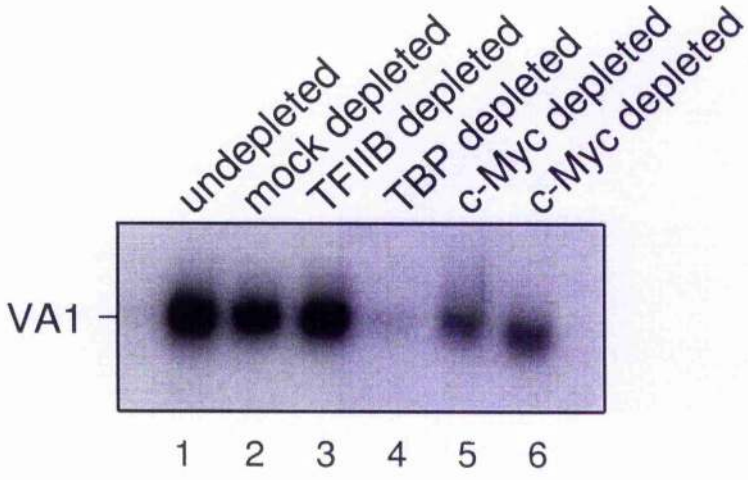

Figure 6.1: Immunodepletion of c-Myc reduces pol III activity. Transcription of the VA1 gene was assayed using HeLa nuclear extracts undepleted (lane 1), mock depleted (lane 2) or previously immunodepleted for 2 hours on ice with antibodies raised against TFIIB (C-18, lane 3), TBP (mTBP-6, lane 4), or c-Myc (9E10, lanes 5 and 6).

6.2 Results

6.2.1 c-Myc Is Required for Pol III Transcription *in vitro*

Although a direct interaction between c-Myc and class III genes was demonstrated (Chapter 5, 5.11), from this evidence it cannot be concluded whether this interaction is functionally significant. To test if the presence of this oncoprotein at the genes transcribed by pol III is required to induce its transcriptional activity, HeLa nuclear extracts were depleted with an anti-Myc antibody and then tested for in vitro transcription (IVT) using the adenovirus VA1 gene as template. As positive control, the nuclear extracts were depleted of TBP, since this protein is essential for pol III transcription, while for the negative control the pol II-specific factor TFIIB was chosen. If c-Myc is having an effect on the pol III machinery, its depletion should affect pol III activity. As expected, extracts depleted of TBP show a dramatic reduction in VA1 transcription (Fig. 6.1, lane 4), while mock-depletion or immunodepletion of TFIIB have little effect (Fig. 6.1, lanes 2 and 3, respectively). When the extracts are depleted of c-Myc with an anti-Myc antibody, they display reduced VA1 transcription (Fig. 6.1, lanes 5 and 6), although to a lesser extent than that observed for TBP. These results suggest the stable binding of endogenous c-Myc to a component of the pol III transcriptional apparatus.

6.2.2 c-Myc Binds to and Depletes a Factor Required for Pol III Transcription

Having established that the depletion of endogenous c-Myc affects pol III transcription, it was investigated whether a pol III factor is codepleted. Since c-Myc cannot bind to a specific DNA sequence in class III genes, its recruitment to these genes must be through a protein-protein interaction with one or more factors from the pol III apparatus. So, when c-Myc is being depleted from the extract, its pol III binding partner may also be removed and therefore pol III transcription is reduced. Another possibility is that c-Myc depletion itself is affecting pol III transcription. To assess if c-Myc depletion is actually removing a factor or factors from the extract, an experiment was designed to see whether recombinant c-Myc could catch this factor and remove it from the extract, without changing the endogenous c-Myc concentration. Whole cell extracts from CHO cells were preincubated with beads carrying GST, or GST fused to residues 1-262 of c-Myc encompassing the TAD (GST-Myc-N262), or GST fused to the C-terminal 92 residues of c-Myc containing the bHLH-LZ domain (GST-Myc-C92) (a recombinant c-Myc protein containing the full length c-Myc could not be used due to the fact that no one in the field has been able to produce it). After preincubation, the extracts were used in an IVT assay using different class III genes as templates, including $tRNA^{Leu}$, $tRNA^{Arg}$, 5S rRNA, VA1 and 7SL (Fig. 6.2). All the class III genes tested exhibit reduced transcription from whole cell extracts preincubated with GST-Myc-N262 (Fig. 6.2, lane 3), compared to those preincubated with GST alone (Fig. 6.2, lane 1) or with the recombinant protein containing the C-terminal domain of c-Myc (GST-Myc-C92) (Fig. 6.2, lane 2). The slightly reduced activity observed for the extracts preincubated with GST and GST-C92-Myc compared to the activity of the input material is probably due to the loss of activity usually obtained during the incubation period, which the input is not exposed to, and not to a specific reduction produced by GST or GST-C92-Myc. These results show that whole cell extracts preincubated with recombinant protein containing the N-terminal domain of c-Myc (GST-Myc-N262) exhibit reduced pol III activity. From this evidence it can be concluded that the extract preincubated with GST-Myc-N262 is depleted of a factor or factors required for pol III transcription.

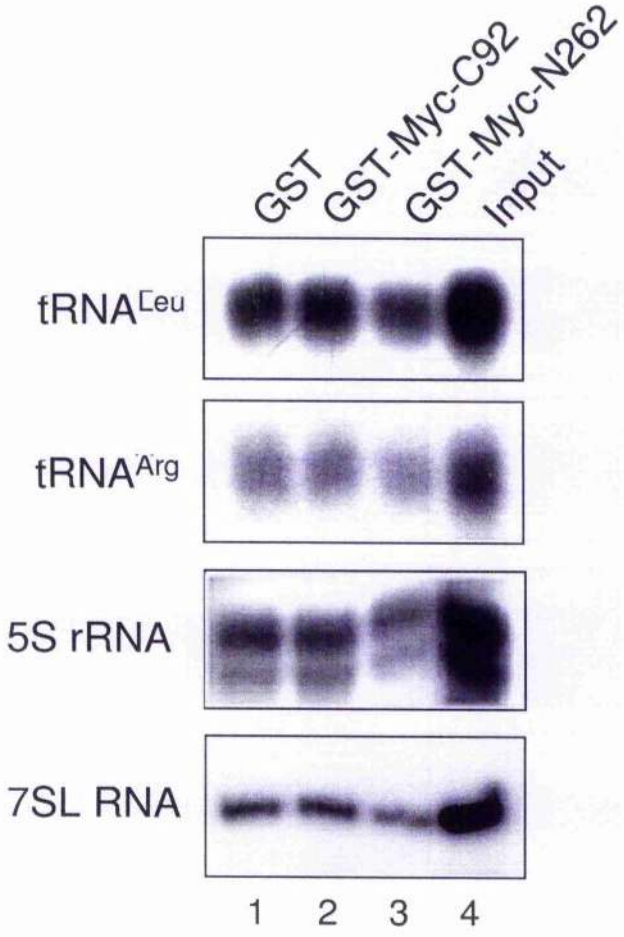

Figure 6.2: Depletion with the N-terminal domain of c-Myc reduces pol III transcription. Transcription of the $tRNA^{Leu}$, $tRNA^{Arg}$, 5S rRNA and 7SL RNA genes were assayed in whole cell extracts from CHO cells previously depleted for 2 hrs on ice of proteins binding to GST (lane 1), GST-Myc-C92 (lane 2) or GST-Myc-N262 (lane 3). Lane 4 is transcription assayed using the same whole cell extract which had not undergone the 2 hrs depletion period.

6.2.3 TBP Does Not Reconstitute Pol III Transcription from c-Myc Immunodepleted Extracts

The experiments presented so far have shown that a factor or factors from the pol III machinery might be associating with c-Myc, specifically with its N-terminal region. However, it is still unclear which factor or factors might be involved in this protein-protein interaction. The most plausible candidate is TBP, which is known to bind the TAD of c-Myc [354,355]. As mentioned before, TBP is a subunit of TFIIIB and is essential for transcription of all class III genes [81,82]. Since transcription from class III genes containing both type I and type II promoters has been shown to be reduced by the preincubation of extracts with the N-terminal domain of c-Myc, it is quite probable that the protein being depleted is involved in the transcription of class III genes with those types of promoters. Therefore, TBP is a good candidate for c-Myc's interaction. To examine this possibility, increasing amounts of recombinant TBP were added to HeLa nuclear extracts previously depleted with an anti-c-Myc antibody and these reactions were used to transcribe the adenovirus VA1 gene as template. Again, extracts depleted with an anti-TBP antibody served as positive control and an anti-TFIIB antibody was used as negative control. Addition of recombinant TBP to TBP-immunodepleted extracts slightly reconstituted pol III transcription (a complete reconstitution would require addition of Brf) from a VA1 template (Fig. 6.3, lane 6) . However, recombinant TBP did not show any pol III activity recovery from c-Myc-immunodepleted extracts. This was not due to the fact that a higher concentration of recombinant TBP was required, since addition of increased concentrations of it only produced a reduction in pol III activity (Fig. 6.3, lane 10). This effect is known as squelching, where an excess of TBP competes for proteins involved in the transcriptional process, and depletes them from the reaction, leaving reduced pol III activity. From these experiments it can be concluded that in the TBP-immunodepleted extract, TBP becomes the limiting factor, thus its addition gives some reconstitution. However, in the c-Myc-immunodepleted extract, addition of TBP did not recover pol III transcription, thus something else (probably Brf) is limiting in this extract. In conclusion, the data presented so far suggest that other factor or factors are also being removed from the extract by depletion with anti-c-Myc antibody or with the preincubation of the N-terminal domain of c-Myc.

6.2.4 c-Myc Does Not Associate with TFIIIC or Pol III

To study which factors, other than TBP, are actually interacting with c-Myc, pull-down experiments were performed using whole cell extracts prepared from CHO

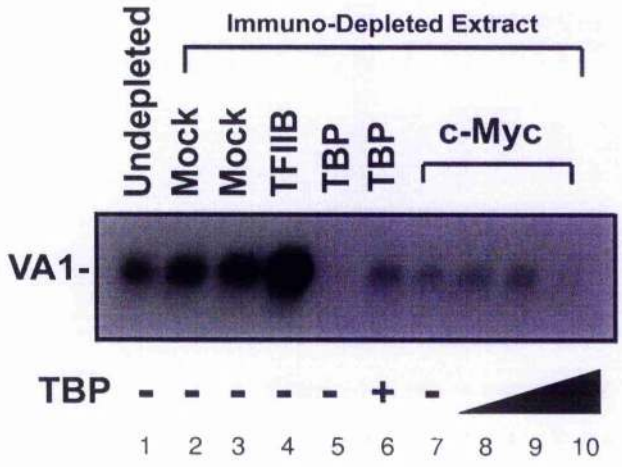

Figure 6.3: Recombinant TBP does not reconstitute pol III transcription from c-Myc immunodepleted extracts. Transcription of the VA1 gene was assayed in HeLa nuclear extracts undepleted (lane 1), mock depleted (lanes 2 and 3) or previously immunodepleted with antibodies raised against TFIIB (C18, lane 4), TBP (mTBP-6, lanes 5 and 6), or c-Myc (9E10, lanes 7 to 10). Immunodepleted extracts were complemented with recombinant TBP at a final concentration of 1 μg (lanes 6 and 8) or 1.5 μg (lane 9) or 2 μg (lane 10).

cells incubated with beads carrying equal amounts of recombinant GST, GST-Max or GST-Myc-N262. After incubation, bound proteins were eluted and analysed by western blotting using different antibodies. The first TF investigated was TFIIIC, which is required for transcription of class III genes containing type I and II promoters. Antisera raised against the subunits TFIIICα and TFIIICβ were used for these experiments. Immunoblotting of the eluted material with the antisera against these two subunits reveals that there is no interaction between either of these TFIIIC subunits and the recombinant proteins (Fig. 6.4 and data not shown). These results suggest that c-Myc does not associate with TFIIIC and therefore is not directly involved in c-Myc's recruitment to class III genes.

The next factor investigated was pol III itself. In this case, an antiserum against the pol III subunit BN51 was utilized. As shown in Fig. 6.5, BN51 appears to be retained by the beads carrying GST-Myc-N262 (lane 4), while GST alone or GST-Max do not (lanes 2 and 3, respectively). This result is in accordance with the reduced pol III activity observed for the extracts preincubated with GST-Myc-N262. Since the concentrations used in the pull-down assays of the GST fusion proteins do not correspond to the endogenous c-Myc protein levels, the result obtained with

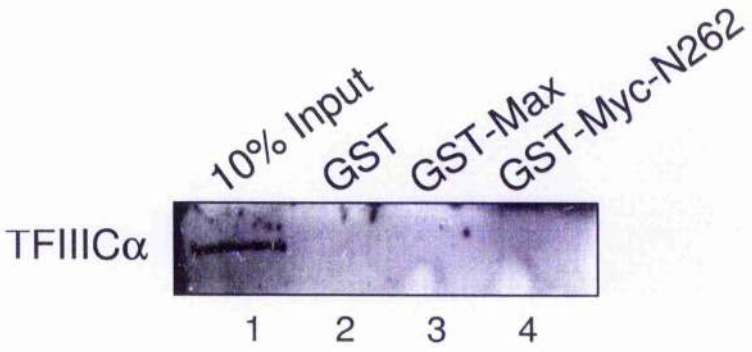

Figure 6.4: TFIIIC does not immunoprecipitate with the N-terminal domain of c-Myc. Proteins from CHO whole cell extracts (250 μg) that remained bound to beads carrying 50 μg of GST (lane 2), GST-Max (lane 3), or GST-Myc-N262 (lane 4) after extensive washing were resolved on an SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting with an antiserum Ab2 raised against TFIIICα. Lane 1 is 10% of input material.

this experiment might not represent an association between these two proteins at physiological ratios. To test whether endogenous c-Myc can associate with pol III, coimmunoprecipitation experiments were performed using HeLa nuclear extracts. Although the pull-down experiments show that there is an interaction between pol III and the N-terminal domain of c-Myc, this interaction could not be observed when material immunoprecipitated with monoclonal antibody against c-Myc was blotted against BN51. In the same manner, an antiserum against BN51 did not coprecipitate c-Myc (data not shown). These results suggest that endogenous c-Myc does not associate with pol III at physiological ratios and that it is not the factor that is being depleted by c-Myc antibody.

