Thesis for the Cand. scient. degree in Molecular Biosciences

Main field of study in biochemistry

Construction of Inducible Cell Lines for the Transcription Factor c-Myb and its Viral Counterpart v-Myb

Model systems for the identification of target genes

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Abstract

The hematopoietic transcription factor c-Myb has been reported to be involved in diverse and important cellular functions such as differentiation, proliferation and apoptosis. It has also been implicated in tumorigenesis, and its viral counterpart v-Myb has been known to cause acute myelogenous leukemia in chickens. The biological mechanisms behind this oncogenic activation of c-Myb are not very well understood, and the list of target genes proposed for this transcription factor is at present too short to properly explain its biological function.

In an effort to reveal some of the secrets of this elusive transcription factor we have established a new model system based on stable transfection of a human cell line with plasmids encoding three different versions of c-Myb: Full-length human c-Myb, a C-terminally truncated version of human c-Myb designated c-Myb$^{1\text{-}443}$ and v-Myb^{AMV}. By use of the Tet-On and Tet-Off gene regulation systems for the generation of these stably transfected cell lines the expression of c-Myb could be induced in a Dox-dependent fashion, allowing for superior reproducibility and control of gene expression compared to what can be obtained in model systems based on transient transfections.

We succeeded in generating two Tet-On HEK293 cell lines with inducible expression of c-Myb and v-Myb^{AMV}. These cell lines were employed in microarray experiments for the determination of potential target genes for these transcription factors. The preliminary results obtained from these experiments were in keeping with observations from other recent studies regarding microarray analyses of c-Myb: The activity of this transcription factor appears to be exceptionally context-specific, indicating that c-Myb is entirely dependent on the correct composition of cooperating factors and post-translational modifications in order to be able to fully exert its potential as a transcriptional activator. In addition it would appear that the truncations and mutations found in v-Myb^{AMV} result in a transcription factor whose activity differs from its cellular counterpart both in a quantitative and qualitative manner.

Contents

Abbreviations

A	log10(average intensity of Ch1 and Ch2)
ADA	Adenosine deaminase
ADORA2B	Adenosine A2B receptor
AMV	Avian myeloblastosis virus
ANPEP	Alanyl aminopeptidase
APS	Ammonium persulfate
ATBF1	AT motif-binding factor 1
ATP	Adenosine triphosphate
BASE	BioArray Software Environment
BCA	Bicinchoninic acid
BCL2	B-cell CLL/lymphoma 2
bp	Base pairs
BSA	Bovine serum albumin
BSAP	B-cell lineage-specific activator protein, corresponds to PAX5
C. elegans	*Caenorhabditis elegans*
C/EBP	CCAAT/enhancer-binding protein
CBP	CREB-binding protein
CCNA1	Cyclin A1
CD34	Hematopoietic progenitor cell antigen CD34
CD4	CD4 antigen
CDC2	Cell division cycle 2
cDNA	Complementary DNA
Ch1/Ch2	Channel 1/channel 2
CHAT	Choline acetyltransferase
ChIP	Chromatin immunoprecipitation
CKII	Casein kinase II
c-Maf	v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog
cMGF	Chicken myelomonocytic growth factor
c-Myb	Cellular Myb
c-myb	Gene encoding the transcription factor c-Myb
c-Myb$^{1-443}$	C-terminally truncated c-Myb, encoded by pTRE2hyg-hcM[1-443]-HA
COL1A2	Collagen, type I, α2
COX2	Cyclooxygenase 2, corresponds to PTGS2
CR	Conserved region, corresponds to the TP-domain
CSF1R	Colony-stimulating factor 1 receptor
Cy3/Cy5	Cyanine 3/cyanine 5
Cyp40	Cyclophilin 40
DBD	DNA binding domain
DMEM	Dulbecco's Modified Eagle Medium
DMP1	Dentin matrix acidic phosphoprotein 1
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
Dox	Doxycycline
dsDNA	Double stranded DNA
E. coli	*Escherichia coli*
env	Gene encoding viral envelope glycoproteins

ERBB2	v-ERB-B2 avian erythroblastic leukemia viral oncogene homolog 2
EtBr	Ethidium bromide
EtOH	Ethanol
EVES	Subdomain of c-Myb NRD
FAETL	Motif within c-Myb NRD
FBS	Fetal bovine serum
gag	Gene encoding the viral core proteins p12, p15 and p19
GATA1	GATA-binding protein 1
GBX2	Gastrulin brain homeobox 2
GTFs	General transcription factors
HEK	Human embryonic kidney
HIPKII	Homeodomain-interacting protein kinase 2
HLH	Helix-loop-helix
HLR	Heptad leucine repeat
HRP	Horseradish peroxidase
HSF3	Heat-shock transcription factor 3
HTH	Helix-turn-helix
IGF1	Insulin-like growth factor 1
Igε GL	Immunoglobulin ε germline
kb	Kilo bases
kD	Kilo Dalton
KIT	v-Kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KITLG (SCF)	Stem cell factor
LB	Luria-Bertani
LCK	Lymphocyte-specific protein-tyrosine kinase
LEF-1	Lymphoid enhancer-binding factor 1
LYZ	Lysozyme
LZ	Leucine zipper
M	log2(ratio Ch1/Ch2)
MACF	Microarray Core Facility
MAPK	Mitogen-activated protein kinase
MAT2A	Methionine adenosyltransferase II α
MCS	Multiple cloning site
MIM1	Myb-induced myeloid protein 1
MRE	Myb recognition element
MW	Molecular weight
MYB	Gene for human c-Myb
MYC	v-Myc avian myelocytomatosis viral oncogene homolog
MyoD	Myogenic differentiation antigen
N-CoR	Nuclear receptor co-repressor
NE (ELA2)	Neutrophil elastase 2
NF-M	Chicken homolog of C/EBPβ
NMC	Norwegian Microarray Consortium
NRAS	Neuroblastoma ras viral oncogene homolog
NRD	Negative regulatory domain
OMIA	Online Mendelian Inheritance in Animals
OMIM	Online Mendelian Inheritance in Man
PAX5	Paired box gene 5, corresponds to BSAP
PAX6	Paired box gene 6
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDCD4	Programmed cell death 4
PKA	Protein kinase A
P_{minCMV}	Minimal CMV promoter
Pol II	RNA polymerase II
PRTN3	Proteinase 3
PTGS2	Prostaglandin-endoperoxide synthase 2
R1, R2, R2	Myb repeat 1, 2 and 3
RAG2	Recombination-activating gene 2
RARα	Retinoic acid receptor α
RCG	Resident chromosomal genes
RLU	Relative luciferase units
RNA	Ribonucleic acid
rpm	Revolutions per minute
RR MC	Rikshospitalet-Radiumhospitalet Medical Center
rRNA	Ribosomal RNA
RT	Room temperature
RT PCR	Reverse transcriptase PCR
rtTA	Reverse tTA
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
Ski	v-Ski avian sarcoma viral oncogene homolog
ssDNA	Single stranded DNA
SUMO	Small ubiquitin-related modifier
SYM	Centre for Occupational and Environmental Medicine
TAD	Transactivation domain
TAFs	TBP associated factors
TCRγ	T-cell antigen receptor γ
TCRδ	T-cell antigen receptor δ
TEMED	*N,N,N',N'*-tetramethylethylenediamine
tetO	Tet operator sequences
TetR	Tet repressor protein
TIF-1β	Transcriptional intermediary factor-1 β
TOM1	Target of Myb 1
TP domain	Threonine and proline rich region of c-Myb
TRAF7	TNF receptor-associated factor 7
TRE	Tetracycline-response element
TRF-1	Telomeric repeat-binding factor 1
TRF-2	Telomeric repeat-binding factor 2
TRHR	Thyrotropin releasing hormone receptor
tTA	Tetracycline controlled transactivator
UiO	University of Oslo
v-Myb	Viral Myb
v-Myb^{AMV}	v-Myb from the AMV virus
v-Myb^{E26}	v-Myb from the E26 virus
WT1	Wilms tumor 1
Znf	Zinc-finger

Amino acids

A	Ala	Alanine	M	Met	Methionine
B	Asx	Asparagine or aspartic acid	N	Asn	Asparagine
C	Cys	Cysteine	P	Pro	Proline
D	Asp	Aspartic acid	Q	Gln	Glutamine
E	Glu	Glutamic acid	R	Arg	Arginine
F	Phe	Phenylalanine	S	Ser	Serine
G	Gly	Glycine	T	Thr	Threonine
H	His	Histidine	V	Val	Valine
I	Ile	Isoleucine	W	Trp	Tryptophan
K	Lys	Lysine	Y	Tyr	Tyrosine
L	Leu	Leucine	Z	Glx	Glutamine or glutamic acid

Bases

A	Adenine
C	Cytosine
G	Guanine
T	Thymin
Y	T or C
N	A, C, G or T

1. Introduction

1.1 Eukaryotic transcription factors

Following the sequencing of the genomes of humans, *Drosophila melanogaster*, *Caenorhabditis elegans* and other model organisms, several previous notions regarding the correspondence between the complexity of an organism and the number of genes in their genome had to be re-evaluated. The numbers simply did not add up to the expectations, the differences were too small. The human genome was found to encode approximately 26 000 genes, the *C. elegans* genome over 19 000 [1, 2]. It would appear that much of an organism's complexity is not based so much on the number of genes as the manner in which the expression of these genes is regulated. Studies of the promoters of genes in yeast, nematodes, mice and humans indicate that the promoters and regulatory sequences in the genome (the cis-elements) are generally more complex the further up on the evolutionary scale the organism is, and the arsenal of trans-acting factors found in higher eukaryotes also support the general idea that the mechanisms regulating gene transcription have evolved towards increasing complexity (reviewed in [3]). The general transcriptional machinery surrounding RNA polymerase II (Pol II) encompasses 200 to 300 different proteins, including the general transcription factors (GTFs), TBP-associated factors (TAFs), several chromatin remodeling complexes and other factors [4]. These proteins are found in all cells, as they form the basis of all Pol II transcriptional activity. The recruitment of this transcriptional machinery to specific promoters, the orchestration of gene regulation in a cell, is organized by another group of trans-acting factors: The site-specific transcription factors.

Transcription factors constitute one of the largest groups of genes in the human genome. Out of a proposed 26 383 genes, of which the molecular function of 12 809 are unknown, 1850 genes are assumed to encode transcription factors [1]. The transcription factors are characterized by the presence of two functional domains: A transactivation domain (TAD) and a DNA binding domain (DBD). The TADs are assumed to mediate interaction with co-activators or parts of the basal transcription machinery. These domains may often have a high fraction of acidic residues or a specific amino acid (like proline, glutamine, serine or threonine), but are otherwise highly divergent in their structure and sequence [5]. Traditionally the transcription factors have been classified on the basis of their DBDs, though a different classification system, based on similarities in function rather than form has been

proposed [4]. This new classification system may be far more useful than the old one in most aspects, as there appears to be little correlation between the type of DNA binding motif and functional relationships, yet at present time there is but a small fraction of the transcription factors we know enough about to be able to classify them properly according to this new system. Some of the most common DNA binding motifs found are homeodomains, helix-loop-helix domains (HLH), zinc-finger domains (Znf) and leucine-zippers (LZ). Depending on the type of DNA binding motif, some bind DNA as heterodimers, some as homodimers, yet others as monomers. The sequence specific DNA interaction is generally facilitated through van der Waals and hydrogen bonds between the major groove of the DNA helix and an α-helical structure in the DNA binding motif, in addition to interactions between the DNA backbone and other parts of the DBD of the transcription factor [6].

A DNA binding motif found in a large group of transcription factors, especially in plants, is the Myb domain. The founding member of this transcription factor family, c-Myb, has been the main subject of study for this thesis, and will be described further in the following chapters.

1.2 The Myb family of transcription factors

The Myb family of transcription factors is characterized by one, two or three repeats of the Myb domain, a highly conserved helix-turn-helix motif of approximately 50 amino acid residues (reviewed in [7-9]). The first Myb transcription factor to be identified was the protein product encoded by the transforming gene of the avian myeloblastosis virus (AMV), a retrovirus isolated in the 1930s, causing acute myelogenous leukemia in chicken. The gene was named *v-myb*, v for viral, *myb* from myeloblastosis. A version of this gene was also found in the E26 virus, an avian leukemia virus isolated in the 1960s and found to cause acute erythroblastosis in chickens. Through molecular cloning and DNA sequencing it was revealed that these two *v-myb* genes were fusion products including parts of a gene of cellular origin, an oncogene given the name of *c-myb* (cellular *myb*). AMV encodes a 45 kD transcription factor (v-Myb^{AMV}) composed of a C-terminally truncated version of c-Myb, with six amino acid residues encoded by the remains of the viral *gag* gene at the N-terminus, and eleven amino acid residues at the C-terminus from the viral *env* gene, in addition to ten amino acid substitutions relative to the c-Myb sequence, mainly in the DBD region. E26 encodes a gag-

Myb-Ets fusion product of 135 kD, v-Myb^{E26}. The Myb sequence of v-Myb^{E26} is shorter than that found in the v-Myb^{AMV}, and has only one amino acid substitution relative to the c-Myb sequence (reviewed in [10]). The interest in c-Myb from the scientific community was in a large part based on these viral proteins and their strong connection to leukemic disease, and c-Myb has been extensively studied over the past few decades. This transcription factor is further discussed in section 1.3.

Vertebrates have two other three-repeat Myb transcription factors that carry significant homology with c-Myb beyond the DBD, named A-Myb and B-Myb, in addition to several more distantly related transcription factors with only one or two Myb-repeats. A-Myb is mainly expressed in male and female reproductive systems and in peripheral blood cells, whilst B-Myb appears to be ubiquitously expressed, with especially high expression levels in rapidly proliferating cells [7, 11]. The 95 kD A-Myb is the least studied of these three Myb-proteins, but knock-out studies in mice indicate that it plays a critical role in spermatogenesis and mammary gland development (reviewed in [7]). The 93 kD B-Myb is a growth-regulated gene involved in cell cycle regulation, apoptosis and cancer. It is assumed to be the ancestral progenitor of the vertebrate Myb family, as it carries the greatest resemblance to the single Myb found in invertebrates. Whereas knock-out studies for A-Myb showed viable mice, B-Myb knock-out mice die at an early embryonic stage due to impaired inner cell mass formation (reviewed in [11]).

In addition to these Myb proteins, vertebrates also have several other proteins with the Myb domains in their structure, but not all of these are transcriptional regulators. For example the telomere binding proteins TRF-1 and TRF-2 have a DBD that carries significant homology to the Myb domain, as does the Cyclin D interacting protein DMP1. In plants the two-repeat Myb (R2R3) family constitutes the largest group of transcription factors, and several three repeat Myb proteins have been found as well [12]. These transcription factors generally carry little homology to the vertebrate three-repeat Myb proteins beyond the DBD, and are involved in diverse cellular functions (reviewed in [13, 14]).

1.3 c-Myb

The human *MYB* gene has been located to chromosome 6, band q22-q23 [15-17], and encodes a 75 kD transcription factor of 640 amino acid residues, c-Myb. An alternative gene product of 89 kD has also been found in some tissues in human, mouse and chicken, the result of an alternative splice variant with an additional exon between exon 9 and 10, named exon 9A [18-20]. The expression of *c-myb* is high in immature hematopoietic cells, but it is also reported to be expressed in colonic crypts and neurogenic zones of mice, in addition to smooth muscle cells, epidermis and melanocytes (reviewed in [21]). It has mainly been studied as a hematopoietic factor, as that was the context in which it was first discovered and brought about the most striking effects in knock-down/knock-out studies in mice [22, 23], but the field of research has lately expanded to include studies of other tissues where c-Myb has been found to be expressed.