6.2.5 c-Myc Associates with TFIIIB

Due to the lack of interaction between c-Myc and TFIIIC or pol III, c-Myc interaction with TFIIIB was investigated. As mentioned in the introduction, TFIIIB is the most probable target for c-Myc binding since it contains TBP, which has been shown to bind c-Myc through its TAD [354,355]. Again, pull-down assays were performed and the eluted samples were analysed by western blotting. All three TFIIIB subunits were tested for interaction with the GST fusion proteins. In the case of Bdp1, instead of using a GST-Myc-C92 fusion protein as negative control, a GST-Max fusion protein was tested; equal amounts of GST or GST-fusion proteins were used for each reaction. As expected, immunoblotting revealed that TBP is retained by beads

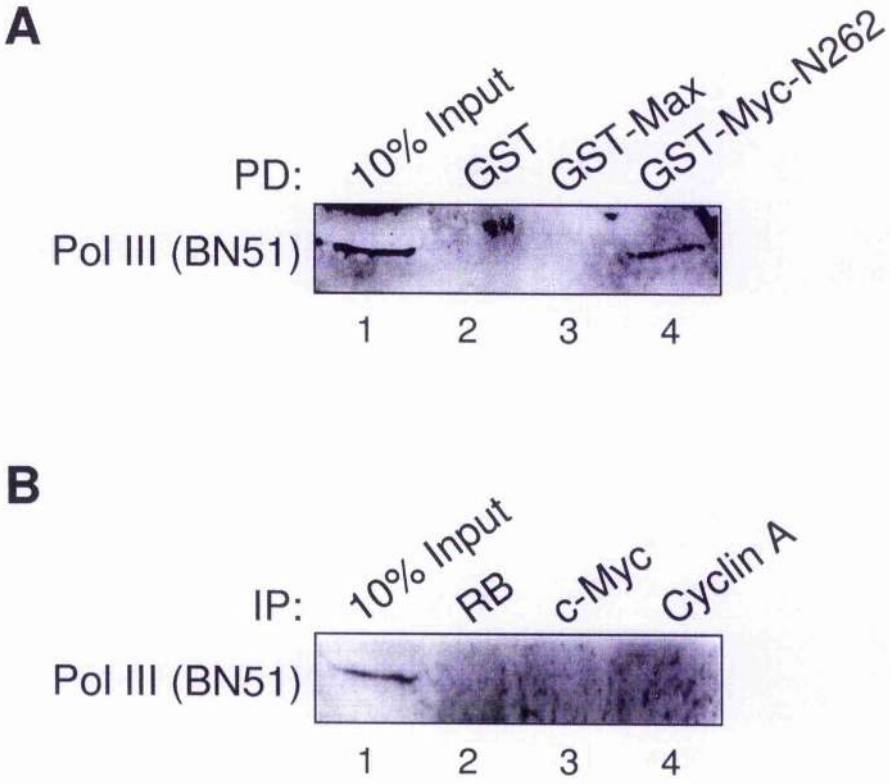

Figure 6.5: Pol III not immunoprecipitate with c-Myc. (A) Proteins from CHO whole cell extracts (150 μg) that remained bound to beads carrying 50 μg of GST (lane 2), GST-Max (lane 3), or GST-Myc-N262 (lane 4) after extensive washing were resolved on an SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting with an antiserum 113 raised against BN51. Lane 1 is 10% of input material. (B) HeLa nuclear extract (187.5 μg) was immunoprecipitated using an anti-c-Myc antibody (9E10, lane 2) or an anti-Cyclin A antibody (BF683, lane 3). Immunoprecipitated material was resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with antiserum 113 against BN51 subunit of Pol III. Lane 1 is 10% of input material.

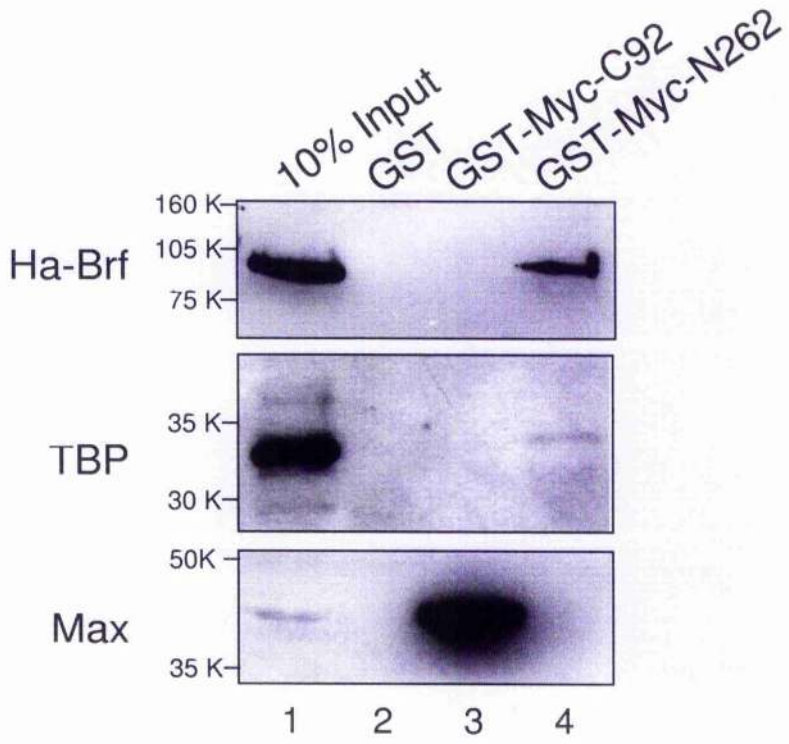

Figure 6.6: The N-terminal domain of c-Myc binds to the TFIIIB subunits TBP and Brf. Proteins from CHO whole cell extracts (150 μg) that remained bound to beads carrying 50 μg of GST (lane 2), GST-Myc-C92 (lane 3), or GST-Myc-N262 (lane 4) after extensive washing were resolved on an SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting with antiserum 128-4 raised against Brf (top panel), with an anti-TBP antibody 58C9 from Santa Cruz (middle panel) or with an anti-Max antibody (lower panel). Lane 1 is 10% of input material.

carrying the GST-Myc-N262 fusion protein (Fig. 6.6, middle panel). In addition, both Brf and Bdp1 also interact with the same N-terminal c-Myc fragment (Fig 6.6, upper panel and data not shown). The binding observed is specific, since neither of the subunits associate with GST alone or with the GST-Myc-C92 fragment. The lack of association between the TFIIIB subunits and the C-terminal fragment of c-Myc is not due to a reduced concentration or inactivity of this recombinant protein, since a clear interaction between this fragment and the protein Max, which is well documented [315, 316], can be observed at the same concentration used for the pull-down assays with TFIIIB. The association between TFIIIB and the N-terminal domain of c-Myc is consistent with the fact that only this fusion protein was able to reduce pol III activity in the IVT assays presented before.

To test whether the association between TFIIIB and c-Myc observed in the pull-down assays still happens at physiological ratios with endogenous c-Myc, coimmunoprecipitation assays were carried out. As positive control, an anti-RB antibody was used, as the RB/TFIIIB interaction is well documented [4, 179, 182], whereas antibodies against E7 and cyclin D1 served as negative controls. Like that of the positive control RB, immunoprecipitations with monoclonal antibody against c-Myc were found to coprecipitate the Brf subunit of TFIIIB (Fig. 6.7A). This interaction is specific, since no coprecipitated Brf could be found in the immunoprecipitated material with antibodies against E7 and cyclin D1 (Fig. 6.7A). Furthermore, coimmunoprecipitation assays also showed an interaction between the TFIIIB subunit TBP and c-Myc (Fig. 6.7B), which was expected as it has been previously described [354]. Again, coimmunoprecipitation with RB served as a positive control for TBP, since the interaction between these two proteins is documented [179], while an anti-cyclin A antibody provided the negative control. From these experiments, it can be concluded that TFIIIB binds specifically to c-Myc, and this interaction appears to be through the TAD.

6.2.6 TFIIIB Reconstitutes Pol III Transcription from GST-Myc-N262 Depleted Extracts

As shown before, whole cell extracts preincubated with GST-Myc-N262 are depleted of a factor required for pol III transcription (Fig. 6.2). Although c-Myc binds to TBP and removes it from the extract, the addition of recombinant TBP alone does not reconstitute transcription of class III genes (Fig. 6.3); thus other factors are being removed by c-Myc's depletion. From the results obtained so far, it can be concluded that c-Myc can associate with two TFIIIB subunits (Brf and TBP, Fig. 6.6). Therefore, a similar experiment as the one using recombinant TBP was designed, but this time it was assayed if addition of a fraction containing TFIIIB could restore pol III transcription of CHO whole cell extracts that had been previously preincubated with beads carrying GST or GST-Myc-N262. These extracts were used in transcription assays with $tRNA^{Leu}$ as template. IVT reactions were supplemented either with a fraction containing pol III, which served as a negative control, or a fraction containing TFIIIB (the fractions used were previously prepared by chromatography from HeLa nuclear extract). Figure 6.8 shows that addition of a fraction containing TFIIIB can fully restore $tRNA^{Leu}$ expression, whereas addition of a fraction containing pol III does not. From the results presented so far, it can be concluded that TFIIIB binds specifically to the TAD of c-Myc and that this binding

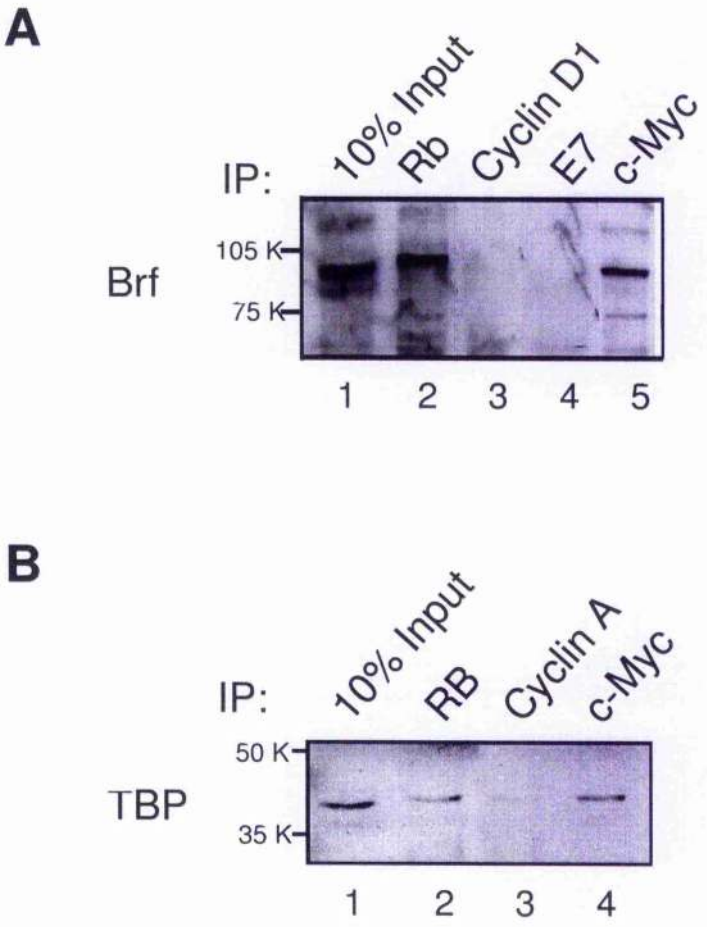

Figure 6.7: c-Myc interacts with the TFIIIB subunits Brf and TBP at physiological ratios. (A) HeLa nuclear extract (187.5 μg) was immunoprecipitated using an anti-RB monoclonal antibody (G3-245, lane 2) as positive control or anti-E7 (TVG7 10Y, lane 3) and anti-Cyclin D1 (R124, lane 4) as negative control. Lane 5 was using an anti-c-Myc antibody (9E10). Immunoprecipitated material was resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with antiserum 128-4 against Brf and lane 1 is 10% of input material. (B) HeLa nuclear extract (187.5 μg) was immunoprecipitated using an anti-RB monoclonal antibody (G3-245, lane 2) as positive control or an anti-Cyclin A antibody (BF683, lane 3) as negative control. Lane 4 was using an anti-c-Myc antibody (9E10). Immunoprecipitated material was resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with antibody 58C9 against TBP and lane 1 is 10% of input material.

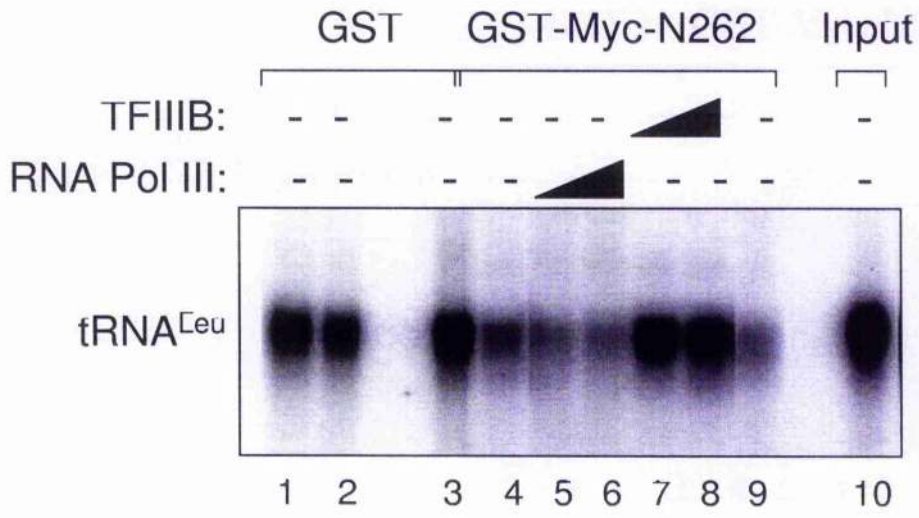

Figure 6.8: Reconstitution of pol III transcription using a PC-B fraction from GST-Myc-N262 depleted extract. Transcription of the $tRNA^{Leu}$ gene was assayed in CHO whole cell extract previously incubated with beads carrying GST (lanes 1 to 3) or GST-Myc-N262 (lanes 4 to 9). Lanes 5 and 6 were supplemented with 2 μl and 4 μl of a pol III fraction, respectively. Lanes 7 and 8 were supplemented with 2 μl and 4 μl of a TFIIIB fraction. Lane 10 shows transcription from whole cell extract that had not undergone any manipulation.

is functionally significant. Furthermore, this interaction is consistent with the fact that induction of tRNA genes *in vivo* requires the TAD of c-Myc (Chapter 5).

6.2.7 Endogenous c-Myc Cofractionates with TFIIIB

The results shown above indicate that there is a physical and functional interaction between c-Myc and TFIIIB. A different way of demonstrating this association between endogenous c-Myc and TFIIIB is by testing for the presence of c-Myc in biochemically fractionated TFIIIB. The different components of the pol III machinery (TFIIIA, TFIIIB and TFIIIC) can be separated from each other by chromatographic fractionation on phosphocellulose (PC) of nuclear extracts. Since the fractions from a PC column very crude, the fractionated material can be further purified using different types of chromatographic resins and gradients to achieve a cleaner sample. The presence of c-Myc was assayed by immunoblotting different TFIIIB fractions purified through different types of resins, and probing them with an anti-c-Myc antibody. Consistent copurification of TFIIIB and c-Myc would be suggestive of a stable interaction between these two factors.

Figure 6.9 demonstrates that c-Myc is readily detectable in TFIIIB fractions

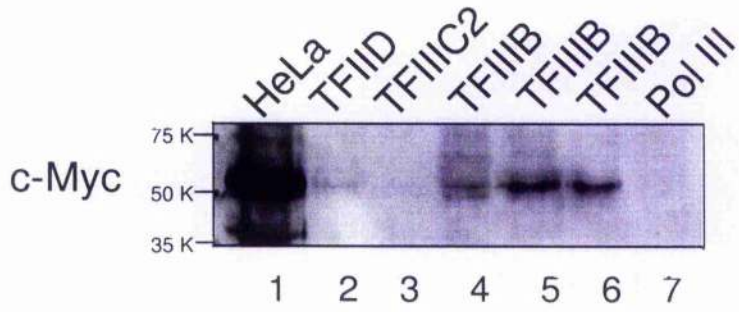

Figure 6.9: A population of c-Myc molecules co-fractionates with TFIIIB. c-Myc is readily detectable in fractions containing TFIIIB and TFIID but not in fractions containing pol III or TFIIIC. Fractionated factors (10 μl) as indicated were analyzed by Western immunoblotting with anti-c-Myc antibody 9E10. The TFIID fraction in lane 2 was prepared by chromatography (Phosphocellulose followed by S-Sepharose). The TFIIIC fraction in lane 3 was oligo-purified (Phosphocellulose followed by a B-block DNA-affinity column). The TFIIIB fraction in lane 4 was prepared by chromatography on Q-Sepharose followed by phosphocellulose. The TFIIIB fractions in lanes 5 and 6 were prepared by step fractionation on phosphocellulose followed by a MonoQ gradient. The pol III fraction in lane 7 was prepared by step fractionation on phosphocellulose and DEAE-Sephadex.

that have been purified through different resins like PC and Q-Sepharose (lane 4) or MonoQ (lanes 5 and 6). In contrast, no c-Myc was detected in fractions containing TFIIIC (lane 3) or pol III (lane 7). As expected, a TFIID fraction also presented c-Myc (lane 2), although at lower levels than those observed for TFIIIB fractions because this TFIID fraction is very dilute.