Though c-Myb has been the subject of intense study for more than two decades, and much has been revealed regarding its structure, post-translational modifications, interaction partners and some of its target genes, there are still many questions that have yet to be answered in order to fully understand how this transcription factor functions in a cellular context. This chapter will present some of the findings from past scientific studies regarding c-Myb structure, interaction partners and biological function.

1.3.1 Functional domains

Like other transcription factors, c-Myb has a modular structure with distinct functional domains separated by flexible linker sequences. The DBD of c-Myb is located near the N-terminus, the TAD in the middle of the protein. In addition to these two functional domains, c-Myb also has a C-terminal region that appears to exert a negative regulatory function on transcriptional activation. This region has therefore been named the negative regulatory domain (NRD).

DNA binding domain – DBD

The DBD of c-Myb is comprised of three imperfect tandem repeats, designated R1, R2 and R3[7]. Each repeat is 51-52 amino acid residues long, and folds into three α-helices, of which the two C-terminal helices constitute an atypical helix-turn-helix (HTH) motif somewhat similar to that found in the homeodomain [24]. A marked feature of the repeats is the

recurrence of three tryptophan residues, each 18-19 amino acid residues apart [25]. These tryptophan residues are highly conserved in the DBD of Myb proteins from yeast to humans, and are found to be critical for DNA binding, forming a hydrophobic scaffold that stabilizes the HTH motif [26]. The latest crystal structure of the c-Myb DBD in complex with DNA confirmed the previous reports that only R2 and R3 form a direct contact with DNA, while R1 appears to only mediate some stabilization of the protein-DNA complex through long-distance electrostatic interactions [7, 27]. R2 and R3 recognize and bind to separate half sites of the functionally bipartite minimal Myb recognition element (MRE) 5'-YAAC[GN/NG]-3' [28], lately extended to the optimal sequence 5'-NNCNTAACGGTTTTT-3' [29]. The role of the R1 repeat is still largely unknown, but has been proposed to play a role in RNA binding [30].

Transactivation domain – TAD

In human c-Myb the TAD has been located to the amino acid residues 275-327 [31], a stretch containing clusters of acidic residues, as is often observed for the TADs of eukaryotic transcription factors [5, 7]. There is still some uncertainties regarding the exact location of the TAD, as results have varied somewhat depending on the cell system used [8]. Interestingly, the transactivation ability does not appear to depend on the overall charge of the region [32]. The KIX domain of the transcriptional co-activator CREB binding protein (CBP) has been reported to interact with the TAD of c-Myb [33], and recently the solution structure of this complex was published, indicating that the minimal TAD forms an amphipathic helix upon binding to CBP, with a critical leucine residue inserted in a deep hydrophobic pocket of the KIX domain [34].

Negative regulatory domain – NRD

The first indications that the C-terminal region of c-Myb exerts a negative regulatory function on the activity of this transcription factor came from studies of the v-Myb proteins and other deletion products of c-Myb that displayed a marked increase in transcriptional activity compared to the full-length protein [32, 35]. The NRD is subject to several post-translational modifications (section 1.3.2) and is also found to mediate interactions with other proteins (section 1.3.3). The NRD encompasses several distinct regions that are highly conserved and assumed to mediate the negative regulatory function of the domain: A heptad leucine repeat (HLR) including a FAETL motif, a TPTPFK stretch (TP-domain, or conserved region (CR)) and SUMO-binding sites, in addition to several phosphorylation sites. The heptad leucine

repeat is proposed to form a leucine zipper motif [36, 37], functioning as a protein-interaction domain for co-regulators that exert a negative effect on c-Myb activity [38-40], and/or the formation of inactive c-Myb homodimers [41]. There is some discrepancy regarding the function of the HLR region and the FAETL motif, as these regions have also been reported to be vital for transactivation by v-Myb and thus considered to be part of the TAD, not the NRD [36, 42]. The leucine zipper motif is disrupted by the insertion of exon 9A in the p89 version of c-Myb. The TPTPFK stretch is conserved between all vertebrate Myb proteins, indicating that it plays an important role in the regulation of c-Myb, and is also subject to post-translational modifications (section 1.3.2 and references therein).

1.3.2 Post-translational modifications

c-Myb is subject to a large variety of post-translational modifications, including phosphorylation, acetylation, ubiquitination, sumoylation and redox-regulation. These modifications regulate the interactions of c-Myb with other proteins, its activation potential, half-life and DNA binding ability.

Phosphorylation

c-Myb has several potential phosphorylation sites, and many of these have been shown to be phosphorylated by kinases *in vitro*, a few also *in vivo*. In the N-terminal region serine 11 and 12 have been reported to be phosphorylated both *in vivo* and *in vitro* by casein kinase II (CKII) or a kinase with similar activity [43]. The effect of this phosphorylation on c-Myb activity and DNA binding ability has been a matter of some dispute, some studies have reported increased DNA binding as a result of phosphorylation [43], others inhibition of DNA binding [44], some no effect at all [45]. The latest reports indicate that these serine residues are constitutively phosphorylated in endogenous c-Myb [46]. Serine 116 has been reported to be phosphorylated *in vitro* by cyclic AMP-dependent protein kinase A (PKA) or a similar kinase activity [43, 47], but the studies do not agree on the effect of this phosphorylation. It has been determined that the adjacent V117D mutation in v-Myb^{AMV} completely abolishes the phosphorylation of this serine residue [47]. The DBD has also been reported to be phosphorylated by the serine/threonine kinase Pim-1 *in vitro* [48], and the interaction with Pim-1 has been reported to enhance c-Myb transcriptional activity in a p100-dependent manner [49]. The C-terminal region is also rich in potential phoshorylation sites, and hyperphosphorylation of the NRD by HIPKII mediated by Wnt-1 signaling has been coupled

to degradation of c-Myb through the proteasome pathway [50]. Phosphorylation of serine 532 is observed in several cell lines *in vivo*, and this residue has been shown to be phosphorylated by p42MAPK *in vitro*, a phosphorylation that may differentially regulate c-Myb activity on different promoters [51].

Acetylation

Acetylation of the lysine residues K471, K480 and K485 in c-Myb has been reported to be mediated by the histone acetyltransferase p300 both *in vivo* and *in vitro* [52]. These lysines are conserved between frog, mouse, chicken and human c-Myb [52], and also among the Myb family members A-Myb and B-Myb [53, 54]. Two other lysine residues, K442 and K445, have also been shown to be acetylated by CBP/p300, and acetylation of these five lysines is reported to increase DNA binding and transactivation activity of c-Myb, probably by increasing c-Myb's affinity for CBP [55].

Ubiquitination

The NRD of c-Myb has been reported to be subject to poly-ubiquitinylation, targeting c-Myb to degradation by the 26S proteasome [56, 57]. The same studies also reported that C-terminally truncated versions of c-Myb, including v-Myb, appear to be more stable than wild-type c-Myb.

Sumoylation

c-Myb can be post-translationally modified by SUMO-1 (small ubiquitin-related modifier-1) on K503 and K527 within the EVES domain of NRD [58]. Stress-induced conjugation of the two other SUMO family members, SUMO-2 and -3, has also been reported, resulting in rapid inactivation of c-Myb [59]. Sumoylation of the two c-Myb sites by SUMO-1 is interdependent [60], and also results in reduced transactivational activity [58, 60]. Sumoylation of c-Myb is shown to be stimulated by TRAF7, leading to an increased fraction of c-Myb located in the cytoplasm, thereby negatively regulating its activity [61].

Redox-regulation

DNA binding by c-Myb is highly dependent on the redox state of a conserved cysteine in R2 of the DBD, cysteine 130 [62]. Several reports have shown that oxidation of this residue has a severe negative effect on the DNA binding ability of c-Myb, possibly due to alterations in the

structure of R2 [45, 63, 64]. Interestingly, some of the mutations found in v-Myb^{AMV} result in decreased redox sensitivity [65], possibly contributing to its transformational activity.

1.3.3 Interaction partners

c-Myb has been found to interact with several proteins, some of which cooperate with c-Myb in the regulation of gene expression at distinct promoters, others that regulate c-Myb activity directly (reviewed in [7, 66]). Most of these protein interacting factors have been found to bind to one of the highly conserved regions of c-Myb; DBD, TAD or NRD. This section will present some of the factors proposed to interact with the c-Myb protein, and the biological consequences of these interactions.

Interactions with the DBD

There are a large number of cellular factors that have been shown to interact with the DBD of c-Myb, indicating that this region has an important function as a "protein docking" domain in addition to its DNA binding function. One of the first cooperating transcription factors determined for c-Myb was C/EBPβ, or NF-M, which was found to cooperate with c-Myb in the activation of specific myeloid genes, including *mim-1* [67]. C/EBPβ and its family members α, δ and ε play an important role in the regulation of hematopoietic development, making their connection to c-Myb quite interesting [68]. The biological relevance of the interaction determined between the co-activator p100 and c-Myb has been more unclear, as p100 is mainly a cytosolic protein [69], yet the results have been confirmed in other studies, and were further supported by the finding that p100 and the kinase Pim-1 interact with each other to enhance the transcriptional activity of c-Myb [49]. Pim-1 is also found to interact with the DBD of c-Myb and phosphorylate it [48], but the biological relevance of these interactions remains to be elucidated. Interaction between c-Myb and the histone H3 tail has been reported to be required for the p300 mediated acetylation of K18 and K23 of H3, a prerequisite for gene transcription [70]. Mutations in the v-Myb^{AMV} protein abolishes this interaction, indicating that v-Myb^{AMV} may play a dominant-negative role in the regulation of genes involved in differentiation, possibly explaining some of its oncogenic potential. Another factor that links c-Myb to chromatin is the chromatin remodeling factor Mi2α, recently found to interact with c-Myb as a co-activator of transcription [71]. Mi2α is reported to form part of the NuRD repressor complex, so the finding that it can act as a co-activator of c-Myb was somewhat unexpected, but it appears that Mi2α has two functions in

transcriptional regulation, a helicase-dependent repressor function and a helicase-independent activation function. Not only is c-Myb activity regulated by interactions with other proteins, c-Myb has also been reported to increase the activity of the heat-shock protein HSF3 in unstressed proliferating cells, creating a link between stress response by heat shock proteins and proliferation. The c-Myb induced activation of HSF3 is negatively regulated by p53, which blocks the c-Myb-HSF3 interaction by binding to HSF3 [72, 73].

In addition to these factors that function as co-activators of c-Myb regulated transcription, there are several factors that interact with the DBD of c-Myb that repress transcription by this transcription factor. Ski, N-CoR, and mSin3A are three co-repressors reported to bind to c-Myb DBD in co-operation with TIF-1β, recruiting the histone deacetylase complex to c-Myb [74]. It was also observed that Ski competes with the co-activator CBP for c-Myb binding, and that mutations found in v-Myb^{AMV} weaken the interaction with these co-repressors. Another interaction where distinct differences between c-Myb and its viral counterpart have been observed is the interactions with the cyclins D1 and D2. Previous studies have reported that these cyclins inhibit the activity of v-Myb, but have little effect on c-Myb activity [75]. However, Lei *et al.* published in 2005 an article reporting that c-Myb activity is also inhibited by cyclin D1, contradicting these previous findings [76]. Cyp40 is a cyclophilin reported to negatively regulate c-Myb, but is unable to bind v-Myb due to the mutation of the binding site, thus evading another potential regulatory mechanism [77]. RARα and c-Maf are transcription factors involved in promoting differentiation, and are both shown to inhibit transcriptional activation by c-Myb (reviewed in [66]). Another factor linking c-Myb to regulation of differentiation is MyoD, a key regulator of myogenesis found to be repressed by direct interaction with c-Myb [78].

Interactions with the TAD and NRD

CREB-binding protein (CBP) and p300 are closely related co-activators that are ubiquitously expressed, have histone-acetyl transferase activity and are presumed to play a vital part in cellular processes such as growth and development, in addition to being implicated in multiple malignancies (reviewed in [79, 80]). They interact with numerous transcription factors as well as transforming proteins, including c-Myb [33, 81]. This c-Myb interaction is reported to be constitutive, rather than phosphorylation dependent, and it has been shown in several studies that CBP or p300 are required for c-Myb transcriptional activity (reviewed in [66]). CBP and p300 are proposed to play a vital part in regulation of differentiation and

proliferation of hematopoietic cells [82], making their connection to c-Myb very interesting. PAX5/BSAP (B-cell lineage-specific activator protein) is reported to cooperate with c-Myb on activation of the *RAG-2* promoter [83], probably in combination with LEF-1 [84], providing another link between c-Myb and gene regulation in hematopoietic cells.

As has been observed for the proteins that interact with the DBD of c-Myb, there are interaction partners that interact with the TAD and NRD that are reported to repress c-Myb regulated transcription. Among these are the adenovirus E1A associated protein BS69 [85], the erythropoietin regulated Rcd1 [86], the AT motif-binding factor termed ATBF1 [40], TIF1β as mentioned above [74] and the nucleolar LZ protein p160 and its proteolytic fragment p67 [38].

Intramolecular interactions

The activity of c-Myb has been proposed to be regulated through intramolecular folding of the protein mediated by an interaction between the DBD and EVES motif [69], quite similar to the interaction between the DBD of c-Myb and an EVES motif found in p100. It has been proposed that this folding of c-Myb reduces its activity by blocking protein-protein interactions with co-activators and/or by obstructing DNA binding [66].

1.3.4 Target genes

Like other sequence specific transcription factors, c-Myb activates its target genes by binding to its recognition sequence (MRE) in the vicinity of the target gene promoter, so one should in theory be able to elucidate whether a gene is a potential c-Myb target simply by studying the regulatory sequences surrounding the gene. Unfortunately, the minimal MRE is very short, only six base pairs (bp) long, so it can be found in just about any random DNA sequence longer than 1 kb. Even so, some target genes for c-Myb have been found in this manner. At present, several target genes have been reported for c-Myb and its viral homologue v-Myb (reviewed in [7]), but activation by c-Myb of most of these proposed target genes has only been studied in reporter assays, not in resident chromosomal genes. In this section, only the target genes in the latter category will be discussed, whereas an extended list of proposed target genes for c-Myb and v-Myb is presented in table 1.

Gene	Species	Function	RCG	References
MIM1 (*mim-1*)	chicken	Chemotactic factor/acetyltransferase	Yes	[87, 88]
GBX2	human	Transcription factor, hematopoiesis	Yes	[89]
CD34	human	Hematopoietic stem cell surface protein	Yes	[90, 91]
LYZ (lysozyme)	chicken	Lysozyme, enzyme degrading bacterial cell walls	Yes	[67]
TOM1 (*tom-1*)	chicken	Endofin binding partner	Yes	[92, 93]
ADORA2B (*A2B*)	chicken	Adenosine receptor 2B, transmembrane	Yes	[94]
Kit (*c-kit*)	mouse	Tyrosine kinase receptor	Yes	[95]
PDCD4 (*Pdcd4*)	chicken	Possible tumor suppressor	Yes?	[96, 97]
RAG2	human	Part of the V(D)J recombinase, lymphoid	Yes	[83, 98]
Igf1 (IGF-1)	mouse	Insulin-like growth factor I	Yes	[99, 100]
MYC (*c-myc*)	hu./mo.	Transcription factor, proliferation-related	Yes	[101-104]
BCL2 (*Bcl2*)	human	Anti-apoptotic regulatory protein	Yes	[105-108]
COL1A2	human	Type I collagen α2 chain, structural protein	Yes	[109]
TCRD (*TCRδ*)	human	T-cell receptor δ, V(D)J recombination	No	[110, 111]
TCRG (*TCRγ*)	human	T-cell receptor γ, V(D)J recombination	No	[112]
CD4	human	Cell surface receptor	No	[113, 114]
ANPEP (*CD13*)	human	Alanyl aminopeptidase	No	[115]
CDC2 (*cdc2*)	human	Cell cycle-related kinase	No	[116]
ADA	human	Adenosine deaminase	No	[117]
MYB (*c-myb*)	human	See section 1.3.5	No	[118]
LCK (lck type I)	human	Lymphoid-specific Src-family tyrosine kinase	No	[119]
PAX6	quail	Transcription factor, nervous system development	No	[120]
Gata1 (GATA-1)	mouse	Transcription factor, hematopoiesis	No	[121]
Ela2 (NE)	mouse	Neutrophil elastase, granule protein	No	[122, 123]
PTGS2 (*COX2*)	human	Cyclooxygenase-2, prostaglandin metabolism	No	[124]
TRHR	human	Thyrotropin-releasing hormone receptor	No	[125]
WT1	human	Transcription factor	No	[126]
CHAT (*hChAT*)	human	Choline acetyltransferase, metabolism	No	[127]
CCNA1 (*Cyclin A1*)	human	Cyclin A1, cell-cycle regulatory molecule	No	[128]
MAT2A	human	Methionine adenosyltransferase	No	[129, 130]
PRTN3	human	Myeloblastin, serine protease, neutrophil	No	[131]
KITLG (*SCF*)	human	Stem cell factor, hematopoietic	No	[132]
Repressed genes				
ERBB2 (c-erbB2)	human	Tyrosine kinase receptor	No	[133]
CSF1R (*c-fms*)	hu./mo.	Macrophage colony-stimulating factor receptor	No	[134]
NRAS (*N-ras*)	human	Small GTPase, intracellular signaling	No	[135]
CD4	human	Cell surface receptor	No	[136]
Igε GL	human	Ig epsilon germline, class switch recombination	No	[137]

Table 1 Proposed target genes of c-Myb and/or v-Myb reported with stronger evidence than simple detection of Myb binding sites in the promoter. The cases where the resident chromosomal genes (RCG) have been shown to be activated by c-Myb/v-Myb are shown in the RCG-column. Repressed genes are shown in the lower part of the table. Gene names listed according to OMIM/OMIA annotation, with gene annotation from reference in brackets. Adapted from [138].

The first Myb target gene to be identified was the chicken gene *mim-1* gene [88], which encodes a myeloid specific protein of 326 amino acid residues designated P33 (or Myb-induced myeloid protein 1, MIM1). It has been shown to be regulated by c-Myb and v-Myb^{E26}, but not by v-Myb^{AMV} [67, 87]. The biological function of P33 is at present not entirely clear, but it has been reported to function as an acetyltransferase [139], and shows significant homology to the human neutrophil chemotactic factor LECT2 [140]. *Gastrulin brain homeobox 2* (*GBX2*) is in contrast a gene that responds to activation by v-Myb^{AMV} in a

constitutive manner, but requires specific signaling events in addition to the presence of the co-activator C/EBP in order to be activated by c-Myb or v-Myb^{E26} [89]. *GBX2* encodes a transcription factor that acts as a regulator of the chicken myelomonocytic growth factor (cMGF), explaining how v-Myb^{AMV} can transform cells in a growth factor independent manner. The hematopoietic stem cell antigen *CD34* has been reported to be regulated by c-Myb at the endogenous level, providing another link between c-Myb and regulation of hematopoiesis [91].

Several target genes for c-Myb and v-Myb have been determined by the use of a fusion protein between the estrogen receptor and v-Myb or a C-terminally truncated c-Myb, including the genes encoding chicken lysozyme (*LYZ*), the putative endofin binding partner known as target of Myb1 (*tom-1*), adenosine receptor 2B (*A2B*), mouse c-Kit (*c-kit*) and the possible tumor suppressor Pdcd4 (*Pdcd4*) (see table 1 for references). The determination of *RAG2* as a c-Myb regulated gene provided an important link between c-Myb and V(D)J-recombination in B and T lymphocytes, emphasizing the important role played by c-Myb in T-cell development. Other genes involved in V(D)J-recombination have also been proposed as c-Myb target genes, including the T-cell receptors γ and δ (table 1). The genes encoding insulin-like growth factor (*IGF-1*), the oncoprotein c-Myc (*c-myc*) and the anti-apoptotic factor Bcl-2 (*Bcl2*) have been reported to be regulated by c-Myb (references in table 1), linking this transcription factor to important cellular functions such as proliferation, tumorigenesis and apoptosis. *COL1A2* is a gene encoding the alpha-2 polypeptide of collagen I, and has been reported to be activated by c-Myb, linking c-Myb to several fibrotic disorders [109].

There has in recent years also been published three articles presenting the results from microarray-based gene expression assays used in combination with virus-based expression systems to determine target genes for c-Myb, v-Myb and the related Myb proteins A-Myb and B-Myb [141-143]. The most striking result reported in these articles is the lack of correlation in Myb regulated gene expression between different cell types; it would appear that c-Myb regulates a different set of genes in each cell type studied.

1.3.5 Biological role

The interaction partners and proposed target genes for c-Myb and its viral counterparts have provided several links between this transcription factor and important biological functions such as proliferation, differentiation, apoptosis and tumorigenesis, and knock-out and knock-down studies in combination with studies of the expression patterns of c-Myb have contributed with additional links to these cellular processes. Knock-out studies have shown that *c-myb* null mice die within day 15 of gestation due to severe anemia after developing normally until day 13 [22], and knock-down studies in which the expression of *c-myb* was reduced to 5-10 % of wild-type level also confirmed that c-Myb plays a vital part in regulation of hematopoiesis [23]. However, *c-myb* is not only expressed in hematopoietic cells, but also in the neural retina [144, 145], lung epithelium [145], colon [146], aortic smooth muscle cells [147], thyroid and hair follicles [148], implying that this transcription factor has a distinct function in these tissues.

The observation that expression of *c-myb* is high in immature proliferating cells of the hematopoietic system but is down-regulated upon terminal differentiation were the first indications that c-Myb plays an important role in the choice between proliferation and differentiation of these cell lines, findings that were supported by the observation that antisense inhibition of c-Myb led to blocked proliferation of several hematopoietic cell lines (reviewed in [7]). Later studies have revealed that low levels of c-Myb are sufficient for the expansion of progenitor cells, but the progression towards terminal differentiation is significantly altered in cells with sub-optimal levels of c-Myb [23], and disruption of the interaction between c-Myb and p300 led to a distinct block in T-cell, B-cell and red blood cell development while the number of hematopoietic stem cells increased significantly [149].

The finding that expression of the anti-apoptotic factor Bcl-2 is regulated by c-Myb provided a link between c-Myb and apoptosis, and it has been shown that down-regulation of c-Myb is connected to decreased expression of Bcl-2 with resulting increased apoptosis [105-107]. Expression of a dominant-negative version of c-Myb in leukemic cells has also been shown to induce apoptosis in these cells, marking c-Myb as a potential candidate for gene therapy treatments of leukemic patients [150]. c-Myb has been implicated in a number of diseases, especially in the hematopoietic system, but also in epithelial cancers of the breast, colon and

gastro-oesophagus, and is considered to be an interesting target for anti-sense therapy for these malignancies (reviewed in [151]).

1.4 Aims of the study

Even though several target genes have been proposed for c-Myb, as described in section 1.3.4, this list of candidates is at present insufficient to explain the role played by this transcription factor in such important biological functions as differentiation and proliferation of the cells in which it is expressed. In previous years the methods available for determination of the target genes of a transcription factor have only allowed for the study of a single or a few targets at a time, but the development of high-throughput methods like microarray analysis has altered this, making it possible to study the expression of several thousand genes in a single experiment. However, the information value of such studies is entirely dependent on the model system used. The most common model systems that can be employed for such experiments are transient or stable transfections of cell lines without endogenous expression of the transcription factor of interest, or knock-down of the transcription factor by the use of siRNA in cell lines with endogenous expression.

The main drawback with the use of transiently transfected cells is the variability of the cells under study and between experiments. Transfection efficiency can change from experiment to experiment and is usually significantly below 100 %, resulting in cell-to-cell variance. In addition, one has limited control over expression levels of the transcription factor, and it is difficult to use short expression times, so that one may discern direct from indirect effects. We therefore wished to generate a model system based on stable transfection of a human cell line with a gene regulation system allowing for the easy induction of *c-myb* expression. The choice of gene regulation system fell on the Tet-On and Tet-Off systems, in which the expression of the gene of interest is induced by the addition or removal of a tetracycline derivative from the growth medium, respectively (section 2.3.1).

Previous studies have indicated that c-Myb's viral counterpart v-Myb^{AMV} is not merely a more active version of c-Myb, but a transcription factor with a different set of target genes and distinct biological function. The truncated v-Myb^{AMV} with its mutations has proven to be a valuable tool for the study of c-Myb function, so we decided to not only generate cell lines

with inducible expression of *c-myb*, but also *v-myb*^{AMV} and a truncated version of *c-myb*, designated *c-myb*$^{1-443}$. c-Myb$^{1-443}$ has the same C-terminal truncation relative to c-Myb as observed in v-Myb^{AMV}, but lacks the N-terminal truncation and the viral mutations. Comparison of the biological function of these three versions of c-Myb could provide an important insight in the molecular mechanisms behind oncogenic activation of c-Myb.

The primary aim of this work thus became to generate and verify cell lines with inducible expression of *c-myb* and its derivatives *v-myb*^{AMV} and *c-myb*$^{1-443}$, which would provide a valuable model system not only in the present thesis but also for the laboratory group in further experiments beyond the scope of this study. The second aim was to employ these cell lines in preliminary microarray experiments with the object of determining new target genes for c-Myb and its viral counterpart v-Myb^{AMV}, in addition to studying whether the effect of a C-terminal deletion of c-Myb on gene expression is mainly quantitative or qualitative by comparison of the microarray results for cell lines with expression of c-Myb and c-Myb$^{1-443}$.

More specifically, the aims were:

1. Generation and verification of stable cell lines with inducible expression of *c-myb* or its truncated counterparts *v-myb*^{AMV} or *c-myb*$^{1-443}$ for the study of c-Myb function and mechanisms responsible for oncogenic activation of this transcription factor.
    i. Construction of response plasmids.
    ii. Generation of double-stable cell lines.
    iii. Verification of double-stable cell lines.
2. Utilization of the cell lines in preliminary microarray experiments to identify potential target genes of c-Myb, v-Myb^{AMV} and c-Myb$^{1-443}$.

2. Materials and Methods

This chapter describes the different methods used in the work on this thesis, providing a short introduction to the theoretic basis of each method and the protocol used. In those cases where commercial kits have been used, the protocol is provided by the supplier and will therefore not be described here, with a few exceptions. Unless otherwise stated, the information presented in this chapter is taken from the suppliers or "Molecular cloning: A laboratory manual" [152]. Solutions, kits and plasmid maps are listed in the appendix, as are web page addresses for the suppliers and collaborators.

2.1 Bacterial methods

The bacterial strain used for all experiments in this work was the DH5α strain of *Escherichia coli* (*E. coli*). All plasmids used carry an ampicillin resistance marker gene for selection of transformed bacteria.

2.1.1 Growth and storage of bacteria

Ideal growth environment for *E. coli* is in LB medium, at 37 °C, preferably under agitation. The medium is usually supplemented with an appropriate antibiotic; in this work only ampicillin was used. *E. coli* may be stored for prolonged periods of time at -80 °C in a solution of LB medium and 15 % glycerol.

2.1.2 Preparation of competent *E. coli*

Competent bacteria are cells that have been treated so as to be more susceptible to transformation, that is, they are more permeable for DNA from their surroundings. Most methods used for generating competent bacteria are based on treating the cells with ice cold solutions of $CaCl_2$ and divalent cations. DMSO and hexamine cobalt chloride have been known to further increase the competence of bacterial cells. In this work, the procedure published in an article by Inoue *et al*. in 1990 [153] was used to generate competent *E. coli*.

2.1.3 Transformation of *E. coli*

Once bacteria have been made competent, they can be transformed with plasmids. A bacterial replication origo in the plasmid will ensure the replication of the plasmid within a bacterial cell, thus providing a source of larger amounts of the plasmid. Transformation of bacteria with plasmids may also be used to express proteins of interest. The method used for transformation of *E. coli* in this work is one in which the uptake of plasmids is induced by submitting the bacteria to heat shock, as described in "Molecular cloning: A laboratory manual" [152].

2.2 General molecular biology techniques

2.2.1 Isolation of plasmid DNA from bacterial cultures

Depending on the amount of plasmid DNA needed, two different kits were used in this work to isolate plasmids from *E. coli*. "GFX Micro Plasmid Prep Kit" from Amersham was used for isolating smaller amounts of DNA, "Genopure Plasmid Maxi Kit" from Roche for the larger amounts needed for purposes such as transfection of mammalian cells. Both kits are based on alkaline lysis of the bacterial cells, precipitation of chromosomal DNA and proteins, followed by column purification of the plasmid DNA from the supernatant. The experimental procedures are described in the user manuals of the kits.

2.2.2 Polymerase chain reaction (PCR)

PCR is an exceptionally efficient, yet simple method for amplifying specific DNA sequences. Two oligos that are complimentary to the 3'ends of short stretches of DNA flanking the sequence to be amplified are used as primers for DNA synthesis by a thermostable polymerase. By raising the temperature, the template is denatured, allowing for the annealing of the primers to the template strand once the temperature is lowered. The annealing temperature is determined by the sequence and length of the primers. The temperature is then raised to the ideal temperature for the polymerase, and the elongation is allowed to proceed for a time determined by the length of the amplification product. Through repeating cycles of template denaturation, primer annealing and elongation, with the amplified product acting as

templates for new copies, an exponential increase in the number of specific DNA fragments is achieved.

Procedure

The components listed in table 2 were mixed in a PCR-tube to a total volume of 100 µl, with the exception of the polymerase that was added after the first denaturation step of the PCR cycle, a 5 minute denaturation at 94 °C. For the PCR program a total of 30 cycles were run of denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 55°C (determined by the melting temperature of the primers), then elongation for 1 minute and 30 seconds (determined by the length of the PCR product) at 72 °C.

	Final
H_2O	-
BSA	1x
10x Thermopol buffer	1x
dNTPs (5 mM)	0.25 mM
Template	0.1 µg
Forward primer	1 µM
Reverse primer	1 µM
Vent DNA polymerase	1 U

Table 2 Components of a typical PCR reaction.