Although the experiment presented above demonstrates that endogenous c-Myc copurifies with TFIIIB, it does not show whether c-Myc is interacting with active TFIIIB. To study if this is happening, a PC fraction containing TFIIIB was chromatographed on Mono Q and gradient eluted fractions were obtained. These fractions were then immunoblotted for the presence of c-Myc using an antibody against it (Fig. 6.10, upper panel), as well as assayed for TFIIIB transcriptional activity using VA1 as template in the presence of TFIIIC and pol III (Fig. 6.10, lower panel). It can be observed from figure 6.10 that both c-Myc and TFIIIB activity are detected in the same fractions (41, 42, 43 or lanes 6 to 8), peaking at fraction 42. Thus, c-Myc cofractionates closely with TFIIIB activity.

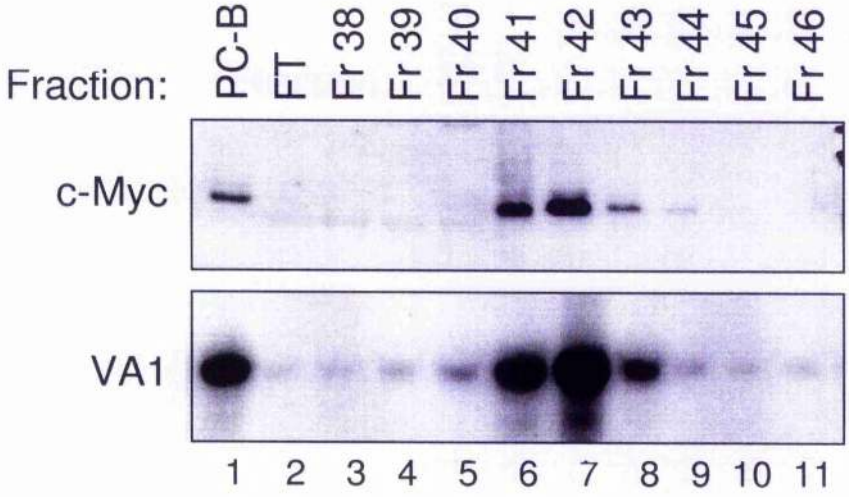

Figure 6.10: c-Myc co-fractionates with TFIIIB activity during gradient chromatography on MonoQ. The upper panel shows c-Myc content using 10 μl of the indicated fractions which were resolved on an SDS-7.8% polyacrylamide gel and analyzed by Western immunoblotting with anti-c-Myc antibody 9E10. The lower panel shows TFIIIB activity of individual fractions. In each case lane 1 contains input material (PC-B), lane 2 contains flow-through, and lanes 3-11 contain fractions 38-46, respectively. TFIIIB activity was assayed using 4 μl of the indicated fraction, 2μl of PC-C and 500 ng of pVAI; after 15 min incubation at 30°C, nucleotides were added to assay transcription (this experiment was performed by Robert White).

These data demonstrate that a population of c-Myc molecules copurifies with TFIIIB on PC, Mono-Q and Q-Sepharose columns. Such copurification supports the data found by coimmunoprecipitation experiments indicating there is a specific stable interaction between endogenous c-Myc and TFIIIB.

6.2.8 Brf Interacts Directly with c-Myc in the Absence of TBP

Once it was established that c-Myc associates stably with TFIIIB, the interaction between the Brf subunit and c-Myc was analysed further. Although the pull-down and coimmunoprecipitation assays show that the TFIIIB subunit Brf associates with c-Myc, from these experiments it cannot be determined whether this association is through interaction of c-Myc with TBP, which is well documented, or whether it is a direct interaction between Brf and c-Myc. To test if this interaction is direct, pull-down assays were performed using *in vitro* translated Brf instead of nuclear extract. Again, equal amounts of GST or GST-Myc-N262 were used. An interaction between recombinant Brf and the recombinant N-terminal domain of c-Myc was detected while beads carrying GST alone could not retain recombinant Brf (Fig. 6.11, lanes 3 and 5, or 2 and 4, respectively). From this experiment, it can be concluded that Brf, like TBP, interacts with the TAD of c-Myc and this interaction does not require the participation of TBP.

6.2.9 TBP Interacts with Max

As mentioned before, c-Myc forms heterodimers with the bHLH-LZ protein Max to induce activation of transcription from class II genes. c-Myc transcriptional activation from genes transcribed by pol II requires its heterodimerization with Max. In these genes, the Myc-Max heterodimer recognizes and binds specifically to a DNA sequence referred to as an E-box; however, c-Myc recruitment to class III genes is not by DNA recognition, but through protein-protein interactions with the pol III-specific transcription factor TFIIIB. Therefore, it was investigated if the c-Myc binding partner Max can also associate with the subunits of this transcription factor. First, pull-down assays were performed with beads carrying equal amounts of recombinant GST, as a negative control, or with the recombinant fusion proteins GST-Max. The recombinant fusion protein GST-Myc-N262 was used as a positive control. As shown in figure 6.12, no interaction can be observed between Brf and the negative control (GST) or with GST-Max, although an interaction between Brf and GST-Myc-N262 is quite clear (Fig. 6.12A, upper panel, lane 4). On the other

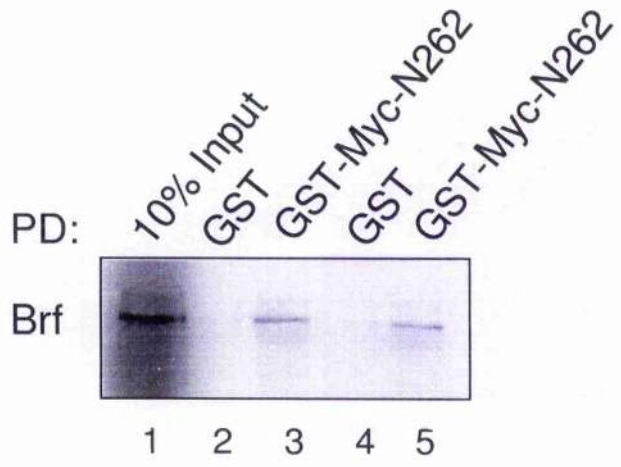

Figure 6.11: Brf binds to immobilized c-Myc. Reticulocyte lysate (5 μl) containing in vitro-translated Brf was added to beads carrying 50 μg of GST (lanes 2 and 4) or GST-Myc-N262 (lanes 3 and 5) and incubated at 4 C with agitation. Proteins retained after extensive washing were resolved on a SDS-7.8% polyacrylamide gel and then visualized by autoradiography. Lane 1 show 10% of the input reticulocyte lysate containing in vitro-translated Brf.

hand, immunoblotting of the eluted material from beads carrying GST-Max appears to contain TBP (Fig. 6.12, lower panel, lane 3), suggesting an interaction between TBP and Max. Again, an interaction between the N-terminal domain of c-Myc and TBP was obtained, while no TBP is retained by beads carrying GST.

To analyse further the interaction between Max and TBP, it was investigated whether these proteins still associate at endogenous ratios. To achieve this, coimmunoprecipitation analysis was performed with an anti-Max antibody. An anti-E7 antibody was used as a negative control, although an interaction between TBP and E7 has been reported [356]. As positive control, an anti-RB antibody was used since its association with TBP through TFIIIB is well documented [179]. Western blot analysis of the immunoprecipitated material was performed using an antibody against TBP. A faint band which might correspond to TBP (albeit with slightly lower molecular weight) was be detected by immunoprecipitation with an anti-E7 antibody (Fig. 6.12, lane 4). On the other hand, an interaction between TBP and Max, like that of the positive control RB, can be observed. These findings demonstrate that an association between TBP and Max is specific, and happens at physiological ratios.

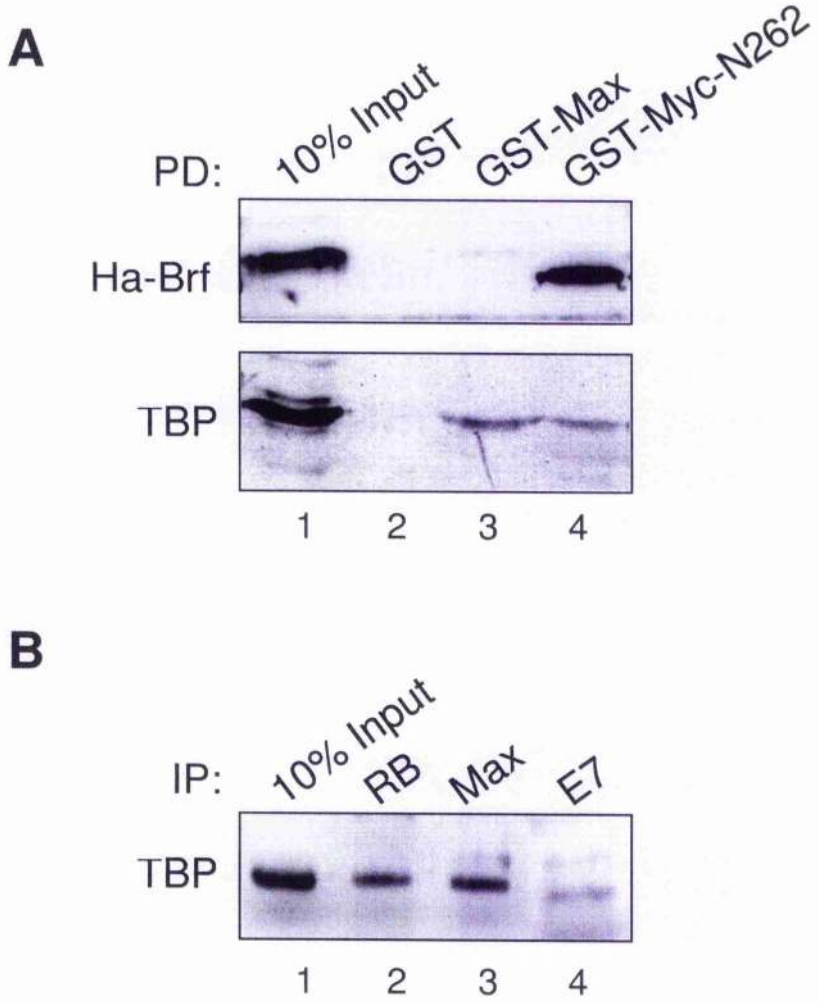

Figure 6.12: TBP binds to the c-Myc binding partner Max. (A) Proteins from CHO whole cell extracts (150 μg) that remained bound to beads carrying 50 μg of GST (lane 2), GST-Max (lane 3), or GST-Myc-N262 (lane 4) after extensive washing were resolved on an SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting with anti-TBP antibody. Lane 1 is 10% of input material. (B) HeLa nuclear extract (187.5 μg) was immunoprecipitated using anti-RB antibody G3-245 as a positive control (lane 2), anti-Max antibody H-2 (lane 3) or anti-E7 antibody TVG7 10Y (lane 4). Immunoprecipitated material was resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with anti-TBP antibody 58C9. Lane 1 is 10% of input material.

6.3 Discussion

It has been previously demonstrated that c-Myc can activate directly pol III transcription, without the induction of proteins involved in this process or the inhibition of the RB family (Chapter 5). Furthermore, c-Myc is localised at different class III genes in vivo. This chapter has addressed the molecular mechanisms of this localisation, which might give an insight on how c-Myc is regulating pol III transcription. Although it has been shown that c-Myc is present at class III genes, in this chapter it was tested how this localisation is achieved. As demonstrated on figure 6.1, depletion of this oncoprotein affects pol III activity; thus c-Myc is interacting stably with one or more components of the pol III machinery.

Once it was established that c-Myc localisation at class III genes is physiologically important, the mechanisms involved in this process were investigated. The first obvious possibility for c-Myc localisation is through the recognition of a specific DNA sequence present in class III genes. However, these genes do not contain the E-box sequence that c-Myc recognizes and binds to. Therefore, protein-protein interactions between the pol III machinery and c-Myc were investigated. In the literature it has been shown that the TAD of c-Myc binds to TBP; thus, this protein seemed to be the most likely molecule to be involved in c-Myc recruitment. Although an interaction between c-Myc and TBP was detected, TBP addition to c-Myc depleted extracts did not restore pol III transcription. These findings suggested that another protein or proteins are also involved in c-Myc's binding to class III genes. TFIIIB contains TBP, therefore it is the most plausible factor to associate with c-Myc. Indeed, a stable association between TFIIIB and c-Myc was demonstrated using several independent lines of evidence. (i) Pull-down experiments demonstrate an association between the Brf and TBP subunits of TFIIIB and the recombinant N-terminal domain of c-Myc. (ii) Addition of TFIIIB reconstitutes pol III transcription from depleted extracts with recombinant c-Myc. (iii) The TFIIIB subunits TBP and Brf coimmunoprecipitate with endogenous c-Myc; this effect is specific, since these subunits are not seen with negative control antibodies. (iv) A population of endogenous c-Myc copurifies with TFIIIB during a variety of distinct fractionation procedures; this is a specific association since no c-Myc could be detected in fractions containing TFIIIC or pol III. From these chromatographic experiments, it cannot be excluded that c-Myc and TFIIIB copurification is fortuitous; however, this seems to be unlikely given the coimmunoprecipitation data. Thus, these experiments suggest that a proportion of endogenous TFIIIB exists in association with c-Myc.

In contrast, no interaction between c-Myc and TFIIIC could be detected. In

the case of pol III, the first experiments performed with recombinant c-Myc suggested that there was an interaction between this oncoprotein and the pol III specific subunit BN51. However, the concentration of the recombinant c-Myc used in these experiments does not represent the physiological levels of the protein. When this interaction was investigated further, at the normal physiological ratios of both proteins, no association could be detected. Furthermore, addition of pol III to a depleted extract with recombinant c-Myc (GST-Myc-N262) did not reconstitute pol III transcription. In addition, no endogenous c-Myc could be found in fractions containing pol III. From this evidence it can be concluded that there is probably no interaction between endogenous c-Myc and pol III. Another TF that is required for pol III transcription is TFIIIA. As mentioned in the introduction of this thesis, TFIIIA is only required for transcription of class III genes containing type I promoters, like that of 5S rRNA. Since c-Myc immunodepletion or depletion with its N-terminal domain not only reduced the transcription of 5S rRNA genes, but also that of tRNA genes, which contain type II promoters and do not require TFIIIA, the participation of this TF in the recruitment of c-Myc was not investigated.

The interaction observed between c-Myc and the TFIIIB subunits, Brf and TBP, appears to be through the TAD of c-Myc. Although these subunits are detected in association with c-Myc, this interaction might be regulated by TBP-c-Myc binding. Further analysis should be performed to study whether c-Myc can bind to B". For this to be achieved, interaction studies have to be performed with purified and separated subunits of TFIIIB and recombinant c-Myc in the absence of other proteins that could mediate their interaction. So far, the experiments presented here demonstrate that c-Myc associates stably and specifically with the TFIIIB subunits TBP and Brf. The interaction of these two proteins with c-Myc appears to be with the TAD of c-Myc; however, further analysis should be performed to study in detail the association of Brf and c-Myc. It is also interesting to note that the region of c-Myc to which TFIIIB binds, the TAD, is required for the induction of tRNA genes *in vivo*. Thus, this association is likely to be physiologically important for c-Myc induction of pol III transcription.

An interesting finding obtained in the experiments presented here is the interaction observed between c-Myc's binding partner Max and the transcription factor TBP (Fig. 6.12). TBP is involved in transcription by all three pols. The fact that Max can associate with it might suggest that Max and its binding partners (the Myc and Mad families) are involved in the regulation of genes transcribed by all three pols. It is already known that c-Myc-Max regulate a wide number of genes

transcribed by pol II; and from the results presented in this thesis, c-Myc can also regulate transcription by pol III. However, the relevance of Max association with TBP and the induction of pol III transcription by c-Myc has still to be determined. In the case of pol I transcription, it will be interesting to study if c-Myc-Max heterodimers activate it through a protein-protein interaction of Max and TBP. Indirect experiments suggest that c-Myc might be involved in the regulation of pol I transcription, since expression of ribosomal RNA genes appears to be reduced in c-Myc null cells [191]. Furthermore, N-Myc, another Max binding partner, has been shown to induce transcription of ribosomal genes in neuroblastomas [357]. Therefore, it will be interesting to study the interaction between Max and TBP, and its possible participation in the regulation of transcription by all three RNA polymerases.

7 Activation of RNA Polymerase III Transcription by Id2

7.1 Introduction

The Id family (inhibitors of DNA binding/differentiation) of helix-loop-helix (HLH) proteins were discovered 10 years ago, as proteins that inhibit both DNA binding and differentiation [358]. They are members of a larger family of transcription factors, referred to as the HLH family which comprises >200 members. The HLH transcription factors form heterodimers with each other through the HLH domain, composed of two amphipathic α helices, each 15-20 residues long, separated by a shorter intervening loop [19]. Adjacent to the HLH domain, a region of highly basic residues is present. The presence of both basic regions from the heterodimer is required for DNA binding to the canonical E Box recognition sequence CANNTG [19, 359]. The bHLH proteins are involved in tissue specific cell differentiation, e.g. myogenin in skeletal muscle differentiation, neurogenin in neurogenesis, and SCL/tal-1 in hematopoiesis [360]. To exert their action, these tissue-specific bHLH proteins require to heterodimerize with ubiquitously expressed bHLH proteins, which include E2-2, and the E2A gene products, E12 and E47, among others. The heterodimers containing two basic regions of each bHLH protein can then bind to DNA and activate expression of genes involved in differentiation. The Id proteins can also bind to the ubiquitously expressed bHLH proteins, however, because they lack the basic region, the heterodimers that are formed are unable to bind to DNA. The Id proteins quench the ubiquitously expressed bHLH proteins, so that they can no longer form dimers with tissue-specific bHLH proteins and differentiation is blocked. Thus, the Id proteins act as negative regulators of bHLH proteins and hence of differentiation (Fig. 7.1) [358, 359, 361].

7.1.1 Cellular functions of Id Proteins

In mammals, the Id family is composed of four members, Id1 to Id4, which apart from the HLH domain, are highly sequence divergent [358]. These proteins play an essential role during development in processes like neurogenesis and lymphopoiesis, as well as organogenesis; they present functional redundancy, so if one gene is missing, the other three can overcome this lost [359]. As mentioned before, the Id proteins inhibit cell differentiation by binding and sequestering bHLH TFs which drive the expression of genes involved in this process [358]. In agreement with this model, Id expression in terminally differentiated cells is low or absent (except for Id2 expression in myelopoiesis, which is upregulated in differentiated cells) [359]. Since differentiation and cell growth are mutually exclusive, these proteins actually act as positive regulators of cell growth and induce cells to continue proliferating, a property which confers on them a wider role in biological processes like cell cycle progression and tumour development. Thus, as it might be expected, expression of Id genes is high not only in undifferentiated cells but also in tumour cells [16].

7.1.2 Role of the Id Family in Tumourigenesis

The overexpression of Id genes has been shown to present oncogenic properties in both primary and immortalized cells. When overexpressed in primary fibroblasts with another oncoprotein like Bcl-2, they produce immortalization of the cells [359]. Deregulation of Id expression has been observed in different types of primary tumours, including overexpression of Id2 in pancreatic cancer [362]; overexpression of Id1, 2 and 3 in astrocytic tumours [363], and colorectal adenocarcinomas [364]; and overexpression of Id1 in breast cancer [364]. In fact, breast mammary tumours that express high levels of Id1 are frequently associated with infiltrating, more aggressive tumours, which suggest a relationship between Id expression and disease progression [359]. Moreover, Id function is required for vascularization and invasiveness of tumour growth *in vivo* [364]. Studies using transgenic mice overexpressing Id genes have also demonstrated their participation in oncogenesis. Transgenic mice that overexpress Id2 protein in thymocytes not only present a block in early thymopoiesis, but also an aggressive T cell hyperproliferation that resembles lymphoma, suggesting that Id2 overexpression predisposes T cells to oncogenic transformation *in vivo* [365].

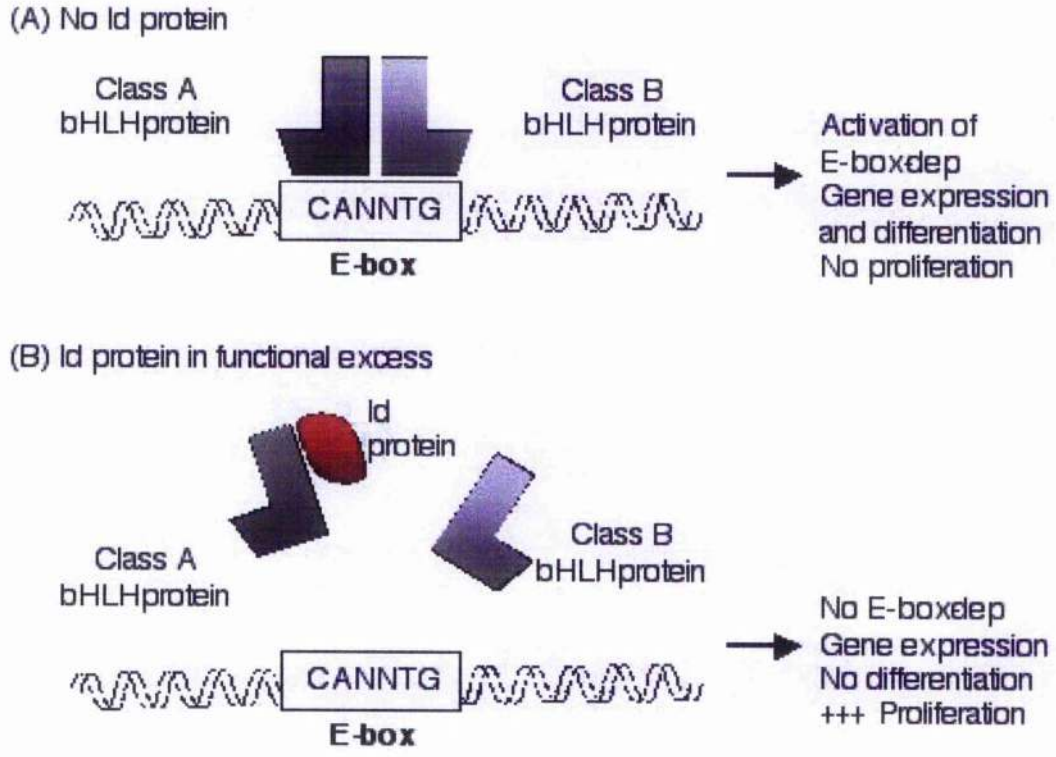

Figure 7.1: Id proteins as dominant-negative antagonists of basic helix-loop-helix (bHLH) transcriptional regulators. (A) bHLH proteins typically bind consensus E-box regulatory sequences in a heterodimeric configuration and function to activate differentiation-linked gene expression. (B) In the presence of excess Id protein, the class A bHLH partner is sequestrated from the heterodimeric complex, resulting in an Id-bHLH heterodimer that is unable to bind DNA, resulting in inhibition of differentiation. This is typically found in proliferating cells. (Taken from [361]).

7.1.3 Functions of Id in Growth Control

A role for Id proteins in cell cycle reentry and induction of cell growth has been observed in mammalian cells using antisense oligonucleotides against the Id mRNAs [366]. From these experiments, it was demonstrated that Id genes are early responsive genes whose expression is strongly dependent on the presence of growth factors. These proteins participate in growth induction following growth factor stimulation of quiescent cells, since if their expression is inhibited by specific Id antisense oligonucleotides, there is a significant delay in DNA synthesis after addition of growth factors, with a specific reduction of the Id mRNA levels [366]. Serum withdrawal (from 20% to 0.5%) in mice fibroblasts reduces Id expression to 50% in <1 h and declines to undetectable levels in quiescent cells [361, 366]. After serum addition, their expression recovers rapidly and peaks at 30 min, followed by a second peak as cells enter the S phase [361, 366]. If these proteins are expressed ectopically, they promote cell growth and therefore inhibit differentiation. Furthermore, indirect evidence of Id regulation of cell growth comes from the observation that these proteins are highly expressed in rapid proliferating cells, like those from early development, while most mature differentiated tissues do not express them [367]. More evidence to show the contribution of Id expression to cell growth has come from experiments performed with smooth muscle cells. Matsumara et. al have demonstrated that overexpression of Id2 promotes cell growth of vascular smooth muscle cells via increased S-phase entry [368]. In this model, Id2 inhibits the expression of the cyclin kinase inhibitor p21, and this regulation depends on phosphorylation of Id2 by cyclin E/cdk2 [368]. All the evidence presented so far demonstrates that these proteins are important positive regulators of cell growth and proliferation.

7.1.4 The Id Proteins and the RB Family

The mechanisms by which the Id proteins regulate cell cycle reentry and cell growth appear to be not only by binding to and antagonising other bHLH protein's function, but also by regulating proteins of the cell-cycle control machinery. In particular, the Id2 protein has been shown to bind the RB tumour suppressor protein and the related proteins p107 and p130 [7, 8, 16]. As mentioned in the introduction of this thesis, the tumour suppressor protein RB, regulates the cell cycle by binding and inhibiting TFs which drive expression of genes required for G1/S phase progression, like E2F-DP1. This inhibition can only be achieved by unphosphorylated RB, since it's phosphorylated form cannot bind to the TFs and therefore cannot inhibit their function anymore. It is interesting to note that Id2 binds to the pocket proteins

in their unphosphorylated active form, through its HLH domain, inactivating their growth inhibitory function. Id2 has been shown to reverse the RB-mediated cell cycle arrest when coexpressed with RB in the human osteosarcoma cell line SAOS-2 which lacks RB [7]. Furthermore, it can also relieve the cell cycle arrest induced by the cyclin kinase inhibitors p16 and p21, in cells containing functional RB (U20S) by inactivating RB [8]. A role for Id1, which might also be used by the other members of the Id family, in cell cycle progression has also been described. Id1 can inhibit the expression of the cyclin kinase inhibitor $p21^{Cip1/Waf1}$ by binding and inhibiting the bHLH TF E2A which drives its expression [369]. A decrease of p21 causes the activation of cyclin E/cdk2 which inactivates RB by phosphorylating it and allowing the expression of genes required for G1/S progression.

The data summarized in this introduction illustrate that the Id proteins are positive regulators of both cell cycle progression and cell growth, while being negative regulators of differentiation. The genes transcribed by pol III are known to be essential for cell growth [10]; moreover, their expression gets downregulated during differentiation [149]. Since Id expression and pol III activity appear to happen in the same conditions, it would be interesting to investigate whether expression of Id proteins might have an effect on pol III activity. This chapter addresses this hypothesis; although only the effect of Id2 on pol III activity was studied.

Transcription by pol III is tightly regulated by RB and the pocket proteins (p107 and p130) [3,182,184]. Similar to RB repression of E2F, the active unphosphorylated form of RB binds to the specific pol III factor TFIIIB, sequestering it from class III genes, hence repressing pol III transcription [182]. Since Id2 has been shown to bind and inhibit the RB family and block their repressive action exerted on TFs like E2F/DP1, it might also participate in the activation of pol III by relieving TFIIIB from RB repression. From this reasoning, it would be interesting to study whether Id2 can actually regulate pol III activity. The following experiments presented in the results section were designed to assess if indeed this is the case. Due to the fact that the other members of the Id family have not been shown to interact directly with the RB family, their participation in the regulation of pol III transcription was not investigated. In the future, it would also be interesting to investigate a role for Id1 in the regulation of pol III transcription, since it has been shown that it can also regulate RB activity through indirect mechanisms [369]. However, in this chapter only the relationship between pol III transcription and Id2 expression is going to be investigated.

7.2 Results

7.2.1 Id2 Competes with Brf for RB Binding

As mentioned in the introduction, Id2 can form heterodimers with RB, sequestering this tumour suppressor from heterodimers formed with transcription factors and therefore, allowing them to activate expression of their target genes and thus cell proliferation [7,8]. One of the transcription factors which RB binds and represses is the specific pol III factor TFIIIB [3,179,182]. Since activation of pol III transcription is also required for appropiate cell growth (and hence cell proliferation), it might be upregulated by Id2 through sequestration of RB from TFIIIB/RB complexes, allowing TFIIIB recruitment to class III genes and hence their expression. To determine if Id2 can actually sequester RB from TFIIIB/RB complexes, immunoprecipitation analysis was performed. Endogenous RB from HeLa nuclear extracts was immunoprecipitated using an anti-RB antibody (C-15) in the presence of *in vitro* translated Id2, or *in vitro* translated Brf, subunit of TFIIIB, or both. As a control for the disruption of the TFIIIB/RB complex upon addition of protein, rabbit reticulocyte lysate was utilized. As previously shown by other authors, RB immunoprecipitates *in vitro* translated Id2, as well as *in vitro* translated Brf (Fig. 7.2, lanes 1 and 2, respectively). However, when both proteins are added at the same concentration to the immunoprecipitation assay, the interaction between RB and Brf diminishes, while the interaction between Id2 and RB remains equal to the one seen when no Brf is present (Fig. 7.2, lane 3). The reduction observed in the interaction between RB and Brf in the presence of Id2 is specific, since addition of rabbit reticulocyte lysate did not have any effect on the binding of these two proteins (Fig. 7.2, lane 4). This result suggests that Id2 has more avidity for RB than the TFIIIB subunit Brf, and thus is able to sequester RB from TFIIIB/RB complexes. If indeed this is happening in vivo, Id2 might be able to activate pol III transcription by sequestering RB and thus releasing TFIIIB from RB repression. To assess this, the following experiments were designed.

7.2.2 Pol III Transcription in Id2 Knockout Cells

Having established that Id2 can successfully compete with TFIIIB for RB, it was investigated whether Id2 expression has any effect on pol III transcription. To assess this, transcription of the pol III transcribed gene B2 was assayed in wild type or *Id2* null mouse embryo fibroblasts. Total RNA extracted from wild type and Id2 knockout cells was analysed by Northern blot. When *Id2* null cells and matched

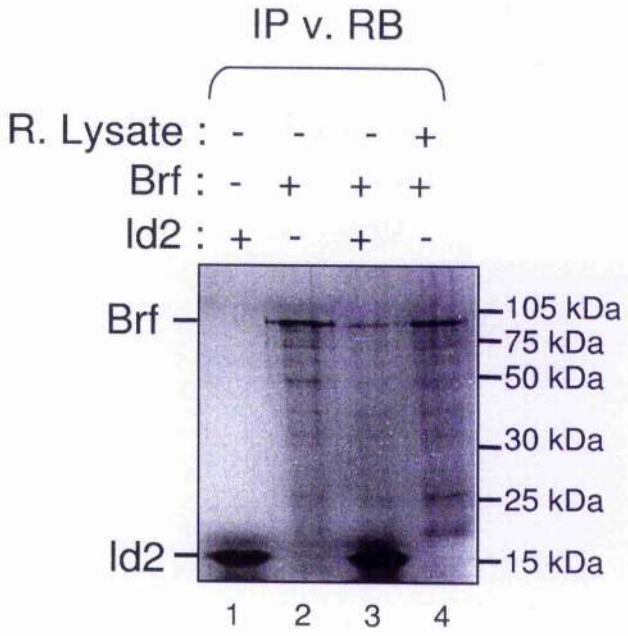

Figure 7.2: *In vitro* translated Id2 binds more avidly to RB than the TFIIIB subunit Brf. HeLa nuclear extract (187.5 μg) was immunoprecipitated using an anti-RB antibody (C-15) in the presence of *in vitro* translated Id2 (lane 1), *in vitro* translated Brf (lane 2), both (lane 3), or *in vitro* translated Brf plus rabbit reticulocyte lysate (lane 4). Immunoprecipitated material was resolved on an SDS-7.8% polyacrylamide gel and then visualized by autoradiography.