2.2.3 Agarose gel electrophoresis

Agarose is a linear polysaccharide that can be used to form gels with a pore size in the region of 100-300 nm, depending on the agarose concentration. The most commonly used concentration is 0.9-1 %, with a separation range of 0.5 to 7 kb. DNA fragments are visualized by staining the gel with ethidium bromide (EtBr) and viewing it in UV light. EtBr intercalates between the DNA base pairs of double stranded DNA, displaying orange fluorescence in UV light. In this work, all agarose gel electrophoresis was performed with a voltage of 100 V, in TAE electrophoresis buffer.

2.2.4 Isolation of DNA from agarose gel

The “GFX PCR DNA and Gel Band Purification Kit” from Amersham was used to isolate DNA fragments from agarose gels. The experimental procedure and theoretic background is described in the user manual.

2.2.5 Restriction enzyme digestion

Restriction endonucleases are enzymes that bind to DNA in a sequence specific fashion, and create a double stranded cut at or near that sequence. There are three groups of restriction endonucleases found, of which the most commonly used in the laboratory are the group II enzymes, as these always create the cut in the DNA in the same place relative to the recognition sequence. The recognition sequence is four to eight bp long, and is usually palindromic. Some restriction enzymes create cuts with either 5' or 3' end overhangs, so-called sticky ends, whereas others cut both DNA strands at the same place, creating blunt ends. The optimal conditions for each enzyme vary with regard to salt concentration, temperature and more, and can be found listed in the catalogues from the suppliers. The enzymes used in this work are listed in the appendix, as are the buffers used for each enzyme.

2.2.6 Ligation of DNA fragments

Bacteriophage T4 DNA ligase is an enzyme that catalyses the formation of phosphodiester bonds between adjacent 3'-hydroxyl and 5'-phosphate termini in DNA, using ATP as substrate. It is commonly used to ligate the DNA strands following restriction enzyme digestion in subcloning experiments. The enzyme is much more efficient at ligating sticky ends than blunt ends. The reactions involving blunt ends were therefore incubated over night, whereas the sticky ends were given a reaction time of only an hour.

2.2.7 Spectrophotometric quantification of nucleic acids

Purine and pyrimidine rings of nucleic acids absorb UV light, with a peak in absorption at 260 nm. With a spectrophotometric reading at this wavelength the amount of RNA or DNA in a solution can be measured. An OD_{260nm} of 1 measured by a standardized spectrophotometer corresponds to 50 µg/ml DNA, 40 µg/ml RNA or 37 µg/ml ssDNA. The purity of the nucleic acid solution can also be measured by comparing the absorption at 260 and 280 nm, as contaminating protein will give a strong absorbance at 280 nm due to their threonine and tryptophane side chains. A pure solution of DNA should have an A_{260nm}/A_{280nm} ratio of 1.8, a pure solution of RNA a ratio of 2.0. In this work, the spectrophotometer NanoDrop ND-1000 was used for measuring the concentration and purity of RNA and DNA solutions.

2.2.8 DNA sequencing

When using PCR amplification, there is always a risk of generating sequences with erroneous incorporation of nucleotides. Such PCR based mutations are most easily found by sequencing the section of the plasmid that includes the PCR amplified sequence. All DNA sequencing performed in the course of this work was done by the staff at the MegaBACE lab at UiO (now known as the ABI-lab). The sequencing instruments from Applied Biosciences used in this lab are based on capillary electrophoresis in combination with the dideoxy chain termination method with fluorescently labeled dideoxy nucleotides.

2.3 Mammalian cells

2.3.1 Tet-On and Tet-Off gene regulation systems

There are several gene regulation systems on the market that can be used for generating stably transfected cell lines with inducible expression of a gene of interest, two of which are the Tet-On and Tet-Off gene regulation systems from Clontech/BD Biosciences. This system is based on the *E. coli* Tet repressor protein (TetR), a protein that in the absence of the antibiotic tetracycline binds to tet operator sequences (*tetO*) and represses the transcription of the genes of the tetracycline-resistance operon on the Tn*10* transposon.

The first part in the generation of a functioning Tet system is the stable transfection of a cell line with a plasmid encoding the regulatory protein. In the Tet-Off system, the regulatory protein is a fusion of the 207 N-terminal amino acids of the TetR protein and the 127 C-terminal amino acids of the VP16 activation domain from the Herpes simplex virus, resulting in a tetracycline controlled transactivator (tTA). The Tet-On system uses a slightly modified version of the tTA, known as reverse tTA (rtTA), in which four amino acids have been altered so that the protein binds to the *tetO* in the presence of doxycyline (Dox), a tetracycline derivative, whereas tTA binds in the absence of tetracycline or Dox. Whereas tTA is responsive to both tetracycline and Dox, rtTA is only responsive to Dox, not tetracycline. Both regulator plasmids (pTet-Off and pTet-On) also carry a geneticin resistance marker gene for selection of stable transfectants.

The second part of the Tet system is the integration of a response plasmid (pTRE2hyg) encoding the gene of interest (Gene X) under the control of a tetracycline-response element (TRE). The TRE is composed of seven direct repeats of the 42 bp sequence containing the *tetO* just upstream of the minimal CMV promoter (P_{minCMV}). A double stable cell line containing both the regulatory plasmid pTet-Off and the response plasmid encoding Gene X will then express Gene X when Dox is removed from the cell culture medium, whereas a double stable cell line with pTet-On as the regulatory plasmid will express Gene X in the presence of Dox in the medium. The response plasmid also contains a hygromycin resistance gene, to allow for selection of stable transfectants.

2.3.2 Growth and storage of HEK293 cells

HEK293 (ATCC number CRL-1573) is a cell line derived from human embryonic kidney cells. They have an epithelial morphology, and fall into the category of adherent growing cells, though they generally adhere only weakly to the growth surface. Tet-Off and Tet-On HEK293 cell lines express the tTA and rtTA, respectively. These were the cell lines used to generate double-stable cell lines with inducible expression of Myb proteins in this work.

Procedure

Frozen stocks of cells were stored in cryotubes in liquid nitrogen vapor. Cells were thawed quickly by stirring the vial gently in 37 °C water, then transferred to a 75 cm^3 flask containing 5 ml of preheated growth medium. An additional 10 ml of medium was added before the cells were incubated in a humidified growth chamber at 37 °C, with an atmosphere of 5 % CO_2. The growth medium was replaced once the cells had adhered sufficiently to the flask. In general, this was done the next day. Medium was then replaced at least every four days or when deemed necessary as determined by the color of the medium. Antibiotics were added to the medium 24-48 hours after seeding, geneticin to all Tet-On and Tet-Off cells, hygromycin only to the double-stable cell lines. Dox was added to the double-stable Tet-Off cells every 48 hours. Once the cells reached ~80 % confluence, they were treated with trypsine/EDTA and split as necessary (generally, 1:4 or 1:8 under normal growth conditions).

New stocks of cells were frozen down at an early passage. The cells were treated with trypsine/EDTA, trypsine was inactivated by the addition of growth medium (twice the volume of trypsine/EDTA used), then transferred to 15 ml tubes and centrifuged at 4 °C, 900 rpm for

5 minutes. The pellet was dissolved in a solution of 90 % FBS, 10 % DMSO to a minimum cell concentration of $1x10^6$ cells per ml. The cell suspension was transferred to cryotubes, 1 ml per tube, and frozen slowly down in an isopropanol container at -80 °C overnight, before they were placed in liquid nitrogen for long term storage.

2.3.3 Counting cells – Determination of cell concentration

To assess the cell concentration in a solution, the Neubauer Improved counting chamber was used. Each main square of the chamber contains 10^{-4} ml of medium; thereby the number of cells per ml can be obtained by counting the cells per chamber. As generally only the number of living cells are of interest, the cell solution is first diluted 1:1 with trypan blue, a dye that only colors the dead cells blue.

Procedure

The cells were treated with trypsine/EDTA, medium was added to inactivate the trypsine, and a small aliquot of the cell suspension was transferred to an eppendorf tube. 50 µl of the aliquot was mixed with 50 µl trypan blue, and a small amount of this solution was then applied beneath a cover slide on the counting chamber. At least 100 cells were counted.

$$\text{Number of cells per ml medium} = \frac{\text{(Dilution factor*Average number of cells per square)}}{(10^{-4}\text{ ml per square})}$$

2.3.4 Transfection of HEK293 cells by use of Lipofectamine

Transfection of mammalian cells is the equivalent of transformation of bacteria, which is the uptake of DNA from the cell's surroundings. Several methods have been designed for this purpose; the most commonly used are electrophoration or different lipid solutions. Lipofectamine (Invitrogen) is a solution of two different lipids that form liposomal structures around DNA, thus enabling the passage of DNA through the cell membrane.

Procedure

Transfections were performed on cells growing both in six-well plates (35 mm wells) and in 10 cm plates. The amount of cells, medium, DNA and Lipofectamine used per well/plate are listed in table 3.

	35 mm well	10 cm plate
Cells	$2x10^5$	$1x10^6$
Growth medium	1 ml	9 ml
DNA	1.25 µg	10 µg
Opti-MEM	100 µl	900 µl
Lipofectamine	5 µl	40 µl
Opti-MEM	100 µl	900 µl

Table 3 Amount of cells, growth medium, DNA and Lipofectamine per well/plate of cells to be transfected. DNA and Lipofectamine were diluted in Opti-MEM medium separately before they were mixed for the final transfection solution. For transfection of multiple wells/plates, a mastermix was made.

The cells were seeded out the day before transfection. Just prior to the transfection the growth medium was replaced by fresh medium without antibiotics. DNA and Opti-MEM were gently mixed and then incubated for 5 minutes at RT, as was Lipofectamine and Opti-MEM. Following incubation, the Lipofectamine/Opti-MEM mixture was added to the DNA/Opti-MEM mixture drop by drop. This solution was gently mixed and incubated for 20 minutes at RT. The solution was then carefully added to the growth medium of the cells and the cell plates were tilted a few times to blend the solutions together. The cells were placed in the growth chamber for four hours before the transfection medium was replaced by normal growth medium with added antibiotics. The cells were harvested 24-48 hours post transfection and potential induction, or in the case of generating stable transfectants, split and seeded out on new 10 cm plates.

2.3.5 Transfection of HEK293 cells by use of FuGENE 6

FuGENE 6 (Roche) is a transfection reagent composed of lipids and several other components. Contrary to Lipofectamine, it does not form liposomes, but a multicomponent DNA complex that can be transported through the cell membrane. In this work, it was used to transfect cells for luciferase assays (section 2.4.6).

Procedure

The day prior to transfection, cells were seeded out in six-well plates, $2x10^5$ cells in 2 ml medium per well. A mastermix was made of 2 µl of FuGENE and 48 µl of medium (DMEM without any additives) per well to be transfected. FuGENE was added very carefully to the medium. The solution was gently mixed and incubated for 5 minutes at RT. DNA was then added to the FuGENE/medium mixture, 1 µg of DNA per well to be transfected. The solution was incubated for 30 minutes at RT before it was added to the cells, 50 µl solution per well.

The cells were incubated with the transfection medium for 4 hours at 37 °C, 5 % CO_2 before the medium was replaced by normal growth medium with selective antibiotics. 1 µg/ml Dox was at this time also added to the medium for induction of Myb expression. Cells were harvested as described in section 2.4.6 16 hours after addition of Dox.

2.3.6 Generation of stable transfectants

Once a mammalian cell has taken up a plasmid, it will usually discard the plasmid within a few days, that is, the transfection is only temporary, or transient. However, a small number of transfected cells will integrate the plasmid into their own genome, making the transfection stable. By growing the cells in the presence of an antibiotic against which the plasmid encodes a resistance gene, one can select out these few stable transfectants, as they will survive whilst the transient transfectants will lose their resistance and die once they discard the plasmid. When allowed to grow, the stable transfectants will form separate colonies of cell clones, which can be isolated and then allowed to reproduce themselves in separate cell cultures. There are several methods that can be used for isolating separate clones. One of the simplest is using Cloning Discs (Sigma-Aldrich), paper discs with a diameter of 5 mm, soaked in trypsine/EDTA. As the trypsine breaks down the extracellular matrix binding the cells to the plate surface and each other, the cells will get soaked into the paper disc, and can then be transferred to separate wells in a 24-well plate.

Procedure

HEK293 Tet-On and Tet-Off cells were seeded out in 10 cm plates and transfected by the use of Lipofectamine as described in section 2.3.4. Prior to transfection each of the plasmids were linearized with restriction enzymes to avoid disruptions of gene or promoter sequences caused by random linearization before insertion into the cellular genome. Linearized plasmids were purified by ethanol precipitation and dissolved in TE-buffer before transfection.

The day after transfection the cells were split 1:20 and seeded out in new 10 cm plates, five plates per construct, resulting in a total of 30 10 cm plates. 48 hours after transfection hygromycin was added to the medium to a final concentration of 100 µg/ml. The medium was replaced every four days, with 1 µg/ml Dox added to the medium of the Tet-Off cells every 48 hours. After three weeks, separate clones were isolated and transferred to 24-well plates using Cloning Discs, with 1 ml medium in each well. For each construct a total of 24 clones

were isolated if possible. Five days after they were transferred to the 24-well plates, the cells were transferred to six-well plates. When the cells were nearly confluent they were transferred to 10 cm plates, and the hygromycin concentration in the medium was increased to 150 µg/ml. When approaching confluence, the cells were frozen down in stock as described in section 2.3.2, with each plate yielding two cryotubes of cells.

2.4 Protein techniques

2.4.1 Isolation of cell lysate for MicroBCA and Western blotting

The MicroBCA protein concentration measurements are very sensitive to reducing agents, so the lysis buffer used for the majority of the Western blots performed in this work should not contain any such agents. With these constraints in mind, RIPA-buffer in combination with sonication was used to lyse the isolated HEK293 cells. The method described here is only applicable on mammalian cells that attach loosely to the growth surface, as HEK293 cells do.

Procedure

Growth medium was removed, and 1 ml of PBS added to each well. Cells were flushed loose by pipetting, and the cell suspension transferred to 1.5 ml eppendorf tubes, which were then centrifuged at 13 000 rpm for 2 minutes. PBS was removed, and the cell pellet lysed in 100 µl RIPA-buffer with added protease inhibitor. The lysate was then sonicated a few seconds at the lowest intensity. The lysate can be stored for a few months at -80°C, and may need to be sonicated anew upon thawing.

2.4.2 MicroBCA protein concentration measurement

The BCA (bicinchoninic acid) method of protein concentration measurement is based on the colorimetric quantification of the purple-colored reaction product formed by the chelation of two BCA-molecules and one cuprous ion (Cu^{+1}). Cu^{+1} is formed when Cu^{2+} is reduced by proteins in an alkaline environment, and the correlation between protein concentration and absorbance of the purple-colored reaction product is linear. In this work, the MicroBCA-kit from Pierce was used to measure protein concentrations of 2-40 µg/ml, with a standard curve in the range of 0-60 µg/ml. The procedure is described in the user manual of the kit.

2.4.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gels are composed of long chains of acrylamide crosslinked by *N,N'*-metylenebisacrylamide. The polymerization of acrylamide and *N,N'*-metylenebisacrylamide is induced by the addition of free radicals, usually ammonium persulfate (APS), and the free radical stabilizer *N,N,N',N'*-tetramethylethylenediamine (TEMED) is also added to the gel mixture. The pore size of the gel is determined by the concentration of acrylamide and *N,N'*-metylenebisacrylamide, and the ratio of these two. Polyacrylamide gels are most often used for the electrophoresis of proteins, where the migration of the proteins is determined by their size, structure and charge, as well as the pore size of the gel. By treating the proteins with the strong anionic detergent sodium dodecyl sulphate (SDS) in the presence of the reducing agent β-mercaptoethanol, all secondary structures of the proteins are removed, disulfide bridges are reduced, and the proteins are given a strong negative charge from the bound SDS. Under these conditions, the migration of the proteins will be approximately proportional to their molecular weight.