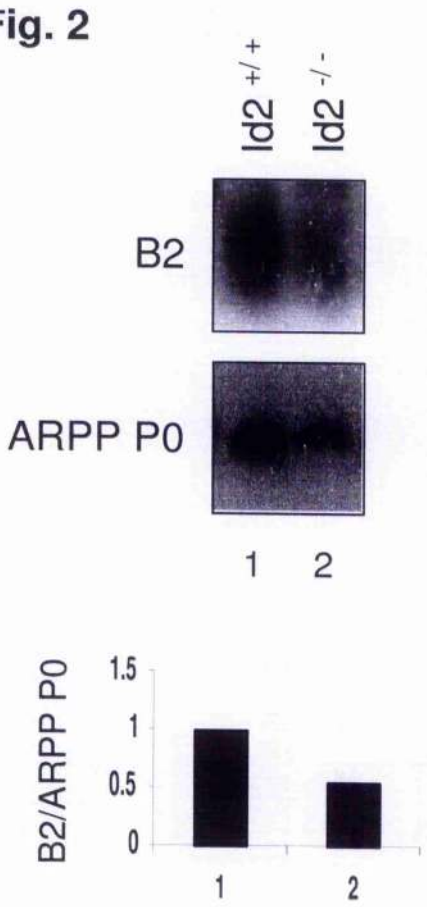

Figure 7.3: Id2 expression affects pol III activity *in vivo*. Total RNA (10 μg) extracted from wild-type ($Id2^{+/+}$) or Id2-null fibroblasts ($Id2^{-/-}$) was analysed by Northern blot using a DNA probe against the pol III transcript B2 (*upper panel*) and ARPP P0 (*lower panel*).

wild-type fibroblasts are compared, the former show lower expression of pol III transcripts from the B2 repetitive gene family (Fig. 7.3, upper panel). Levels of B2 RNA provide a convenient and reliable indication of pol III transcription activity, since these transcripts are rapidly degraded after being transcribed [10], thus reflecting pol III transcriptional activity at the time of extraction of RNA and not build up products. mRNA encoding acidic ribosomal phosphoprotein (ARPP) P0 was used as loading control, and although there is a slight decrease of ARPP P0 mRNA in the Id2 knockout cells compared to the wild type, the decrease observed for the B2 transcript is considerably more striking (Fig. 7.3). From the results obtained here, it can be concluded that pol III activity is indeed sensitive to the presence of endogenous Id2 *in vivo*.

7.2.3 Id2 Is Required for Pol III Transcription *in vitro*

To analyse further if the presence of endogenous Id2 stimulates pol III transcription, in vitro transcription of the pol III template VA1 from immunodepleted extracts was performed. HeLa nuclear extracts were incubated for 2 hours, prior to the in vitro transcription, with different antibodies, including an anti-Id2 antibody, to deplete them of the particular protein that the antibody would recognise. As positive control, the nuclear extract was depleted of TBP, since this protein is essential for pol III transcription, while for the negative control the pol II-specific factor TFIIB was chosen. Depletion of the bHLH protein E47 was also used as a negative control, since it shares the same HLH motif as Id2 and has not been shown to produce any effect on pol III transcription so far. If Id2 is having an effect on pol III transcription, as observed for the B2 transcript in the Id2 knockout mouse embryo fibroblasts compared to wild type, its depletion should affect its activity. As previously demonstrated (Chapter 6), extracts depleted of TBP show a dramatic reduction in VA1 transcription (Fig. 7.4, lane 3). In contrast, mock-depletion or immunodepletion of TFIIB have little effect on pol III transcription (Fig. 7.4, lanes 2 and 4, respectively). When the extracts are depleted of Id2 with an anti-Id2 antibody, they display reduced VA1 transcription (Fig. 7.4, lane 5), although to a lesser extent than that observed for TBP. However, depletion of the bHLH transcription factor E47 does not have any effect in VA1 transcription, proving that the reduction in pol III activity obtained from the Id2-depleted extracts is specific (Fig. 7.4, lane 6). The evidence presented so far demonstrates that endogenous Id2 has a positive effect on pol III activity.

7.2.4 Overexpression of Id2 Activates Pol III Transcription

Id2 has been shown to be overexpressed in different types of cancers, e. g. neuroblastomas, pancreatic carcinomas and astrocytic tumours [16, 362, 363]. Moreover, it has been shown to transform primary cells when overexpressed, acting as a co-operating oncogene [359], and to predispose T cells to oncogenic transformation *in vivo* [365]. Pol III transcription is upregulated in different types of cancer, as well as in transformed cell lines [10]. Transformed cells grow faster than normal cells. In rapid growing cells, pol III transcripts, including tRNAs and 5S rRNA, must be produced in bulk to meet the need for protein synthesis. Since Id2 overexpression has been shown to induce cell growth, as well as being highly expressed in rapidly proliferating cells [367], it would be interesting to study if it can also upregulate pol III activity. To achieve this, mouse fibroblasts (Balb/c 3T3) were transiently

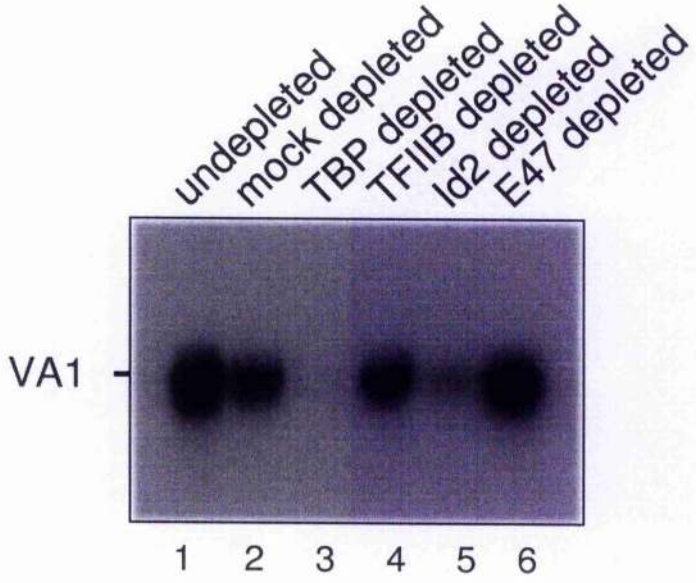

Figure 7.4: Immunodepletion of Id2 reduces pol III activity. Transcription of the VA1 gene was assayed using HeLa nuclear extracts undepleted (lane 1), or previously immunodepleted for 2 hours on ice with antibodies raised against TBP (MTBP-6, lane 3), TFIIB (C-18, lane 4), Id2 (C-20, lane 5), or E47 (N-649, lane 6) or mock depleted (lane 2).

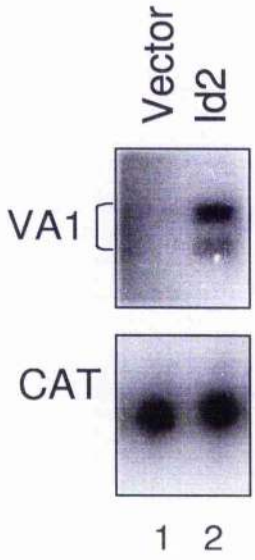

Figure 7.5: Activation of pol III transcription by Id2 overexpression. Balb/c 3T3 cells were transiently transfected with pVA1 (0.5 μg), pCAT (0.5 μg) and pId2 (2 μg), encoding wild-type Id2 (lanes 2) or empty vector (lane 1). RNA was extracted after 48 hrs of transfection. VA1 (*upper panel*) and CAT (*lower panel*) RNA levels were assayed by primer extension analysis.

transfected with empty vector or an Id2 expression vector, together with the class III gene, adenovirus VA1, and the SV40-CAT vector as control. Total RNA was extracted and analysed by primer extension. As seen in figure 7.5, transfection of Id2 expression vector stimulated pol III transcription of the adenovirus VA1 gene in fibroblasts, compared to empty vector. This effect is specific, since the transcription of the cotransfected SV40-CAT reporter remained the same in both conditions (Fig. 7.5, lower panel). This result demonstrates that overexpression of Id2 induces pol III transcription.

7.2.5 Id2 Overexpression and Pol III Transcription in RB Knockout Cells

RB represses pol III transcription in a phosphorylation-dependent manner. Id2 can bind to the hypophosphorylated form of RB. In figure 7.2, it was clearly shown that Id2 has greater avidity for RB than the TFIIIB subunit Brf in an in vitro system. However, from this evidence it cannot be concluded whether the sequestration of RB is the mechanism that Id2 is using to activate pol III transcription. To assess

if this is the case, immortalized fibroblasts from wild-type or RB knockout mice were subjected to the same transient transfection as the one performed above. As expected, transient transfection with an Id2 expression vector stimulated pol III transcription of the adenovirus VA1 gene in the wild-type fibroblasts, compared to empty vector (Fig. 7.6, upper panel, lanes 2 and 1, respectively). If sequestration of unphosphorylated RB is the sole mechanim by which Id2 is inducing pol III activity, then no upregulation of VA1 would be expected from transfection of this protein into RB knockout cells. However, when Id2 was transfected in RB null fibroblasts, a robust and specific induction was still obtained (Fig. 7.6, upper panel, lane 4). This is a specific effect on VA1 expression, since the cotransfected SV40-CAT reporter was unaffected (Fig. 7.6, lower panel). The induction of VA1 transcription observed in the $RB^{-/+}$fibroblasts is stronger than that observed in the $RB^{-/-}$ fibroblasts. This implies a participation of RB in the Id2 activation of pol III transcription, since when RB is not present, the induction observed is smaller than that obtained when it is. However, if the sequestration of RB from TFIIIB/RB complexes were the sole mechanism by which Id2 activates pol III transcription, there would not have been any stimulation of the VA1 transcript in the $RB^{-/-}$ cells. Therefore, from these results it can be concluded that the sequestration of RB does not appear to be the sole mechanism by which Id2 triggers pol III activity.

7.2.6 Pol III Activity in Id2/RB Knockout Cells

From the results obtained from transfection into RB knockout cells, it appears that Id2 is stimulating pol III activity not only through the sequestration of hypophosphorylated RB, but also through a different unknown mechanism. In this experiment, Id2 is overexpressed under artificial conditions and might not reflect an accurate physiological role. To analyse further whether Id2 is using a secondary mechanism to upregulate pol III activity in addition to relieving TFIIIB from RB inhibition, pol III transcription of the B2 repetitive gene family was studied in mouse embryo fibroblasts carrying variable Id2/RB genotypic backgrounds ($Id2^{-/-}/RB^{-/-}$, $Id2^{+/+}/RB^{+/+}$, $Id2^{+/-}/RB^{-/-}$, $Id2^{+/1}/RB^{-/-}$). Northern blot analysis of total RNA extracted from the four types of mouse embryo fibroblasts was performed. If Id2 is activating pol III transcription only through RB inhibition, then the $Id2^{-/-}/RB^{-/-}$, $Id2^{+/+}/RB^{-/-}$should present the same levels of the B2 transcript. As shown n Fig. 7.7, this does not appear to be the case. The $Id2^{-/-}/RB^{-/-}$ presents lower B2 RNA levels than the $Id2^{+/+}/RB^{-/-}$ (Fig. 7.7, lanes 1 and 2, respectively). Furthermore, when there is only one Id2 gene present in an RB null

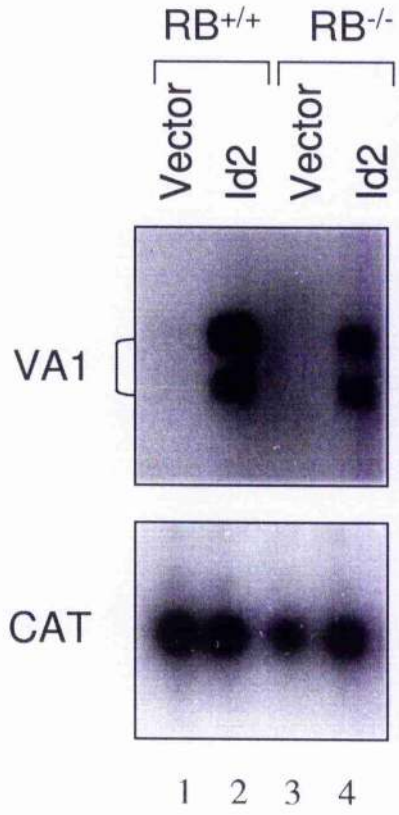

Figure 7.6: Activation of pol III transcription by Id2 does not require neutralisation of RB. Immortalized fibroblasts from RB null ($RB^{-/-}$, lanes 3 and 4) or wild-type ($RB^{+/+}$, lanes 1 and 2) were transiently transfected with pVA1 (0.5 μg), pCAT (0.5 μg) and pId2 (2 μg), encoding wild-type Id2 (lanes 2 and 4) or empty vector (lanes 1 and 3). RNA was extracted after 48 hrs of transfection. VA1 (*upper panel*) and CAT (*lower panel*) RNA levels were assayed by primer extension analysis.

context, the B2 RNA levels are higher than those of the Id2/RB knockout cells. When both Id2 genes are present in an RB knockout background, the highest expression of the B2 transcripts is observed (Fig. 7.7, lane 4), which confirms that Id2 upregulation of pol III activity is RB-independent. The mRNA encoding ARPP P0 was used as a loading control, since it is known not to be modulated by mitogens or serum growth factors, cell cycle or the RB pathway [287]. These findings are in agreement with the data obtained from the transfections, as well as for the Id2/RB wild type compared to Id2/RB knockout mice. Taken together, they indicate that the positive effect of Id2 on pol III transcription is through both RB-dependent and RB-independent mechanisms.

7.2.7 Id2 Overexpression and Pol III Transcription in RB/p107/p130 Knockout Cells

In the literature, it is well documented that Id2 not only binds to RB but also to the two RB-related proteins, p107 and p130 [8, 16]. As with RB, Id2 binds only to the hypophosphorylated active forms of p107 and p130 [16]. As mentioned in the introduction of this thesis, pol III transcription is also tightly regulated by the hypophosphorylated forms of these proteins and, like RB, they bind to TFIIIB and repress its function, hence downregulating pol III activity [184]. Since Id2 induction of pol III activity appears to be partially RB-independent, and this HLH protein can bind and sequester the pocket proteins which are able to repress pol III transcription, it was tested whether Id2 overexpression continues to activate the pol III transcript VA1 in the absence of the entire RB family. To achieve this, the same transient transfection experiment as the one described before was performed in primary fibroblasts from wild-type or RB-p107-p130 triple knockout mice (TKO). After the transfection, total RNA was extracted and analysed by primer extension. As expected, transfection of Id2 expression vector stimulated pol III transcription of the adenovirus VA1 gene in the primary fibroblasts, whereas the cotransfected SV40-CAT reporter remained unaffected. In the TKO cells, overexpression of Id2 still produced a specific induction of the VA1 transcript compared to empty vector. This effect is specific since the SV40-CAT reporter stayed constant. Although the TKO cells present higher pol III activity, the overexpression of Id2 produced a similar increase in both the wild-type and the TKO cells ($\sim$1.5 fold increase in the wild-type compared to $\sim$1.25 increase in the TKO cells). From these findings it can be concluded that Id2 can stimulate pol III transcription in the absence of the entire RB family.

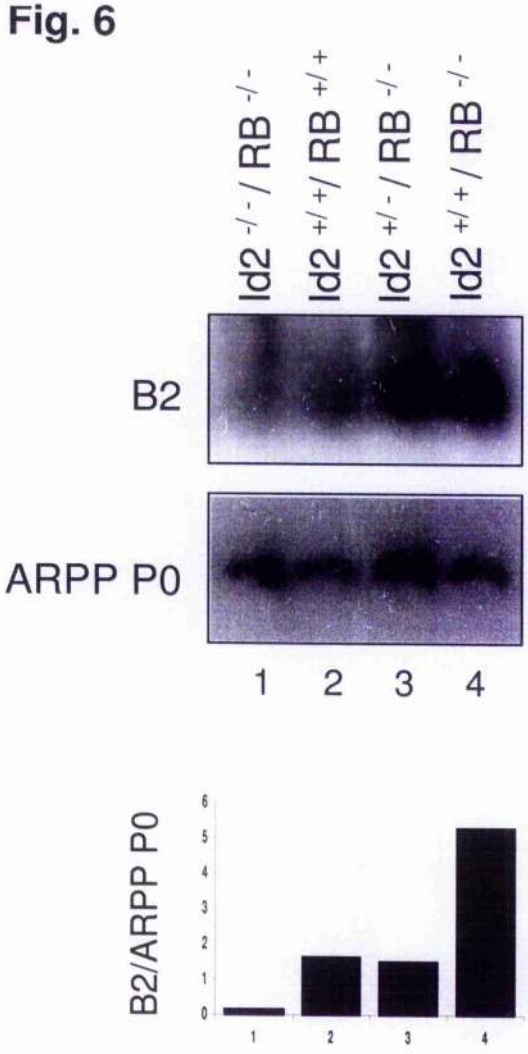

Figure 7.7: Pol III activity in different Id2/RB genotypic backgrounds. Total RNA (10 μg) extracted from Id2/RB null fibroblasts ($Id2^{-/-}/RB^{-/-}$, lane 1), wild-type fibroblasts ($Id2^{+/+}/RB^{+/+}$, lane 2), heterozygous Id2/RB null fibroblasts ($Id2^{+/-}/RB^{-/-}$, lane 3), or wild-type Id2/RB null fibroblasts ($Id2^{+/+}/RB^{-/-}$, lane 4) was analysed by Northern blot using a DNA probe against the pol III transcript B2 (*upper panel*) and ARPP P0 (*lower panel*).

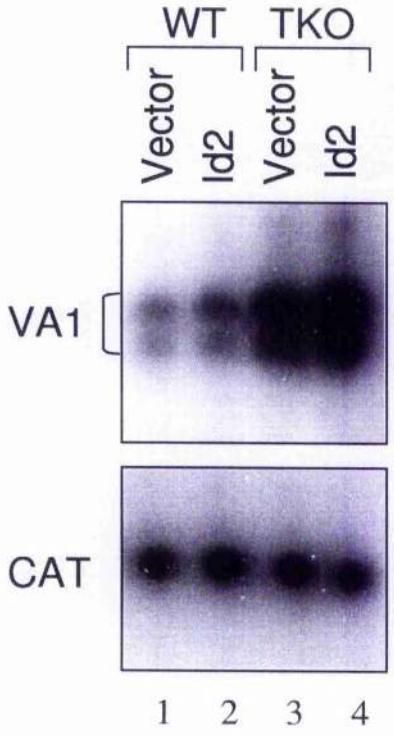

Figure 7.8: Activation of pol III transcription by Id2 does not require neutralisation of pocket proteins. Primary fibroblasts from p107-p130-RB triple knock-out cells (TKO, lanes 3 and 4) or wild-type (WT, lanes 1 and 2) were transiently transfected with pVA1 (0.5 μg), pCAT (0.5 μg) and pId2 (8 μg), encoding wild-type Id2 (lanes 2 and 4) or empty vector (lanes 1 and 3). RNA was extracted after 48 hrs of transfection. VA1 (*upper panel*) and CAT (*lower panel*) RNA levels were assayed by primer extension analysis.

7.2.8 Id2 Localizes at Pol III Genes *in vivo*

The experiments presented so far have shown that Id2 induces pol III transcription. Although this upregulation seems to be in part by relieving TFIIIB from RB repression (Fig. 6.7 and 7.6), another novel mechanism must be also involved, since Id2 overexpression can still induce pol III function when the entire RB family is absent. A possible scenario might be that Id2 is actually interacting with the pol III apparatus associated with class III genes, and its presence on pol III templates might contribute to their transactivation. To test if endogenous Id2 is actually able to associate with genes transcribed by pol III *in vivo*, chromatin immunoprecipitation was performed in mouse fibroblasts (Balb/c 3T3), since overexpression of Id2 in these cells stimulated pol III activity (Fig. 7.5). Antibodies against Id2, the TFIIIB subunit TBP, the pol III subunit BN51 and the negative control TAF_I48 were used to precipitate their respective proteins that had been previously cross-linked to chromatin. As expected, TBP and the pol III subunit BN51 are detected at $tRNA^{Tyr}$, $tRNA^{Leu}$, and 5S rRNA genes, whereas the pol I-specific transcription factor TAF_I48 is not (Fig. 7.9). It is interesting that endogenous Id2 was found to localise at the three pol III templates tested (Fig. 7.9, lane 6). This interaction is specific and not due to over cross-linking of proteins to DNA, since no signal was observed from either beads alone or TAF_I48. These results demonstrate that Id2 can associate with class III genes *in vivo*.

7.2.9 Endogenous Id2 Co-fractionates with TFIIIB Activity

From the chromatin immunoprecipitation assay, it can be concluded that Id2 is located at pol III templates *in vivo*. The finding that Id2 can be found associated with class III DNA is quite novel, since it does not contain a DNA binding domain; in fact, the Id proteins are not considered to be associated with DNA, but rather act as negative regulators of DNA binding by sequestering other transcription factors from it [358]. Thus, the association observed of Id2 with class III genes must be regulated through protein-protein interactions with one or more factors from the pol III apparatus. Since TFIIIB has been shown to be a transcription factor that can associate with a broad range of regulatory proteins, including the RB family, c-Myc, p53, among others, it was investigated whether endogenous Id2 could co-fractionate with it. A way of investigating if there is an association between endogenous Id2 and TFIIIB is by testing for the presence of Id2 in biochemical fractions containing TFIIIB activity. The fractions containing TFIIIB activity were prepared by chromatography. First, the constituents of the pol III apparatus are separated by

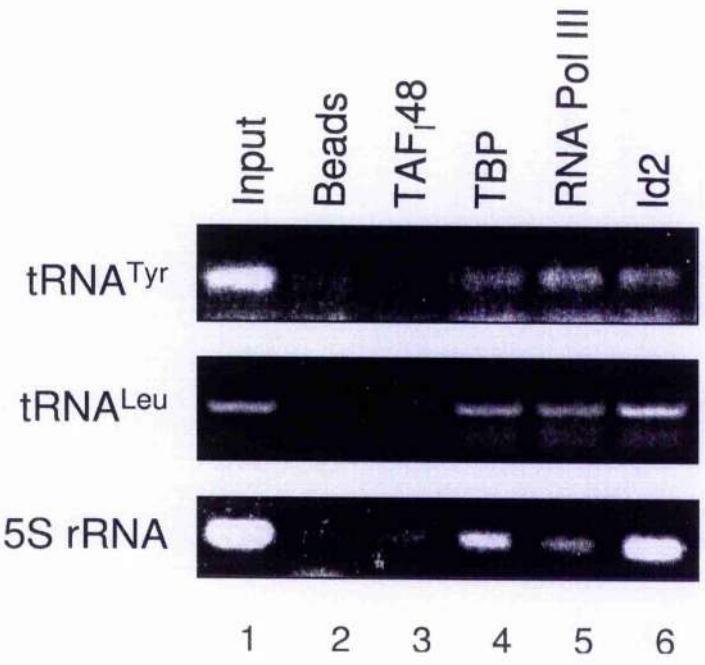

Figure 7.9: Id2 interacts with pol III templates *in vivo*. Chromatin immunoprecipitation of Balb/c 3T3 cells ($2x10^7$ per immunoprecipitation) with 20 μl of the respective antibodies (M-19 against TAF_I48, mTBP-6 against TBP, serum against the pol III subunit BN51 and C-20 against Id2). The immunoprecipitated DNA was quantified by PCR performed on the input (diluted 1:200) and bound fractions, using primers against $tRNA^{Tyr}$ (*upper panel*), $tRNA^{Leu}$ (*middle panel*) and 5S rRNA (*lower panel*).

chromatographic fractionation of HeLa nuclear extracts (on phosphocellulose (PC)). Having a PC fraction containing TFIIIB, a second chromatogrophy on Mono Q is performed and gradient eluted fractions are obtained. These fractions were analysed by western blot for the presence of Id2 (Fig. 7.10, upper panel), as well as assayed for TFIIIB transcriptional activity using VA1 as template in the presence of TFIIIC and pol III (Fig. 7.10, lower panel). As shown in figure 7.10, endogenous Id2 is detected in the fractions that present the highest TFIIIB activity (fractions 41 and 42).

Further confirmation of the association between endogenous Id2 and TFIIIB came from the detection of Id2 in TFIIIB fractions that had been purified through different chromatographic conditions (like chromatography on Q-Sepharose followed by PC, data not shown). Taken together, these data indicate that a population of Id2 molecules copurifies with TFIIIB. However, more experiments to study the interaction between Id2 and TFIIIB should be performed to confirm that a specific physical and functional interaction between these proteins is occurring. In addition, it would be interesting to test whether TFIIIC or pol III can also associate with Id2.

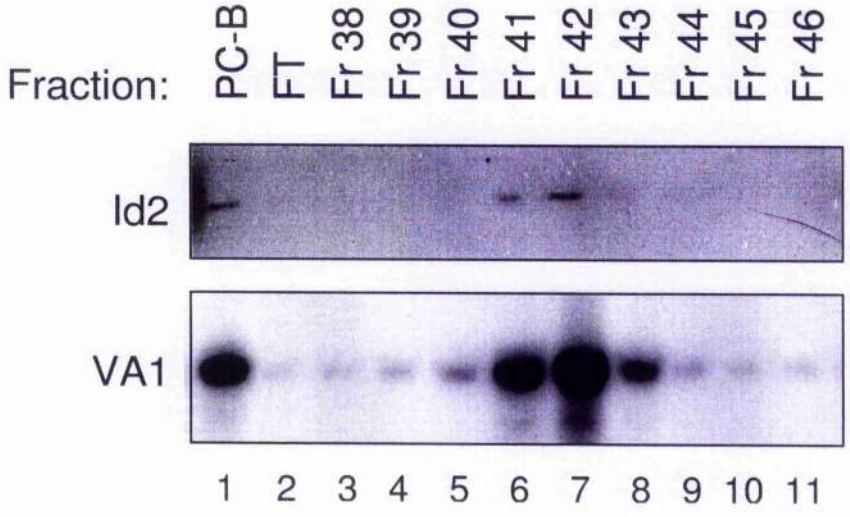

Figure 7.10: Id2 cofractionates with TFIIIB activity during gradient chromatography on Mono-Q. The *upper panel* shows Id2 content using 10 μl of the indicated fractions which were resolved on an SDS-7.8% polyacrylamide gel and analyzed by Western immunoblotting with anti-Id2 antibody C-20. The *lower panel* shows TFIIIB activity of individual fractions. In each case lane 1 contains input material (PC-B), lane 2 contains flow-through, and lanes 3-11 contain fractions 38-46, respectively. TFIIIB activity was assayed using 4 μl of the indicated fraction, 2 μl of PC-C and 500 ng of pVA1; after 15 min incubation at 30°C, nucleotides were added to assay transcription (the in vitro transcription was performed by Robert White).

7.3 Discussion

The genes transcribed by pol III are essential for protein synthesis, which is usually upregulated when cells are cycling and growing at fast rates. Furthermore, it has been reported that down-regulation of pol III transcripts, like that of B2 gene, can cause a direct reduction in growth rate of up to 80% [224]. Therefore, the rate of pol III transcription is an important determinant of cell growth. Cells overexpressing Id2 have a faster growth rate than normal controls, with a larger proportion of the population in the S phase of the cell cycle [7, 365]. This suggests that Id2 overexpression confers an enhanced growth potential to the cells and hence has a positive function in growth regulation. Since pol III transcripts are essential to achieve a steady rate of cell growth, it is quite plausible that positive regulators of this process would be activating pol III transcription. The data presented in this chapter indicate that transcription by pol III is upregulated by the expression of the HLH protein Id2, thus providing another mechanism by which Id2 enchances cell growth.

One of the mechanisms by which Id2 has been shown to promote cell growth and cell cycle progression is by neutralizing the inhibitory effects of the tumour suppressor protein RB and its related proteins p107 and p130 [7, 8, 16]. The RB family (or pocket proteins) regulate the cell cycle by fluctuating from an active hypophosphorylated form at the beginning of G1 phase, which represses transcription of genes required for G1/S transition by all three RNA pols [3, 179, 370], to an inactive hyperphosphorylated form at late G1 and the rest of the cycle [262]. In particular, the pocket proteins down-regulate pol III transcription by binding to TFIIIB and sequestering it from pol III templates [182, 184]. Since Id2 binds to the active underphosphorylated forms of the pocket proteins, one possible mechanism of activation of pol III transcription would be by releasing TFIIIB from the inhibitory actions of the RB family. Coimmunoprecipitations with *in vitro* translated proteins showed that Id2 can compete effectively avidity for RB with TFIIIB, and can actually disrupt the interaction between RB and the TFIIIB subunit Brf (Fig. 7.2). When Id2 was overexpressed *in vivo* in wild-type cells, pol III transcription was clearly upregulated. It is interesting that when Id2 was overexpressed *in vivo* in RB knockout or in triple knockout cells (RB-p107-p130), it could still induce pol III transcription, suggesting that a different mechanism is also involved. In the RB knockout cells, the activation of pol III transcription is lower than that in the wild-type cells, implying that RB neutralization does play a role in Id2 induction of pol III activity. However, the results demonstrate that the activation of pol III by Id2 is through a

pocket proteins-independent mechanism as well.

Due to the fact that Id2 can stimulate pol III transcription even in the absence of the RB family, it was investigated whether this protein could be interacting directly with pol III templates and the pol III apparatus to induce class III genes. The results presented here demonstrate that Id2 is localized at different pol III templates *in vivo* and that there might be a physical interaction between Id2 and the pol III-specific factor TFIIIB. The finding that Id2 is bound to DNA presents an exiting new function for this protein, since there is substantial evidence showing that the Id proteins function by forming complexes with other transcription factors and sequestrating them, with the consequent inhibition of their ability to bind DNA. Thus, to find that Id2 is present at pol III templates strikes as surprising. There is one report showing that an Id protein, Id1, can actually stimulate the DNA binding of another transcription factor and is present at the DNA-MIDA1 complex. In this study, Inoue et. al. demonstrated that the association of Id1 with MIDA1 (mouse Id associated 1) potentiates the sequence-specific DNA binding activity of this transcription factor to a 7-bp sequence, GTCAAGC, present in the 5' flanking region of several growth-factor/cytokine genes [371, 372]. MIDA1 has been shown to be required for growth of erythroleukaemia cells andtherefore is considered to act as a positive regulator of cell growth [371].

The fact that an Id protein can stimulate the DNA binding properties of another transcription factor presents a new insight into how Id might be regulating cell growth. Since the products made by pol III are important determinants of this process, Id2 localisation at pol III templates might be acting by potentiating the DNA binding properties of one or more pol III-specific factors. Preliminary studies presented in this chapter expose a possible interaction between Id2 and the pol III-specific factor TFIIIB, although this association has to be investigated further. TFIIIB has been shown to be regulated by a number of different tumour suppressor proteins (like RB and p53, [3,187,188]) as well as oncoproteins (like c-Myc and ERK [269,373]). It therefore appears to be a crucial factor involved in the determination of the rate of pol III transcription. Thus, it is important to confirm that there is an interaction between Id2 and TFIIIB, and if this association is responsible for the upregulation of pol III activity. Furthermore, it would be interesting to study whether Id2 can increase TFIIIB DNA binding or if there are other novel mechanisms participating. In addition, the interaction between Id2 and other pol III-specific factors or pol III itself has to be examined.

In conclusion, the results presented here expose a new function for Id2, the

upregulation of pol III transcription, and might provide a more complete picture of how Id proteins regulate cell growth. Whether the other Id proteins can also regulate pol III activity remains to be determined, although this might be quite plausible since they have been shown to have growth-promoting functions.