Procedure

The gel slab of a 10 % polyacrylamide gel with an upper stacking gel was placed in an electrophoresis apparatus filled with electrophoresis buffer (1xSDS-PAGE), and all air bubbles beneath the gel were removed. The upper chamber was also filled with 1xSDS-PAGE, and the wells of the gel were washed thoroughly with the buffer. To one well 10-15 µl of BioRAD Precision Plus Protein Standards was added, protein samples/cell lysates to the other wells of the gel. The electrophoresis was run at 25-30 mA for 2-3 hours, or until the marker had migrated at least 2/3 of the gel.

2.4.4 Western blotting – Semidry

Western blotting (also referred to as immunoblotting) is a method commonly used for the detection of specific proteins in cell lysates. Following a separation of the proteins by SDS-PAGE, the proteins are transferred, by blotting, to a PVDF membrane or nitrocellulose filter which binds proteins tightly. The membrane is washed in a blocking solution containing nonspecific proteins to block excess absorption sites in the membrane. An antibody specific for the protein of interest is then added, binding to its specific target protein. The presence of

this primary antibody is visualized by incubating the membrane with a secondary antibody (specific for the primary antibody) conjugated to the enzyme horseradish peroxidase (HRP). HRP will in the presence of its substrate catalyze a reaction emitting light which can be detected on a photographic film. The membrane used in this work was Hybond-P and the detection kit ECL Plus kit, both from Amersham.

Procedure

The SDS-polyacrylamide gel was cut to size, and the orientation marked by cutting off one corner of the gel. A piece of membrane (Hybond-P) was also cut to the same size as the gel, as were six sheets of 6 mm paper. The membrane was first soaked in methanol, then in plus(+) buffer. Three sheets of paper were soaked in plus(+) buffer, three in minus(-) buffer. The different sheets were then stacked on the anode of the blotting apparatus (TE 70 SemiPhor Semi-Dry Transfer Unit, Amersham) in the following order: Three sheets of paper soaked in plus(+) buffer, membrane, gel and finally three sheets of paper soaked in minus(-) buffer. All air bubbles were removed, the lid (cathode) put on, and extra weight was put on the lid. The electrophoresis was run for an hour, with 1 mA per cm^2 membrane and a maximum voltage of 50 V. The membrane was then fixated in methanol for 2 minutes, while the apparatus was washed with distilled water and left to dry.

Optionally, the membrane may at this point have been dyed with Ponceau S. to control whether the blotting process had been satisfactory. Washing the membrane for 5 minutes in a Ponceau S. solution would dye the proteins in the membrane red, and excess color would be washed away with distilled water.

The membrane was gently shaken in 1xTBS-T with milk for 30 minutes at RT to block all protein binding sites. The primary antibody was diluted to the optimized concentration in the amount of 1xTBS-T with milk that just covered the membrane, and the membrane was incubated in this solution for at least two hours at RT or overnight at 4 °C, with constant agitation. The membrane was then washed at least 3x20 minutes in 1xTBS-T with milk at RT. The HRP-conjugated secondary antibody was diluted in 1xTBS-T with milk to the optimized concentration, and the membrane was incubated in this solution for one hour at RT, with constant agitation. The membrane was then washed six times in 1xTBS-T, then for 20 minutes in 1xTBS-T with constant agitation. The membrane was kept in 1xPBS until detection.

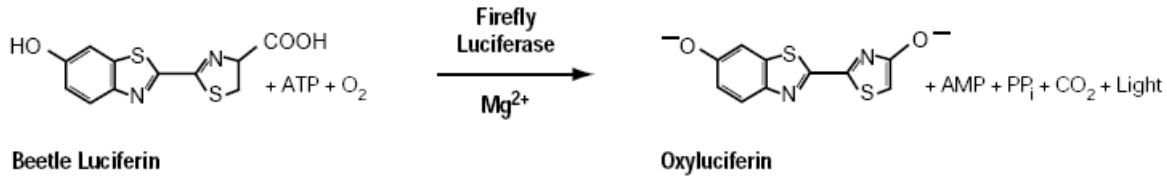
The procedure for the ECL Plus kit (Amersham) is described in the user manual.

2.4.5 Stripping and reprobing Western blot membranes

Occasionally, there may be need for stripping and reprobing Western blot membranes, whether to use a different antibody or a different concentration of the antibody previously used. The membrane is then washed at 50 °C in a stripping buffer for 30 minutes while under rotation, removing antibodies and detection solution, leaving the membrane ready for a new round of antibody incubation and detection. Due to the rather harsh process of stripping the membrane, which often removes some of the blotted protein from the membrane, one should not use this method for blots for quantitative analysis. The procedure is described in detail in the user manual of the ECL Plus kit from Amersham.

2.4.6 Luciferase reporter gene assay

Firefly luciferase is an enzyme that catalyzes the following reaction:



The amount of light produced will give a measurement of the amount of luciferase expressed in the cells, and this can be measured by means of a luminometer. By constructing a vector where expression of the luciferase gene is under the control of a c-Myb responsive promoter (TRHR or 3xGG), the amount of luciferase will be determined by the activity of c-Myb in the cells, and the luminometer measurements will thereby give an indirect measurement of this activity.

Procedure

The cells were seeded out and transfected with FuGENE 6 as described in section 2.3.5. 16 hours after changing the medium, the cells were harvested. The medium was removed, the cells were washed in PBS, 1 ml per well. PBS was removed, and 250 µl of Passive lysis buffer was added to each well. The tray was then placed on a shaker for 10 minutes at RT. The lysates were transferred to 1.5 ml eppendorf tubes. 50 µl of lysate was mixed with 50 µl

of "Luciferase assay reagent" in a tube, which was then quickly placed in the luminometer for measurement of the luciferase activity.

2.5 RNA techniques

To avoid contamination of RNA samples by RNases or other contaminants that could disrupt the following analyses, special precautions were made. In addition to heeding general guidelines for working with RNA, such as using gloves at all times, the water used for all experiments with RNA was Molecular biology grade water from Eppendorf, the pipette tips were RNase/DNase free filter tips and only single-use sterile plastic equipment was used.

2.5.1 Trizol RNA-isolation

Trizol (Life Technologies/Invitrogen) is a solution consisting of phenol and guanidine isothiocyanate, used for the isolation of total RNA from cells and tissues. Upon addition of Trizol, the cells are lysed, and further addition of chloroform followed by centrifugation separates the solution into an upper aqueous phase and a lower organic phase, with RNA in the aqueous phase, proteins and DNA in the organic phase. RNA is precipitated from the aqueous phase using isopropanol.

Procedure

Cells were seeded out on 15 cm plates two or three days prior to RNA isolation at a density so as to be 50-80% confluent upon harvesting. Dox was added to the medium to a final concentration of 1 µg/ml 8 or 24 hours before harvesting to induce expression of Tet-regulated genes. On each plate the medium was removed before addition of 4 ml Trizol. The lysates were scraped together on each plate and then transferred to 15 ml tubes. To each tube 0.8 ml of chloroform was added before the tubes were shaken vigorously for 15 seconds, and then incubated at RT for 3 minutes. The tubes were centrifuged 20 minutes at 3000 rpm, 4 °C in a swing-out rotor (JS-rotor), brakes turned off. The upper aqueous phase in each tube was transferred to four 1.5 ml eppendorf tubes, 600 µl per tube. To each tube 500 µl isopropanol was added, and the tubes were incubated for 30 minutes at RT to precipitate RNA. Following a 10 minute centrifugation at 14000 rpm and 4 °C the supernatants were removed, and the pellets washed in 1 ml 70% ethanol. The samples were centrifuged at 14000 rpm for 5

minutes at RT, ethanol was removed, and the pellets dried completely before they were dissolved in 20 µl of water each. The RNA samples were quickly frozen down and stored at -80 °C.

2.5.2 RNeasy – Purification of RNA

Some methods, such as real-time RT PCR and microarray analysis, require a very high quality of RNA, one that is higher than that generally achieved by Trizol isolation. DNase treatment is of particular importance, as traces of genomic DNA can wreak havoc on the results of such methods. The kit used in this work for the purification of isolated RNA was the "RNeasy Mini Kit" in combination with the "RNase-Free DNase Set" (both from Qiagen). The kit is based on DNase treatment of the RNA sample on a silica-gel column, where RNA with a length greater than 200 bp is bound to the silica-gel while smaller RNA fragments and other sources of contamination are washed away before the bound RNA is eluted with water. The procedure is described in the user manual.

2.5.3 Bioanalyzer – Determination of RNA quality

RNA is quite susceptible to degradation, and one way to measure whether the total RNA has been degraded or not is to compare the amount of 28S and 18S ribosomal RNA in the test sample. In this work the Agilent 2100 Bioanalyzer with the kit "RNA 6000 Nano Assay" was used for this purpose. The system is based on electrophoresis of the samples through glass capillaries filled with a gel matrix, and fluorescence is used to detect the RNA components. The main perks of this method compared to traditional gel electrophoresis are the small amounts of RNA necessary, a shorter, simpler work procedure as well as better, more standardized data. In this work the RNA was analyzed after the RNeasy purification step described in section 2.5.2, with 12 samples per chip used. The procedure is described in the user manual of the kit.

2.5.4 Real-time RT-PCR

Conventional RT-PCR (reverse transcription PCR) is one of the most sensitive methods available for the detection of gene expression at the mRNA level, but it is also a complex technique, and how quantitative it can be is rather questionable. Some of these major

drawbacks have in recent years been overcome by the introduction of fluorescent techniques for the quantification of synthesized DNA during the PCR process in combination with the use of a thermal cycler with inherent detection instruments, resulting in the real-time (or kinetic) RT-PCR method (reviewed in [154]).

In the first step of this method cDNA is synthesized from total RNA using a reverse transcriptase enzyme with oligo-dT as a primer. Depending on the nature of the experiment and the RNA sample, other primers can also be used, such as random hexamers or gene specific primers. In the second step of the process a PCR reaction is run with primers for the gene of interest, and for each PCR cycle the amount of synthesized DNA is measured by a fluorescent detector in the thermal cycler in which the PCR reaction is run. In this work the Lightcycler apparatus from Roche was used, in combination with the SYBR Green dye for quantification of double stranded DNA (dsDNA). The SYBR Green dye emits a strong green fluorescence when bound to dsDNA, but very little in its free form or when bound to ssDNA. Based on how many cycles are needed to reach a determined threshold level of synthesized DNA, and determination of the efficiency of the PCR reaction through the use of a standard curve based on dilutions of the original sample, the original amount of a specific mRNA in a sample may be determined. The measurement is always made relative to another DNA source, in this work the level of the housekeeping gene β-actin was used as a reference. The procedure is described in the user manual for the Lightcycler apparatus.

2.6 Microarray analysis

Though many people may think of microarray analysis only in terms of the microarrays used for transcriptional profiling, this is but one of many applications for the method principle. Since the first articles on DNA microarrays (for transcriptional profiling) were published in the mid-nineties, the microarray term now also embraces methods for studying proteins, DNA mapping, RNA/RNAi and more (reviewed in [155, 156]). In addition to new microarray based methods emerging, the use of the technology has also shown an exponential increase over the past decade. In 1995, a search for the word “microarray” in PubMed gave a list of only three articles, only one of which was an article on DNA microarrays [157]. In 2000, the number of articles for that year was 411. For 2005 the number had soared to 5029 articles, at this time

the term not only referring to DNA microarrays, but also protein microarrays and other microarray methods.

In this work two different DNA microarray platforms were used; the Affymetrix system (section 2.6.1) and in-house spotted oligoarrays from the Norwegian Microarray Consortium (NMC) at Rikshospitalet-Radiumhospitalet Medical Center (RR MC) hybridized with Cy3 and Cy5 labeled cDNA (section 2.6.2-2.6.4). These two methods are both for transcriptional profiling and share some of the same theoretic principle, but are very different when it comes down to the experimental procedure. They exemplify the most widely used types of DNA array manufacturing: The photolithographic method and deposition (spotting) of DNA fragments (oligos) on a glass slide.

2.6.1 Affymetrix microarray system

The Affymetrix GeneChip arrays are an example of so-called high-density microarrays. Rather than depositing DNA fragments in spots on a glass slide, as is done in the production of spotted arrays, the features on a GeneChip array are synthesized directly on the array, nucleotide by nucleotide. This process begins by coating a quartz wafer with a light sensitive coating compound, preventing coupling of nucleotides to the surface of the wafer. A photolithographic mask is then applied before the wafer is exposed to light, deprotecting the features that are to have the same nucleotide at this position. When the wafer at this point is subjected to a solution of for instance thymine, this nucleotide will be coupled to the wafer only at the features that have been deprotected, making the oligo at this feature one nucleotide longer. The nucleotides that are washed over the wafer carry their own light sensitive protection group, preventing coupling of new nucleotides to the oligo until it has been subjected to light. The wafer must be washed with all four nucleotides for each nucleotide added to the oligos on the entire wafer, giving a total of $4n$ cycles of deprotection and coupling for a wafer with oligos of n nucleotides length. Usually the oligos are 25-mers, resulting in a total of 100 cycles of deprotection and coupling per wafer [158]. The density of the features becomes very high with the use of this production method, and each wafer can be diced into tens or even hundreds of separate arrays, depending on the number of features required per array. The arrays also become far more uniform than arrays produced by the deposition method, allowing for a different form of hybridization of the samples to be studied. Rather than the two-color cross-hybridization used for the deposition arrays, where two

samples are hybridized on the same slide, the samples analyzed with high density microarrays are hybridized to separate arrays. This also allows for all samples to be coupled to the same dye, usually biotin, eliminating another source of variation.

The Affymetrix GeneChip system is a completely closed system, requiring the use of Affymetrix scanners and computer programs for data analysis. In this work, RNA samples were delivered to the Affymetrix lab at Centre for Occupational and Environmental Medicine (SYM) at RR MC, now a part of the Microarray Core Facility (MACF) at RR MC, and all further work including the data analysis was done by graduate engineer Siv Haugen Tunheim at this lab. The array used was the Human Genome Focus Array, an array representing over 8 500 human genes.

2.6.2 FairPlay – Synthesis and labeling of cDNA

There are two different methods that can be used for labeling cDNA with Cy3 or Cy5, either direct incorporation of the dyes by using Cy3- or Cy5-linked dUTP in the cDNA synthesis reaction, or indirect incorporation, in which the cDNA is synthesized using amino allyl linked dUTP and the dyes with a NHS-ester leaving group are chemically coupled to these modified nucleotides at a later step in the process. Though the indirect method is somewhat more laborious and time consuming, it does have some rather important preferential qualities when compared to the direct labeling method. The direct method struggles mainly with the large size of the Cy-dye molecules, which can lead to the reverse transcriptase struggling with the incorporation of them, thereby impeding the enzyme's efficiency as well as causing a low incorporation rate of these dye-coupled dUTP nucleotides. There is also a significant difference in the molecular size of Cy3 and Cy5, a factor that may lead to a higher rate of incorporation of Cy3 compared to Cy5, as Cy3 is the smaller molecule. Both of these factors are avoided in the indirect labeling method, as the amino allyl groups are rather small, and the size difference between Cy3 and Cy5 is insignificant for the coupling reaction. The indirect method also uses less RNA than the direct labeling method (10-20 µg versus 20-100 µg). The indirect labeling kit used in this work was the FairPlay II Microarray Labeling Kit from Stratagene, with NHS-ester containing Cy3 and Cy5 dyes from Amersham. Several minor tweaks were made to the procedure compared to the one given in the instruction manual of the kit. The procedure is therefore described in full below.