8 Discussion and Conclusions

8.1 Discussion

8.1.1 Pol III Transcription and Cell Growth

Growing cells require to duplicate both their genetic material as well as their cell mass before they divide. While duplication of the genetic material occurs at one point during the cell cycle, duplication of the mass is a gradual and continous process that requires activation of diverse proteins, each responsible of specific processes that overall ensure constant growth. Because a cell's dry mass is mainly constituted of proteins (70%), one of the main processses that must be activated for sustainable cell growth is protein synthesis; in fact, growth rate is directly proportional to the rate of accumulation of protein (see [11] for review). High rates of protein synthesis require an increase of the constituents of the translational machinery, i.e. ribosomes and tRNAs. Ribosomes are composed of proteins, encoded by genes transcribed by pol II; and rRNA, encoded by genes transcribed by pol I and pol III; whereas tRNAs are encoded in genes transcribed by pol III. rRNA availability mainly depends on the rate of rRNA synthesis, which has been shown to decrease in response to amino acid starvation and in stationary phase cells, demonstrating regulation coupled to growth (for review see [15]). rDNA from the 45S gene is transcribed by pol I, and the transcription factor regulated by growth has been identified to be TIF-IA. Moreover, the tumour suppressor protein RB appears also to be involved in pol I transcriptional regulation .

On the other hand, 5S rRNA, as well as tRNAs genes, which are also components of the protein synthetic apparatus and therefore essential for protein synthesis, are synthesized by pol III. The rate of pol III transcription has been shown to be regulated by a diverse range of oncogenes and tumour suppressors, including Ras oncoprotein as well as several viral oncoproteins, and the tumour suppressors RB and p53 [10, 11, 166, 170]. In addition, some of the constituents of the pol III machinery

have been observed to be upregulated, which will ultimately induce class III gene transcription [12,168,170]. Evidence suggesting a possible regulation of pol III transcription by cell growth has come from experiments performed on rapidly growing cells like tumour and transformed cell lines. These cells present higher rates of pol III transcription than their normal counterparts and thus overexpress pol III products [11]. Further evidence of the requirement for a high level of pol III transcription in cell growth comes from the observation that mitogenic stimulation induces tRNA and rRNA, as well as ribosomal proteins and translation factors [10,11]. From these observations, a possible requirement for the activation of pol III transcription in the process of tumourigenesis is suggested.

8.1.2 The receptor ErbB2/neu and Pol III Transcription

Mitogens usually bind to and activate receptors at the plasma membrane that will ultimately transfer the signal to the nucleus via the activation of signalling cascades. Activation of pol III transcription by mitogens suggest the participation of a receptor or receptors at the plasma membrane in this induction. One receptor that has received a lot of interest due to its oncogenic properties is the ErbB2/neu receptor. Notably gene amplification and overexpression of ErbB2/neu occurs in 25-30% of breast and ovarian carcinomas and is associated with poor prognosis [206,207]. Overexpression of pol III products have been detected in ovarian cancers when compared with noncancerous tissue isolated in parallel from the same individuals [12]. In these samples, the levels of both mRNA and protein of the TFIIIC2 subunits were higher in the tumour tissue than the healthy tissue. Furthermore, microextracts prepared from tumour samples presented an increased TFIIIC2 activity when compared to the normal ovarian tissue [12]. In addition, pol III activity can be stimulated by raising the amounts of TFIIIC2 in extracts prepared from ovarian epithelial cell extracts, confirming that an increase in TFIIIC2 concentration does indeed affect expression of class III genes in ovarian tissue [12]. It would be interesting to analyse the expression of the ErbB2/neu receptor in these ovarian epithelial tumours, and test if there might be a correlation between ErbB2 overexpression and the stimulation of pol III transcription observed; however, this research was not performed in Winter et. al.'s study. Chapters three and four of this thesis address this hypothesis. Instead of using ovarian tumour samples, an immortalized rat epithelial ovarian cell line stably transfected with a constitutive active form of the ErbB2/neu receptor (ROSE-neu) or the β-galactosidase protein (ROSE-βgal) as control was used. As shown in chapter three, ErbB2/neu constitutive activation does indeed stimulate

pol III transcription, with overexpression of all the pol III products tested; however, no overexpression of the TFIIIC2 subunits could be observed. Although no overexpression of TFIIIC2 was found in the ROSE-neu cells, previous studies have shown that its activity is slightly elevated when compared to that of ROSE-βgal [226]. The fact that TFIIIC2 activity is increased with no apparent increment in its expression levels suggests that an increase in the expression levels of TFIIIC2 is not required as long as its activity is elevated.

Because no overexpression of the TFIIIC2 subunits could be found in the neu-overexpressing cells, and the induction in pol III transcription in these cells was so striking, other routes of transcriptional regulation were investigated. Due to the fact that the ErbB2/neu receptor is known to activate several signalling cascades which have been shown previously to stimulate pol III transcription, their activation in the ROSE cells was analysed. The ROSE-neu cells presented higher activation of the Ras signalling pathway, as well as of the kinase $p70^{S6K}$. There are three main mechanisms by which ErbB2/neu could be targeting pol III transcription through the activation of these pathways. The first one is through the induction of the *cyclin E* and *D* genes, which will ultimately affect RB function [5, 170, 278, 282, 283]. An increase in these two cyclins was observed in the neu-overexpressing cells. Furthermore, the RB tumour suppressor protein is highly phosphorylated in ROSE-neu cells compared to control; and as the co-immunoprecipitations showed, the interaction between RB and TFIIIB is reduced (Fig. 8.1). These findings present one mechanism by which ErbB2/neu constitutive expression might induce pol III activity.

The second mechanism involves an induction of the TFIIIB subunit TBP. Results in Chapter 3 demonstrate that in the ROSE-neu cells, the expression of TBP is increased compared to control cells. In addition, both TBP and Brf appear to be phosphorylated in the neu-overexpressing cells (Fig. 8.1). In a recent report, Scott's group have shown that the Ras pathway, particularly through Erk, is responsible for phosphorylating the Brf subunit of TFIIIB, and this phosphorylation appears to upregulate pol III activity [269]. These findings present a possible explanation for the doublets present on the TBP and Brf proteins of the ROSE-neu extracts, and might account for the activation of pol III transcription in these cells. However, it still remains to be analysed whether the doublets observed in these proteins do correspond to phosphorylation or to a different modification. Also, it still needs to be determined what role this modification plays in the ErbB2/neu activation of pol III transcription.

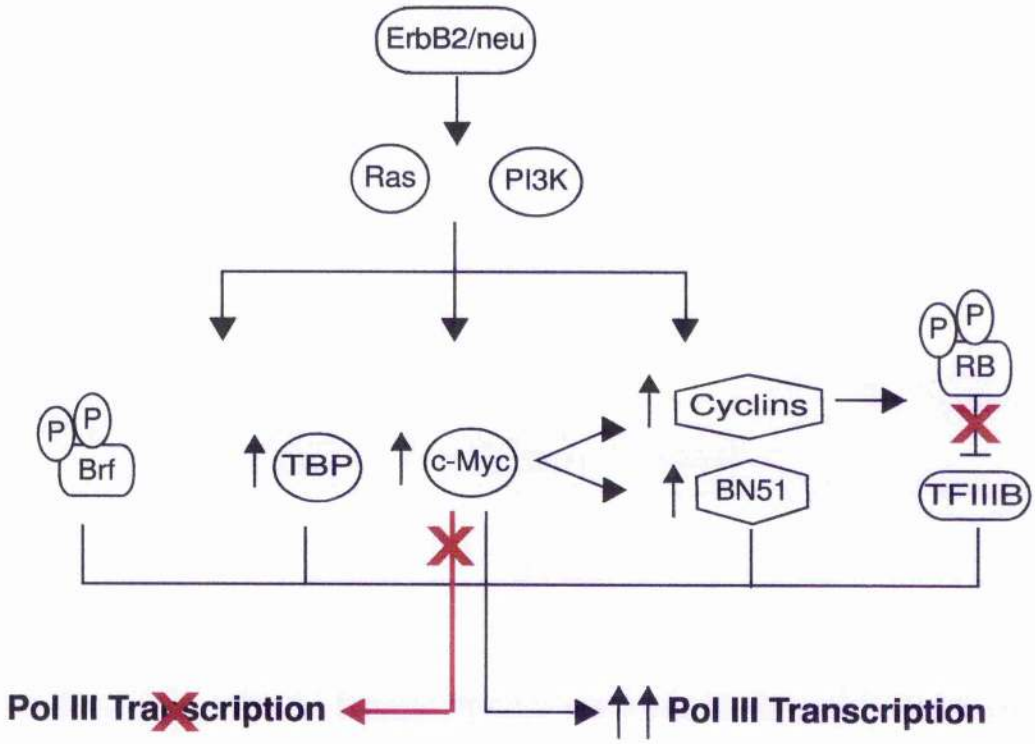

Figure 8.1: Pathways involved in ErbB2/neu upregulation of pol III transcription. Activation of the ErbB2/neu receptor stimulates signalling pathways including Ras and PI3K which will ultimately affect expression of different genes including TBP, c-Myc, cyclins E and D, and the pol III subunit BN51, as well as phosphorylation of Brf and RB which will act as positive effectors of pol III output. However, if c-Myc expression is compromised (by antisense oligonucleotides), the induction of class III genes observed by constitutive activation of ErbB2/neu does no longer occur, suggesting c-Myc as a main effector of ErbB2/neu.

The third mechanism by which the activation of the Ras and $p70^{S6K}$ signalling pathways by ErbB2/neu could affect pol III activity is through the induction of the transcription factor c-Myc. Both these pathways are involved in the upregulation of c-Myc activity by increasing its half life (through phosphorylation by Erk, [282,283]) as well as by increasing its translation (through $p70^{S6K}$, [285]). This transcription factor was investigated due to the fact that in a recent report it has been shown to mediate ErbB2/neu oncogenicity. In this report, when c-Myc activity was blocked, the cells were not longer malignant eventhough ErbB2/neu was still overexpressed. From these findings, it could be concluded that ErbB2/neu requires the induction of this transcription factor to exert its oncogenic effects. Although no previous data have shown a link between pol III transcription and c-Myc, the work described in this thesis presents a clear demonstration that this transcription factor actually upregulates pol III transcription. As shown in Chapter 4 and as previously documented in breast cells [5], ErbB2/neu overexpression in the rat ovarian epithelial cells induces c-Myc expression. To analyse whether c-Myc played any role in the induction of pol III transcription, c-Myc antisense oligos were tested to reduce c-Myc's expression. When the neu-overexpressing cells were grown with the c-Myc antisense oligos, an unexpected decrease of pol III activity was obtained, which was now comparable to the normal levels usually observed in control cells (Fig. 8.1). A similar result was also obtained from *in vitro* transcription assays with whole cell extracts that were immunodepleted of c-Myc (Chapter 4). The fact that a reduction of this transcription factor could bring down pol III activity to normal levels is quite surprising, since neu-overexpression in the rat ovarian epithelium not only presented increased levels of this transcription factor, but also changed other conditions (e.g. RB-phosphorylation and increased TBP levels) which are known to affect pol III activity. From these results, it could be concluded that downregulation of c-Myc can override the other conditions, and is a crucial determinant in the activation of pol III transcription caused by neu-overexpression.

To better understand the mechanism used by ErbB2/neu to upregulate pol III, each of the different processes involved should be studied further. To address this, it would be interesting to perform experiments in which the Ras pathway on one hand, and the $p70^{S6K}$ kinase on the other are downregulated by specific inhibitors and study what effects are obtained concerning RB activity, TFIIIB and c-Myc induction. Furthermore, c-Myc's role in RB activity, TFIIIB protein modifications and induction of the pol III subunit BN51 should also be addressed.

8.1.3 Activation of Pol III Transcription by c-Myc

The finding that c-Myc induction by neu-overexpression produces an increase in pol III transcription, presents a novel mechanism by which pol III activity can be upregulated. The exact mechanism by which c-Myc is exerting its positive effect on pol III was investigated in Chapters 5 and 6, using systems where only c-Myc expression was affected, avoiding other conditions that could interfere with this study (however, it would be interesting in the future to investigate whether downregulation of c-Myc in the ROSE-neu cells has any effect on RB-phosphorylation and TFIIIB and BN51 induction). To achieve this, the experiments to analyse c-Myc regulation of pol III were performed on c-Myc-knockout cells and arrested primary cells transfected with the fusion protein c-Myc-ER. These systems confer the advantage of studying the regulation of c-Myc in an environment where there is only one variable changed, that is, c-Myc expression; thus, the changes observed in pol III activity are going to result only from the change in c-Myc. The results presented in Chapter 5 demonstrate that c-Myc positively regulates expression of different pol III transcripts.

Because c-Myc is responsible for increasing the expression of cyclins D and E, which will ultimately induce phosphorylation of RB, this mechanim seemed to be the most plausible involved in c-Myc regulation of pol III transcription. However, contrary to what was expected, RB expression did not play any role in c-Myc upregulation of pol III, since its overexpression in RB null fibroblasts, as well as RB-p107-p130 null fibroblasts, still managed to produce activation of pol III transcription to a similar extent to that observed in wild-type cells. The next possibility considered was that c-Myc could be upregulating constituents of the pol III apparatus. However, no increase in the expression of mRNAs encoding several proteins belonging to the pol III apparatus was observed. Furthermore, pol III activity still responded to c-Myc expression even in the absence of pol II transcription. From these findings, it was concluded that c-Myc did not require any intermediary to acheive regulation of pol III activity; instead it must be working directly on it.

Although in the primary fibroblasts no induction was observed for the pol III subunit BN51, in immortalised and transformed cells c-Myc can increase BN51 expression (Chapter 4 and [287]). Preliminary data by Felton-Edkins using c-Myc RNA interference in transformed cervical cells show that the mRNA encoding of this pol III subunit decreases in response to a reduction in c-Myc expression. This evidence implies that in transformed cells c-Myc might also be targeting other routes to achieve high levels of pol III products, and suggest that in rapid growing cells like cancer cells further mechanisms are involved in the upregulation of pol III tran-

scription by c-Myc to ensure pol III output (see Fig. 8.2).

As observed in Chapter 5, c-Myc is capable of activating pol III transcription through a direct mechanism, and as the chromatin immunoprecipitation experiments showed, it is localised at class III genes in both primary as well as immortalised and transformed cells [373]. Having established that c-Myc utilises a direct mechanism to target pol III, the recruitment agent was investigated. Chapter 6 addressed this question, and the results show that c-Myc interacts with the pol III-specific transcription factor TFIIIB. In addition, an interaction between Max and TBP was detected, which might increase the efficiency of the binding between c-Myc and TFIIIB. These experiments demonstrate that c-Myc is being recruited to class III genes through protein-protein interactions with TFIIIB.

8.1.4 Possible Mechanism of Induction of Class III Genes by c-Myc

The mechanism by which c-Myc is capable of transactivating of its target genes had remained unclear until recent years. In the literature, there is now evidence showing that c-Myc may activate transcription through chromatin remodelling [322]. In eukaryotes, the DNA is packaged into nucleosomes composed of histones. The histones have been shown to be modified through acetylation by histone acetyltransferases (HATs) and deacetylation by histone deacetylases (HDACs), having a positive or negative effect, respectively, on transcription. Acetylation levels are controlled by sequence-specific activators or repressors which will recruit HATs or HDACs, respectively, and will ultimately mediate transcriptional activity. c-Myc has been shown to recruit HATs and induce histone acetylation of several target genes [318, 319]. The recruitment of HATs is through the N-terminal transactivation domain of c-Myc, in particular MBII, that binds to TRRAP (TRansactivation/tRansformation-domain Associated Protein), a large ATM-related protein that is a component of several different multisubunit HAT complexes [322, 353].

As presented in this thesis, transactivation of class III genes by c-Myc is through recruitment of this protein to promoters by TFIIIB (Chapters 5 and 6). However, it did not address the mechanism involved in the transactivation. Due to the fact that regulation of gene expression of other c-Myc target genes appears to be modulated via histone acetylation, it seems fairly likely that the same mechanism might be involved in the regulation of pol III transcription. Previously, it has been documented that chromatin remodelling does exert an effect upon pol III activity [374]; thus c-Myc function might be the recruitment of HATs to class III genes . Evidence that

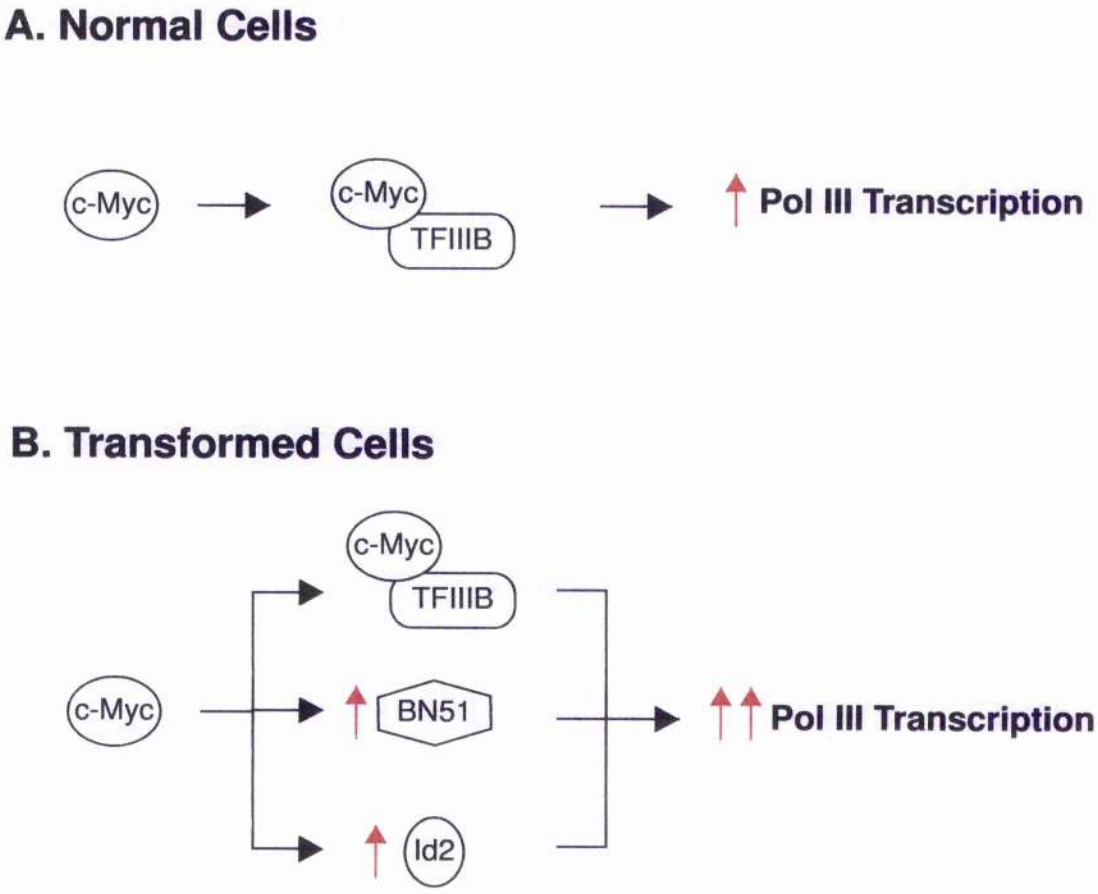

Figure 8.2: Possible mechanisms of pol III activation by c-Myc in normal cells compared to transformed cells. (A) As demonstrated in Chapter 6, activation of pol III transcription in primary fibroblasts is through direct interaction with proteins of the pol III appparatus (TFIIIB) and thus localization of it at class III genes. (B) Possible induction of pol III activity in transformed cells by c-Myc through interaction with TFIIIB as well as induction of constituents of pol III (BN51), and other positive regulators of its activity (Id2).

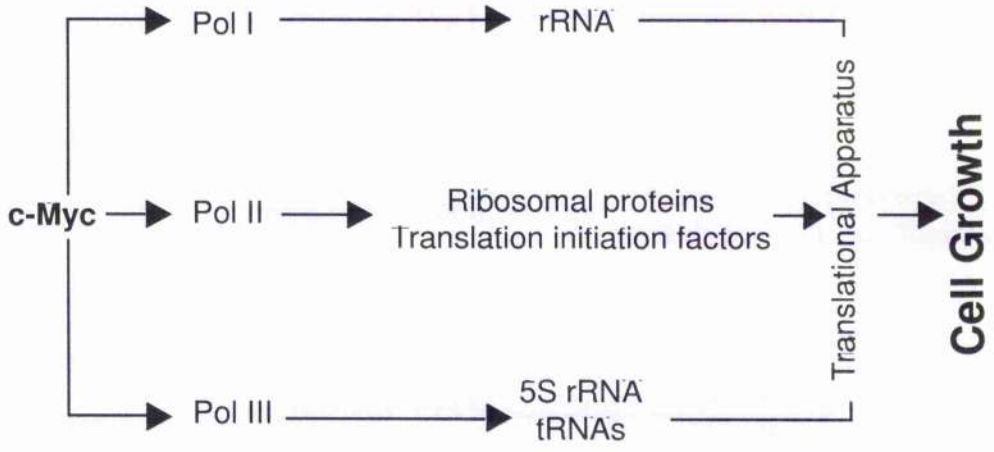

Figure 8.3: c-Myc as a key regulator of cell growth. The transcription factor c-Myc has been recognised to transactivate expression of genes whose products are part of the translational apparatus, and thus affect the biosynthetic capacity of cells.

suggests this to be the possible mechanism used by c-Myc comes from the results obtained with the mutant ΔMyc-ER, which lacks a region within the TAD, from aa 106 to 143, that includes MBII, and therefore is incapable of binding to TRRAP. As shown in Chapter 5, ΔMyc-ER failed to activate both tRNA and cyclin D2 transcription upon addition of OHT. In addition to these findings, data that supports this mechanism comes from the experiments using *c-myc* null cells, where the natural occurring mutant c-MycS did not manage to rescue the *c-myc* null phenotype for class III genes. Although c-MycS contains the MBII domain, McMahon *et al.* demonstrated that c-MycS does not bind to TRRAP; for this binding to occur, the N-terminal domain must be almost complete [352]. These data support the hypothesis that recruitment of TRRAP and complexes with HAT activity and modification of histone acetylation may contribute to the regulation of class III gene expression by c-Myc *in vivo*.

In the recent years, the product of the proto-oncogene *c-myc* has been shown to participate in the regulation of both cellular proliferation and growth, and is known to be involved in tumourigenesis. It can regulate RB phosphorylation state by inducing the expression of proteins involved in this process (i.e. cyclins and cdc25A), allowing progression of the cell cycle from G1 phase to S phase, as well as increasing the expression of molecules (both ribosomal proteins and rRNA) involved

in translation (see Chapter 5, Table 5.1, see Fig. 8.3) allowing an increase in cell mass. Since some of the products synthesized by pol III are constituents of the translational apparatus, it would be economical for the cell to utilise the same protein to regulate the expression of all the molecules involved in this process, and ultimately in cell growth. The results in this thesis demonstrate that c-Myc is able to induce the products synthesized by pol III, thus presenting this transcription factor as a key regulator of cell growth. In the future it would be interesting to investigate what role does the increase of pol III output by c-Myc play in tumourigenesis. The pol III product 7SK RNA has been suggested to participate in the transformation process by regulating transcriptional initiation of the *c-myc* gene [375]. In fact, the induction of c-myc gene expression by 7SK RNA appears to be in direct proportion to transformation and tumourigenicity [375]. These results suggest that the regulation by c-Myc of class III genes may act as a positive feedback towards its own expression, as well as having an effect on cell growth and tumourigenesis.

8.1.5 Induction of Pol III Transcription by Id2

A third protein that was shown to regulate transcription by pol III was the product of the proto-oncogene Id2. The HLH Id2 protein was considered because it is a target gene of N-Myc as well as c-Myc [7,16], as well as being an inhibitory binding partner of the hypophosphorylated form of RB, which is a known repressor of pol III activity. Although Id2 had been found previously to be a target gene of N-Myc, no induction of its transcription could be observed in the quiesced primary fibroblasts transfected with c-Myc-ER upon addition of OHT. One possible reason for the discrepancy in the results obtained here with those previously documented could be the cells chosen for the assay. In the work presented here, primary fibroblasts previously quiesced were used, while Id2 induction by N-Myc has been documented in neuroblastomas and immortal fibroblasts [16]. It seems that Myc transactivation of the Id2 gene might depend on other factors that are present in transformed and immortal cells, thus no upregulation is aparent in normal cells. Further investigation into this subject is required to understand the exact mechanism involved in Myc transactivation of Id2.

As demonstrated in Chapter 7, Id2 can upregulate expression of class III genes. Although the obvious mechanism used by Id2 to regulate pol III activity seemed to be through inactivation of RB, the fact that transfection of Id2 in RB null and RB-p107-p130 null cells still managed to induce pol III activity indicated that this was not the only mechanism involved. The experiments presented in this thesis suggest

that Id2 can actually exert its positive effects through interaction with proteins of the pol III transcriptional apparatus, specifically with TFIIIB, and can be found localised at class III genes. This is the first time that the HLH Id2 protein has been documented to localise at DNA, which came as a great surprise since Id2 lacks DNA-binding domains and none of the known pol III factors has an HLH domain. Although the data presented here seems quite encouraging, further research into this matter should be performed to confirm these results.

8.1.6 TFIIIB as a Possible Determinant of Pol III Activity

From the findings presented in this thesis an obvious conclusion that can be drawn is the fact that all three oncoproteins, i.e. ErbB2/neu, c-Myc and probably Id2, appear to control pol III output through regulation of TFIIIB. ErbB2/neu stimulated TFIIIB activity through several mechanisms, including the increase in the expression of the TFIIIB subunit TBP, protein modifications in TBP and Brf, by inducing phosphorylation of RB and thereby reducing RB binding to TFIIIB, and finally, through induction of c-Myc. The fact that TFIIIB is being upregulated at so many different levels confirms its significance as a regulatory hub controlling the expression of class III genes. TFIIIB has received a lot of interest in the past since its function has been shown to be the target of tight control by tumour suppressors, like RB and p53, as well as being frequently activated by viral and cellular oncoproteins, like Ras (reviewed in [11]). This evidence suggests that TFIIIB is a crucial determinant of the rate of pol III transcription, which is not surprising since it is responsible for recruiting pol III to the start site and positioning it for initiation [121]. Furthermore, it provides an indication that its activation may be a significant step towards tumour development.

8.2 Conclusions

RNA Polymerase III synthesizes a variety of essential cellular products, including 5S rRNA, tRNA and U6 snRNA, which play an essential role in the biosynthetic capacity of cells and, ultimately, of growth. The work described in this thesis demonstrates that three different oncoproteins, ErbB2, c-Myc and Id2, which have been clearly implicated in tumour development and induction of cell proliferation and growth, participate in the positive regulation of class III gene expression.

Although ErbB2/neu appears to deregulate multiple factors to achieve activation of pol III, including phosphorylation of RB and induction of the pol III subunit BN51, all three oncoproteins target TFIIIB to achieve upregulation of class III genes,

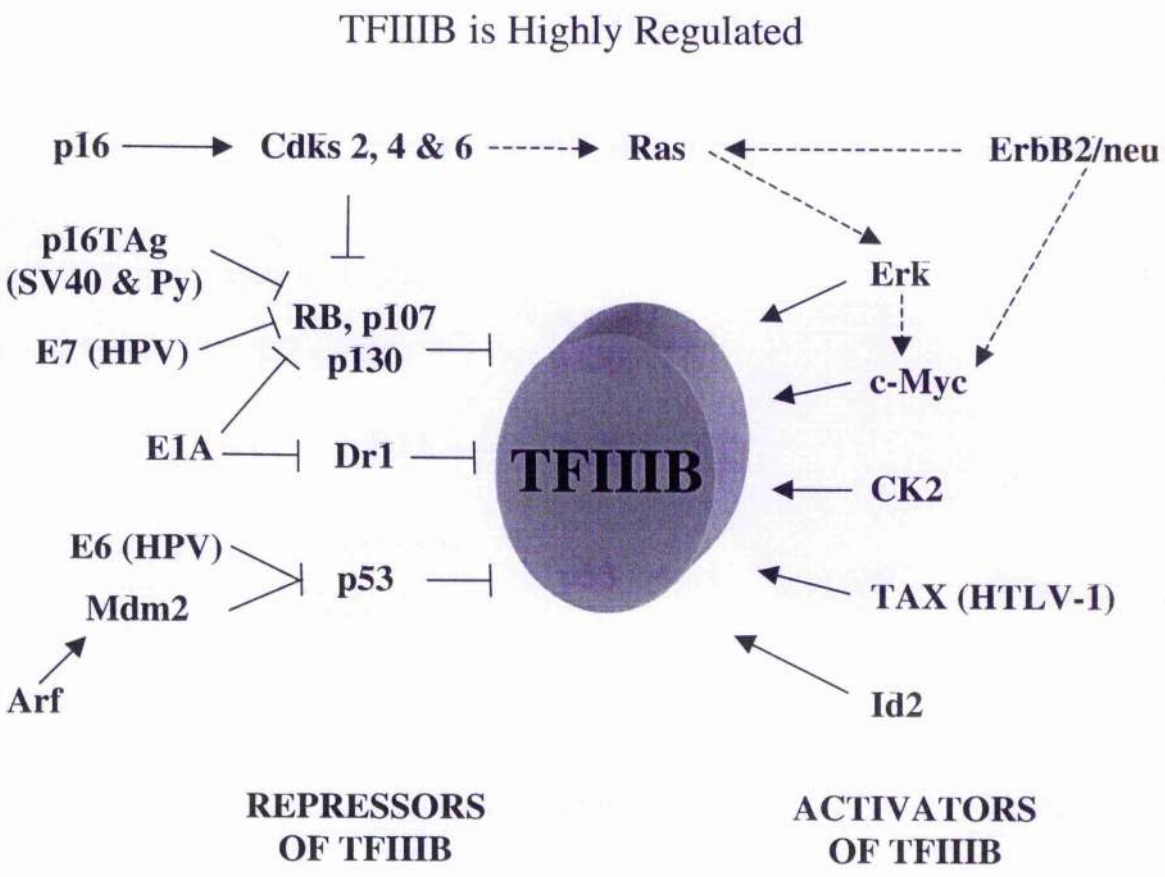

Figure 8.4: TFIIIB is highly regulated by several different proteins. The growth repressors RB, p107, p130, Dr1 and p53 can all repress TFIIIB by direct interactions. The function of these repressors can be blocked by several viral proteins including the large T antigen (TAg) from SV40 and poliomavirus (Py), the Adenovirus E1A, the HPV E7, as well as the cellular proteins Cdk 2, 4 and 6, which relieve TFIIIB from repression by RB; adenovirus E1A also relieves repression of Dr1; and HPV E6 and the cellular protein Mdm2 which block p53. In contrast, a variety of oncoproteins have been shown to stimulate TFIIIB including Ras, Erk, ErbB2/neu, c-Myc, CK2, Id2 and the viral protein TAX from human T-cell leukaemia virus type 1 (HTLV-1) (for review see [11]).

implying that TFIIIB is a major determinant for dictating the rate of pol III transcription. Both c-Myc and Id2 can interact directly with TFIIIB, as well as being localised at class III DNA. This interaction might allow recruitment of coactivators and chromatin-modifying activities, or it might block effects of negative regulators of the pol III machinery. Because all three proteins are positive regulators of cell growth, it seems that in order to control this process, they target transactivation of pol III products which are major determinants of the rate of translation, and therefore essential for growth. Given the frequency with which these three oncoproteins get deregulated in human cancers, it seems highly likely that pol III transcription will become deregulated in a large proportion of malignancies. This also suggests that deregulation of TFIIIB may be an important requirement in the process of tumourigenesis.

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