Procedure

cDNA synthesis

A single labeling reaction does not always produce sufficient labeled cDNA for slide hybridization, so two parallel labeling reactions were performed for each slide, giving a total of four labeling reactions per slide.

1) 10 µg total RNA was dissolved in water to a total volume of 12 µl in a 0.5 ml PCR tube.
2) 1 µl 500 ng/µl oligonucleotide d(T)12-18 was added. The solution was incubated at 70 °C for 10 minutes, and then cooled on ice.
3) A master mix was made of the following components (volumes are for a single reaction) in a PCR tube:
   2 µl 10x StrataScript reaction buffer
   1.5 µl 0.1 M DTT
   1 µl 20x dNTP mix
   0.5 µl RNase Block (40 U/µl)
4) 5 µl of the master mix from step 3 was added to each of the RNA tubes from step 2. The tubes were given a brief spin to collect the contents.
5) 3 µl StrataScript HC RT was added to each tube, and the tubes were incubated at 42 °C for 120 minutes (in a PCR machine to ensure constant temperature).
6) 10 µl 1 M NaOH was added to each tube. The tubes were then incubated at 70 °C for 10 minutes to hydrolyze RNA. The 1 M NaOH was made fresh for each day of labeling by dilution of a 10 M NaOH solution.
7) The samples were cooled slowly to RT (important *not* to place the samples on ice), and were then given a quick spin.
8) 10 µl 1 M HCl was added to each tube to neutralize the solution.
9) The samples were transferred from the PCR tubes to clear 1.5 ml eppendorf tubes.

cDNA purification

10) 4.8 µl 2.5 M sodium acetate, pH 4.5 was added to each tube.
11) 1 µl 20 mg/ml glycogen was added.
12) 100 µl ice-cold 95% EtOH was added.
13) The tubes were incubated at -20 °C for at least 30 minutes, usually overnight. The samples may at this point of the labeling reaction be stored for up to two months.

14) Samples were spun down at 13-14 000 x g, 4 °C for 30-45 minutes. The supernatant was gently removed.
15) The pellet was washed in 0.5 ml ice-cold 70% EtOH, and then spun down for 15 minutes at 13-14 000 x g, 4 °C.
16) The supernatant was gently removed with a pipette, the tubes were given a quick spin, and the last remnants of the supernatant were removed with the tip of a 10 µl pipette. The pellet was allowed to air dry until it had just turned translucent. Should the pellet overdry, it would be nearly impossible to dissolve it again.

NHS-ester containing dye coupling reaction

17) The cDNA pellet was resuspended in 5 µl 2x coupling buffer and vortexed to mix. The buffer was incubated a short time at 37 °C before use, as it can form a precipitate at low temperatures. The cDNA solution was incubated at 37 °C for 15 minutes to aid in the resuspension process.
18) The fluorescent dyes were resuspended in 45 µl DMSO from the kit, and vortexed. Dye that would not be used the same day was dispensed into single use aliquots of 5 µl in brown 1.5 ml eppendorf tubes and stored at -20 °C in the dark. When such aliquots were used, they were allowed to reach RT before the tubes were opened.
19) 5 µl of dye was added to the dissolved cDNA pellet. The solution was mixed gently by pipetting.
20) The solution was incubated for 60 minutes at RT in the dark.

Dye-coupled cDNA purification

21) 90 µl of TE buffer was added to the labeled cDNA.
22) 210 µl DNA binding solution was thoroughly mixed (vortexed) with 210 µl 70% EtOH per reaction.
23) 200 µl of the mix from step 22 was added to the cDNA solution, which was then mixed by vortexing.
24) The solution from step 23 was transferred to a microspin cup seated in a 2 ml receptacle tube. The cap of the tube was snapped into place, and the tubes were spun down at maximum speed for 30 seconds. The cup was retained while the solution in the tube was discarded, then the cup was placed back into the tube.

25) 200 µl of the solution from step 22 was added to the cup, the cap of the tube was snapped into place, and the tubes were spun down for 30 seconds. The solution in the tube was discarded, the cup retained, and placed back into the empty tube.
26) 750 µl 70% EtOH (washing buffer) was added to the cup. The tubes were spun down for 30 seconds, the washing buffer discarded. This washing step was performed twice.
27) The cup was placed back into the tube, which was then spun down for 30 seconds to remove any last remnants of washing buffer.
28) The cup was transferred to a clean 1.5 ml eppendorf tube, the receptacle tube was discarded.
29) 50 µl of 10 mM Tris, pH 8.5 was carefully added to the fiber matrix of the cup. After 5 minutes incubation at RT, the tube was spun down for 30 seconds at full speed.
30) The elution from step 29 was reapplied to the fiber matrix, incubated for 5 minutes at RT and spun down for 30 seconds. This step was repeated so that the buffer with the labeled cDNA was eluted through the matrix a total of three times.

Analysis of labeled cDNA

31) The labeled cDNA was analyzed using the NanoDrop ND-1000 spectrophotometer described in section 2.2.7. This spectrophotometer has a separate function for microarray samples, one that gives a measurement of the amount of cDNA as well as incorporated Cy3 and Cy5 from a sample of 1-2 µl. For each slide 600 ng of Cy3- and Cy5-labeled cDNA was used, corresponding to 20-60 pmol of dye.

Concentration of labeled cDNA

32) The 600 ng samples of Cy3- and Cy5-labeled cDNA from step 31 were mixed together, and the solution was concentrated to 59 µl using a SpeedVac vacuum centrifuge.

2.6.3 Oligoarrays – Hybridization, wash and scanning

The oligoarray type used in this work was the 21K human oligonucleotide array v2.3, produced by the NMC group at RR MC. These arrays are Corning GAPS2 glass slides spotted with oligonucleotides with a length of approximately 70 bp (70-mers) from the AROS Human oligo v2.0 set from OPERON. This gene set represents 21.521 human genes (UniGene

clusters). In addition to the gene spots, there are also several different types of validation spots, as well as some empty spots, giving a total of 23 232 spots per slide.

Prehybridization of the slide is a method for minimizing background, and must be optimized for the slide surface and printing method used in the production of the array. Reactive groups on the slide surface are inactivated to suppress background, unbound DNA from the printing process is washed away, and the remaining DNA on the slide surface is denatured. Large amounts of BSA are generally used to block any reactive groups on the slide surface.

There are several methods that can be used for hybridization of the slide, in this work both manual and automated hybridization were tried out, but only the results from the automated hybridization station were used.

In this work the prehybridization of the slides, hybridization, wash and scanning was performed by the NMC division at RR MC due to difficulties with the hybridization station at our lab. The labeled cDNA was delivered after being concentrated to a volume of 59 µl as described in section 2.6.2. The scanned images were received the following day, ready for data analysis.

2.6.4 Software analysis of microarray data with GenePix and BASE

Microarrays generate huge amounts of data, and in order to make these data comprehensible and useful specialized software is needed. Different programs may be used for this purpose; in this work GenePix v6.0 and BASE v1.2 were used.

First the scanned images must be linked to their respective GAL-files, files that give information on the identity of each spot on the slide. Then flawed spots must be flagged, whether the flaws are the results of wrong morphology, dust particles, salt residues or due to other reasons. This was all done in GenePix. The resulting data set was used to create a GPR file, a file that contains all the necessary information for further data analysis, which is the intensity, diameter, etc for each spot as well as the spot identity.

BASE (BioArray Software Environment) is a database wherein one can store not just the scanned images and resulting data from them, but also all other relevant information from the

microarray experiments performed, such as RNA isolation and labeling method used. The database allows for the upload of several slide images with their respective GPR files into a single experiment, so that the information from multiple hybridizations may be compared and analyzed together [159]. The processing of the microarray data in this work was done according to the recommendations from NMC, as described below, with Lowess normalization used to correct for systematic differences in Cy3 and Cy5 intensities [160].

Procedure

The following list represents the filtering options used in the BASE analysis of the microarray experiments performed in this work. The spot filters used ensure that the spots flagged in GenePix due to bad morphology or suchlike and the spots that were too small to give a reasonable measurement were removed from the data set. The reporter filters used removed any control spots and empty spots that should not form a part of the final data set, and the normalization method used in the final step was a pin-based Lowess normalization.

Measurement to use:

Mean foreground – Median background.

Spot filter:

[Raw] Flags ≥ 0

[Raw] Spot diameter ≥ 60

Reporter filter:

Gene name not in %YF%

Gene name not in %SP%

Gene name not in Neg-HS

Gene name not in Cy3-Cy5-landmark

Gene name not in empty

Gene name not in SC%

Gene name not in Ctrl%

Normalization:

Lowess

3. Results

As described in section 1.4, the main goal of this work was to generate a new model system for the analysis of c-Myb function, with a secondary goal of utilizing this new model system in preliminary microarray experiments for the determination of potential target genes of this hematopoietic transcription factor and its viral counterpart v-Myb^{AMV}. The work was divided into three sequential main parts, as follows:

- Construction and verification of plasmids for the Tet-systems (chapter 3.1).
- Generation and verification of double-stable cell lines with inducible expression of three different versions of c-Myb (chapter 3.2).
- Microarray analysis of the double-stable cell lines (chapter 3.3).

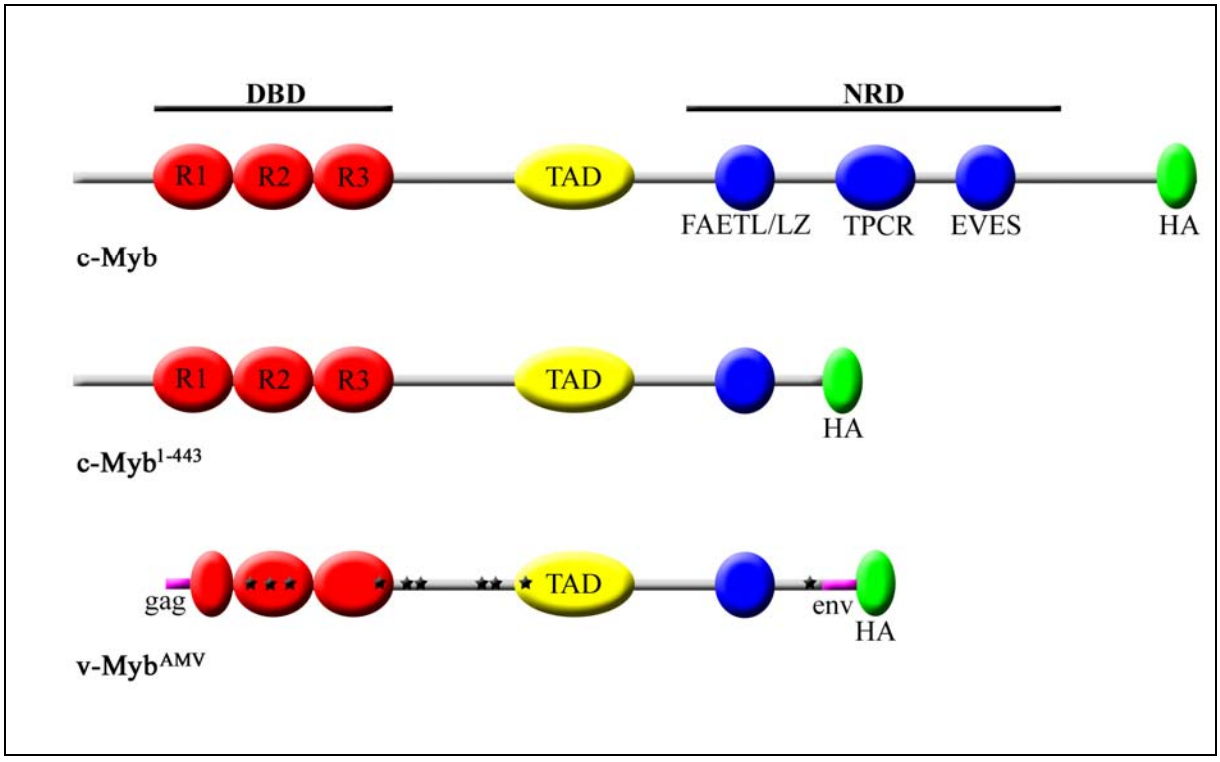


Figure 1 Overview of the three c-Myb proteins encoded by the pTRE2hyg constructs. Viral parts of the v-Myb^{AMV} protein (gag and env) are colored purple, viral mutations relative to the sequence of chicken c-Myb are indicated by black stars. DBD=DNA binding domain; TAD=Transactivation domain; NRD=Negative regulatory domain; R1, R2, R3=Myb repeat 1, 2, 3; FAETL/LZ=Leucine zipper region; TPCR=Threonine/proline rich domain or conserved region; EVES=Motif within the NRD; HA=HA-tag.

3.1 Construction and verification of plasmids for Dox-regulated expression of c-Myb

As described in section 2.3.1, the basis of the Tet-On and Tet-Off gene regulation systems are the integration of two plasmids into the chromosomal DNA of a cell, one plasmid expressing the Dox-regulated transactivator rtTA or tTA, respectively, and a response plasmid (pTRE2hyg-geneX) encoding the gene of interest under the control of a TRE, and generating a new cell line from this double-stable clone. The cell lines used in this work already had the regulator plasmids integrated in their genome, thus simplifying the process from a two-step to a one-step transformation and selection procedure. The first step in the process of generating our double-stable cell lines was to subclone the gene of interest into the MCS of the pTRE2hyg vector.

Rather than simply studying the effect of full-length c-Myb, we also wanted to explore the changes in gene expression following induced expression of two other versions of c-Myb: A truncated version lacking the NRD and v-Myb^{AMV}. Experiments performed with these two particular Myb proteins might contribute with information on the changes in gene regulation that occur during oncogenic activation of c-Myb. Therefore, three different pTRE2hyg constructs were made (figure 1):

- pTRE2hyg-AMV-HA, encoding the viral version of chicken c-Myb, v-Myb^{AMV}, with a C-terminal HA-tag.
- pTRE2hyg-hcM[1-443]-HA, encoding a C-terminally truncated version of human c-Myb (the N-terminal 443 amino acid residues) with a C-terminal HA-tag (c-Myb$^{1-443}$).
- pTRE2hyg-hcM-HA, encoding full-length human c-Myb with a C-terminal HA-tag.

Construction and verification of each plasmid are described below. All plasmid maps, sequences of the primers used as well as the amino acid sequence for human c-Myb can be found in the appendix.

pTRE2hyg-AMV-HA

The plasmid pCIneo-AMV-HA was digested with the restriction enzymes *Nhe*I and *Not*I, and the resulting fragment encoding v-Myb^{AMV} was then subcloned into the corresponding restriction enzyme sites of the pTRE2hyg-vector, as described in figure 2 A. The resulting plasmid pTRE2hyg-AMV-HA was verified by restriction enzyme analysis with *Nhe*I and *Not*I. The construction and verification of this plasmid was done by post.doc. Vilborg Matre.

pTRE2hyg-hcM-HA

The plasmid pCIneo-hcM-HA was digested with the restriction enzymes *Sal*I and *Nhe*I, and the fragment encoding c-Myb was then subcloned into the pTRE2hyg vector by digesting this vector with the same restriction enzymes before ligating the fragments, resulting in the 7.3 kb plasmid pTRE2hyg-hcM-HA, as shown in figure 2 B. The *Sal*I and *Nhe*I restriction enzyme recognition sites in pTRE2hyg are separated by only 25 bp, therefore the digestion of this vector with these restriction enzymes had to be performed in two steps. First the vector was digested with *Sal*I, and an aliquot of the mixture was taken for agarose gel analysis to ensure proper digestion of the vector. Then *Nhe*I was added, and the reaction was left overnight. The plasmid was verified by restriction enzyme analysis with *Sal*I+*Nhe*I, *Kpn*I, and *Xho*I.

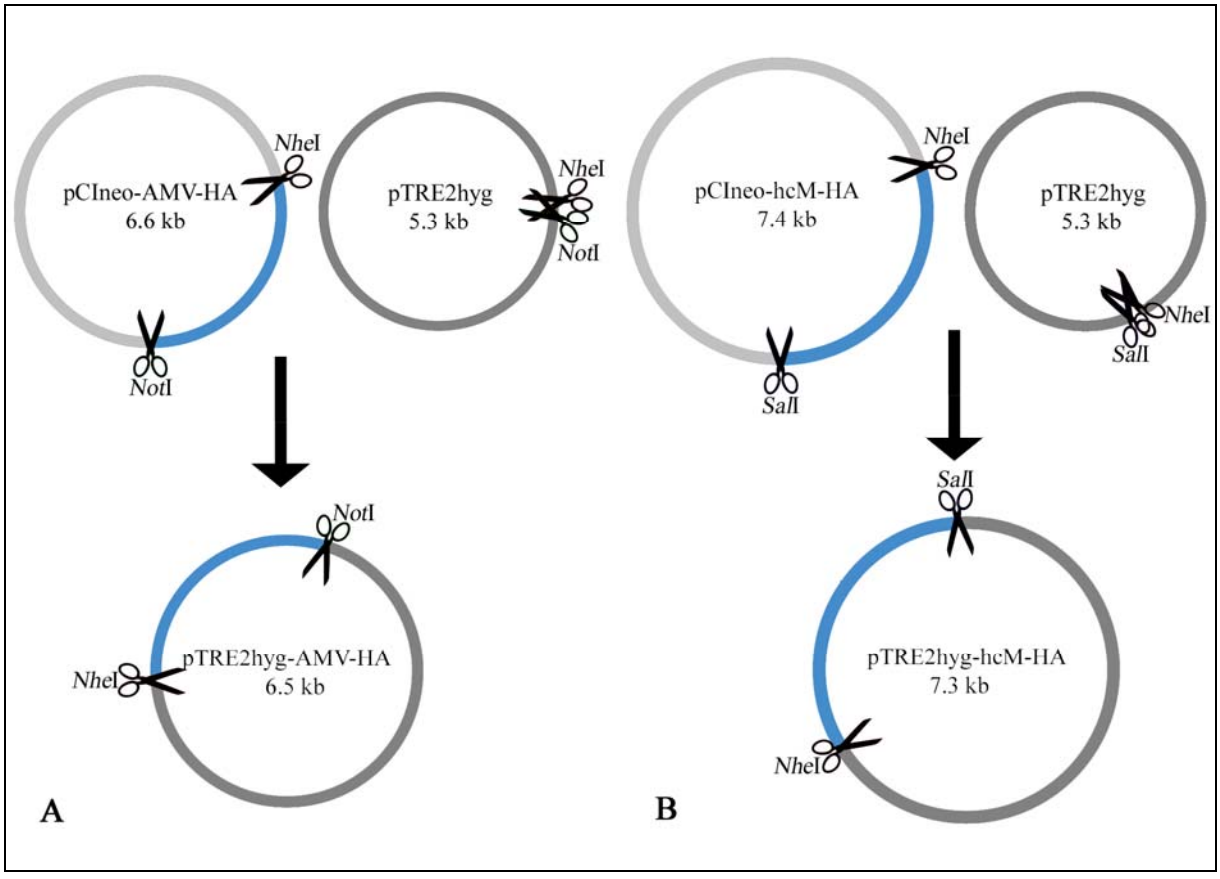


Figure 2 Construction of the plasmids pTRE2hyg-AMV-HA and pTRE2hyg-hcM-HA. **A)** Subcloning of the fragment encoding v-Myb^{AMV} from the pCIneo-AMV-HA plasmid into the pTRE2hyg vector. **B)** Subcloning of the fragment encoding c-Myb from the pCIneo-hcM-HA plasmid into the pTRE2hyg vector.

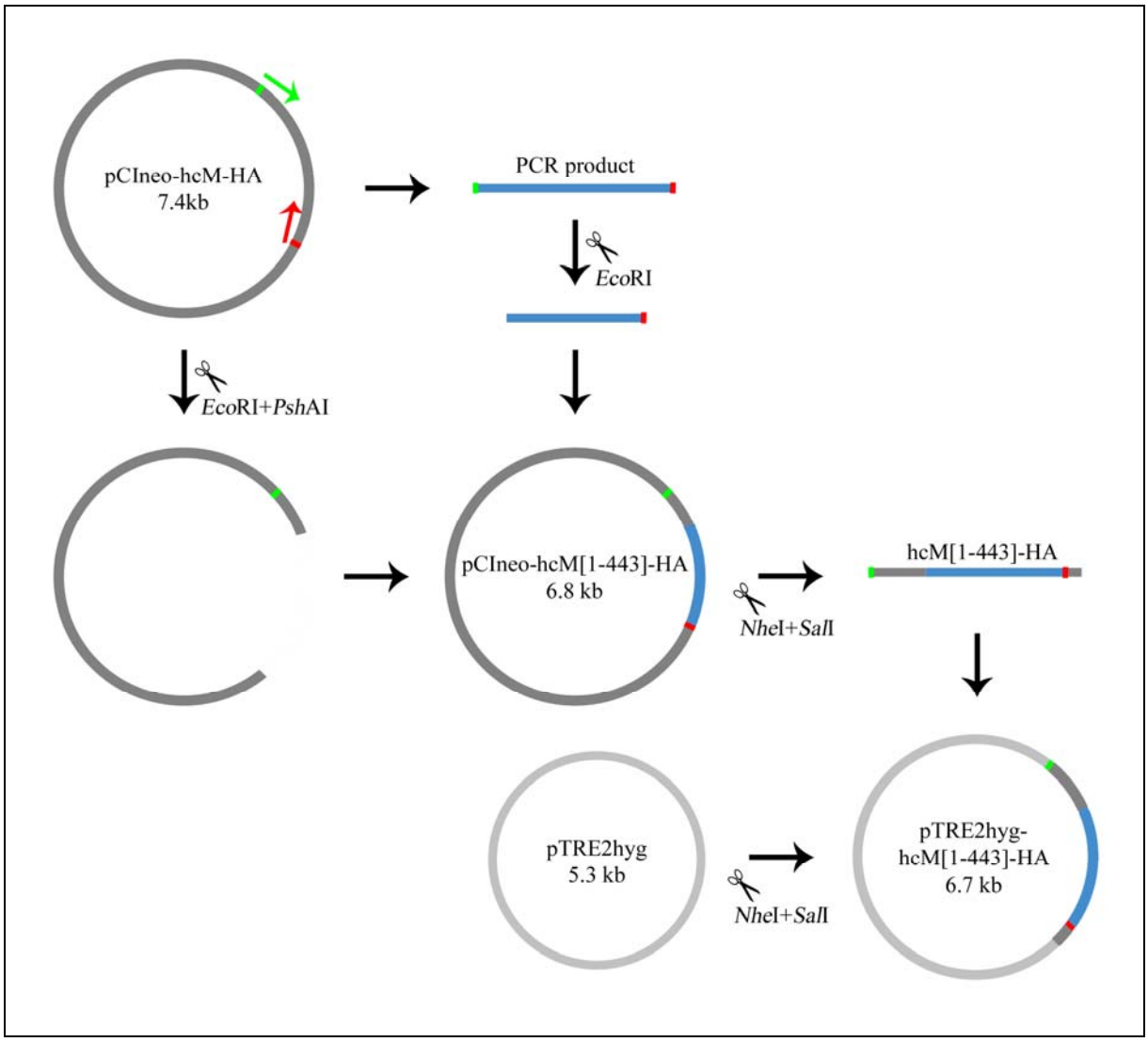


Figure 3 Construction of the plasmids pCIneo-hcM[1-443]-HA and pTRE2hyg-hcM[1-443]-HA. Green and red arrows represent the forward and reverse primers C158 and C177, respectively. Blue areas represent sequences from the PCR product, dark gray areas sequences from pCIneo-hcM-HA. The PCR product was digested with *Eco*RI and ligated into the corresponding sites of pCIneo-hcM-HA, resulting in a plasmid encoding a C-terminally truncated version of human c-Myb, c-Myb$^{1-443}$. The fragment encoding this transcription factor (hcM[1-443]-HA) was then subcloned into the pTRE2hyg vector, resulting in the plasmid pTRE2hyg-hcM[1-443]-HA.

pCIneo-hcM[1-443]-HA and pTRE2hyg-hcM[1-443]-HA

The plasmid pCIneo-hcM-HA encodes a full length version of human c-Myb with a C-terminal HA-tag. In order to create a truncated version of this gene, encoding only the first 443 amino acid residues of the protein with a C-terminal HA-tag, PCR was used, with pCIneo-hcM-HA as template (section 2.2.2). The forward primer (C158) was one complementary to the sequence just upstream of the MCS of the pCIneo vector, while the reverse primer (C177) was designed specifically to create a PCR product terminating 197 codons of the c-*myb* sequence that are lacking in AMV *v-myb*. By digestion of the pCIneo-hcM-HA plasmid with *Eco*RI+*Sal*I and replacement of the fragment with the *Eco*RI digested

PCR product, the reading frame would be maintained, yielding a plasmid encoding the N-terminal 443 amino acid residues of human c-Myb, plus three amino acid residues, an SGSG linker and a C-terminal HA-tag (figure 3). This plasmid, named pCIneo-hcM[1-443]-HA, was verified with restriction enzyme digestion with *Xho*I+*Sal*I and *Nhe*I+*Sal*I, then sent to be DNA sequenced to reveal any potential PCR-generated mutations.

While awaiting the results of the DNA sequencing of pCIneo-hcM[1-443]-HA, pTRE2hyg-hcM[1-443]-HA was constructed (figure 3). Both pTRE2hyg and pCIneo-hcM[1-443]-HA were digested with *Nhe*I+*Sal*I, and the pCIneo-hcM[1-443]-HA fragment encoding c-Myb$^{1\text{-}443}$ was subcloned into the pTRE2hyg vector as had been done in the construction of pTRE2hyg-hcM-HA from pCIneo-hcM-HA. The plasmid was verified with restriction enzyme digestion with *Eco*RI, then sent to DNA sequencing, as the results from the sequencing of pCIneo-hcM[1-443]-HA had not been sufficient.

A BLAST search following the sequencing of the plasmids revealed three deviations from the sequence of human *MYB* (numbering from ATG):

9: A/G: Silent mutation already known, caused by the use of a forward primer for chicken *c-myb* in the construction of the plasmid pCIneo-hcM-FLK, which had been used for the construction of pCIneo-hcM-HA.

39: C/G: Aspartate to glutamate. This mutation was not within the sequence that was replaced by the PCR product, and must therefore have originated from the template sequence. Some time after the work on this thesis was finished several other c-Myb-encoding plasmids used by the research group were sequenced, revealing that this mutation was indeed found in all of them. It may well be a polymorphism.

1039: A/T: Serine to cysteine. Highly likely a PCR generated mutation, and under normal circumstances this would have been reason to construct the plasmid anew. However, it was judged to be a conservative mutation in a region without any important functional domains, and considering the restricted time limits of this work it was decided to go ahead with the plasmids regardless of this mutation.

Summary

The three pTRE2hyg plasmids for the generation of stable cell lines with inducible expression of c-Myb had been constructed as according to plan, one for each of the proteins v-Myb^{AMV},

c-Myb$^{1-443}$ and full length human c-Myb. A PCR-generated mutation in pTRE2hyg-hcM[1-443]-HA had occurred, but was considered to be of minor importance.

3.2 Generation and verification of HEK293 cell lines with inducible expression of c-Myb

The cell line used for the generation of stable transfectants with inducible expression of c-Myb had to be one that did not have any endogenous expression of this transcription factor. The choice fell on the HEK293 cell line, a cell line derived from human embryonic kidney fibroblasts. The decision was made to try out both Tet-systems, as we had both HEK293 Tet-On and Tet-Off cell lines available in our lab. The Tet-Off cell line had been tried out previously, without much success, whilst the Tet-On cell line was a new investment. Comparing these two systems in this manner would also help to determine which one should be used for later projects in the group. In total, six different cell lines were to be generated: Tet-Off and Tet-On cell lines expressing the proteins v-Myb^{AMV}, c-Myb$^{1-443}$ and c-Myb, respectively. The generation and verification of these cell lines are described in the following chapter.

3.2.1 Kill-curves – Determination of selective concentration of hygromycin for the cell lines HEK293 Tet-On and Tet-Off

Transfected mammalian cells generally rid themselves of the plasmids in a few days time, but a small fraction of the cells integrate the plasmids into their own chromosomal DNA, they become stably transfected. Some plasmids harbor an antibiotic resistance marker gene to allow for the selection of these few stably tranfected cells. In the case of the pTRE2hyg plasmids, the antibiotic resistance gene encodes Hygromycin B phosphotransferase, a kinase that inactivates the antibiotic hygromycin. Growing transfected cells in the presence of hygromycin will therefore kill all the cells that are not harboring the pTRE2hyg construct, whereas the stably transfected cells will survive, and form separate colonies. Diverse cell lines have a variable level of tolerance for different antibiotics, so the ideal selective concentration of hygromycin had to be determined for the HEK293 Tet-On and Tet-Off cell lines.

The ideal selective concentration of the antibiotic is that which causes massive cell death within five days, and kills all the cells within fourteen days. In order to determine this ideal concentration, cells were seeded out in 6-well cell culture plates, and hygromycin was added to the medium to a final concentration of 0, 25, 50, 75, 100 or 150 µg/ml. The experiment was performed twice, both times the ideal concentration of hygromycin was found to be 100 µg/ml (results not shown). This concentration was therefore used in the following selection of stably transfected clones of the HEK293 Tet-On/Tet-Off cell lines.

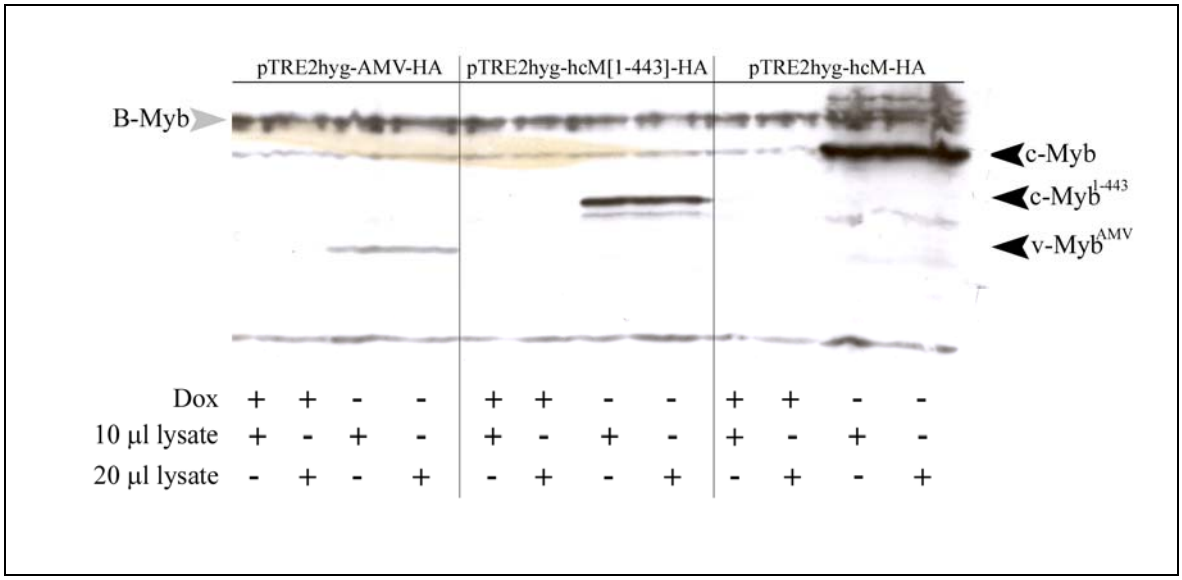


Figure 4 Transient transfection of HEK293 Tet-Off cells. Western blot shows lysates for cells with or without 16 hours induction following transient transfection of the cells with the plasmids pTRE2hyg-AMV-HA, pTRE2hyg-hcM[1-443]-HA and pTRE2hyg-hcM-HA.

3.2.2 Transfection of 293 Tet-On and Tet-Off cells and clonal selection

Prior to the generation of stable transfectants, a few tests were performed with transient transfections to ensure that every part of the system was working as expected. First, the transfection method had to be optimized for the cell line. HEK293 cells are easy to transfect, but bind very loosely to the growth surface, a trait that makes gentle handling and very few medium changes important for a successful transfection. The Lipofectamine reagent was used for these transfections, with a procedure in accordance with the recommendations of the supplier, as described in section 2.2.6 (35 mm wells). Western blots of the lysates of transfected Tet-Off cells with and without addition of Dox to the medium showed that the transfection had indeed worked satisfactorily and the plasmids expressed c-Myb proteins of the expected size after induction (figure 4), thus indicating that both the Tet-systems and the pTRE2hyg-constructs appeared to be in order. Real-time RT-PCR (section 2.5.4) of Tet-Off

cells transfected with pTRE2hyg-AMV-HA indicated a four-to-five fold increase in expression of v-Myb^{AMV} upon removal of Dox (results not shown), perhaps somewhat lower than expected, but according to the Tet system manual, transient transfections generally result in far lower expression levels than can be achieved with stable transfections, so it was not a great cause for concern. As the transfection method, plasmids and cell lines seemed to be all in order, the stable transfectants could be generated.

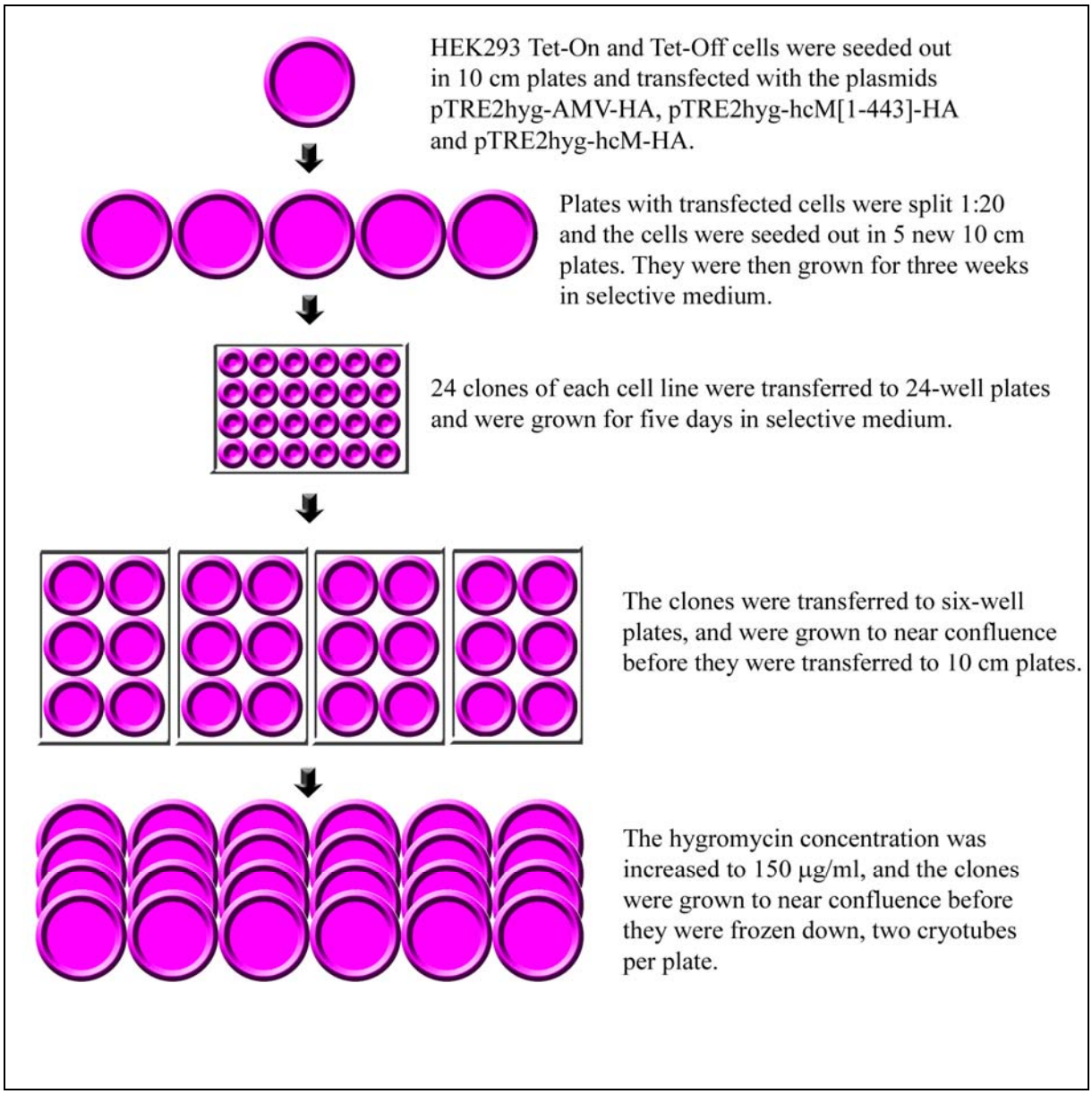


Figure 5 Overview of the selection process for the generation of stable transfectants.

Fresh cultures of Tet-On and Tet-Off cells were seeded out and transfected with the pTRE2hyg-constructs as described in section 2.3.4, then split, seeded out in new plates, and allowed to form separate colonies while growing in selective medium. These colonies were

then transferred to separate wells in a 24 well plate, as described in section 2.3.6 and summarized in figure 5. The number of clones originally picked and surviving each selection step are summarized in table 4 for each cell line/construct. The reason for the lower number of Tet-Off clones isolated and transferred to 24-well plates was simply that there were not that many healthy, separate clones available on the plates.

Cell line and construct	24 well plate	6 well plate	10 cm plate	Frozen down
Tet-On v-Myb^{AMV}	24	24	24	22
Tet-On c-Myb$^{1-443}$	24	24	24	23
Tet-On c-Myb	24	24	22	21
Tet-Off v-Myb^{AMV}	18	18	18	18
Tet-Off c-Myb$^{1-443}$	12	12	12	10
Tet-Off c-Myb	16	16	15	13
Total	118	118	115	107

Table 4 Tet-On and Tet-Off clonal selection. The table shows the number of clones that were isolated and survived each selection step from the first transfer of the clones to the 24 well plates until they were finally frozen down after they had grown to near confluence in the 10 cm plates.

The process took about two months from the time of transfection until the last clones were frozen down, with large clone to clone variations observed. While all clones were transferred from the 24- to the 6-well plates after five days, the time it took them to reach near confluence before transfer to 10 cm plates varied from four to 22 days. For the majority of the Tet-On clones, the time needed was four to seven days, with a few requiring 10-12 days. However, the Tet-Off clones generally needed 7-14 days growth, a few clones even a week more than that. The Tet-Off cells were also more prone to let go of the growth surface, tended to form big lumps of cells rather than grow in the typical monolayer expected of HEK293 cells, and were generally smaller than the Tet-On cells. Therefore, the number of cells needed for a confluent layer was generally greater for the Tet-Off than the Tet-On cells, perhaps explaining some of the added growth time needed.

As a total, 107 clones were frozen down, two cryotubes per clone. Unfortunately, there was not enough free space in the N_2 tank for all these cells, so they were stored at -80 °C. For a short period of storage, this does not pose a great problem, but the storage period for these cells became much longer than originally expected.

With the clones selected and isolated, they were ready to be tested for expression of the genes encoding c-Myb, c-Myb$^{1-443}$ or v-Myb^{AMV} now integrated in their chromosomal DNA under the control of a TRE.

3.2.3 Expression of c-Myb in isolated clones

Depending on the integration site of the plasmid, stably transfected clones display very different levels of gene expression upon Dox-regulated induction. Clones with the plasmid integrated in areas of the genome with low transcriptional activity will not show proper induction of the gene encoded by the plasmid, whereas clones with the plasmid integrated in areas of high transcriptional activity (for example close to enhancers) might have such high expression that the regulator protein will not be able to quench it in the un-induced state, thus making the promoter leak. The ideal clone will be one that exhibits high expression of the integrated gene in the induced state, but no expression in the un-induced state. To select the clones with these traits Western blots were used to analyze the induction of the c-Myb genes in the isolated clones. Several other methods could have been used for this purpose, including northern blots and real-time RT PCR that display the level of induction at the mRNA level. Western was chosen mainly because this method shows the protein level of induction, which is more relevant in most cases, in addition to the possibility of revealing any erroneous protein products due to truncations or suchlike.

The different clones were thawed and resuspended in growth medium in batches of six, with the total number of cell cultures in use at one time kept at a maximum of eighteen, usually twelve. Upon the first passage, new aliquots were frozen down, two cryotubes per clone. In total, 54 Tet-On clones were tested, 18 of each of the v-Myb^{AMV}, c-Myb$^{1-443}$ and c-Myb clones, as well as 12 Tet-Off clones, six of v-Myb^{AMV}, six of c-Myb$^{1-443}$. For the Western blots, 7.5x10^5 cells were seeded out in six-well plates, two wells per clone, one well with 1 µg/ml Dox, one well without added Dox. After 16 hours the cells were harvested as described in section 2.4.1, the protein concentration of the lysates determined by the MicroBCA method (section 2.4.2), and a volume of cell lysate corresponding to 30 µg of protein was used for SDS PAGE (section 2.4.3). A standard gel had 13 wells, allowing the analysis of six clones, as one well was used for the protein standard, leaving 12 wells for cell lysates. The Western blots were performed as described in section 2.4.4, with the primary antibody 5e11 at a dilution of 1:200 and the secondary antibody HRP conjugated α-mouse IgG at 1:5000. Figure 6 shows two typical blots of the Tet-On clones with expression of c-Myb$^{1-443}$ and c-Myb, with clones showing unwanted leaking, low expression or truncated protein products, in addition to clones that had the desired qualities. B-Myb is also visible in these blots, as the 5e11 antibody

used is specific for the DBD region of all three Myb-proteins c-Myb, A-Myb and B-Myb [161].

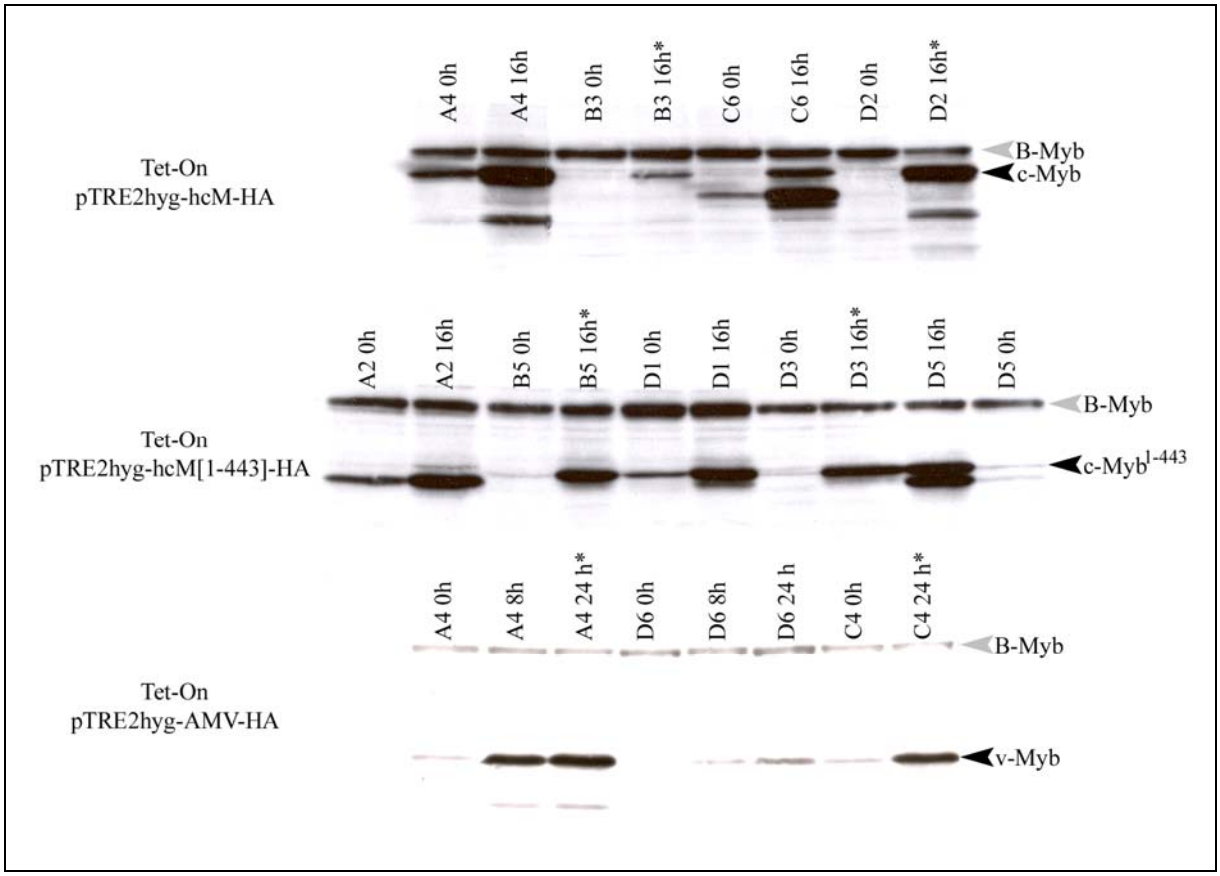


Figure 6 Western blot of selected Tet-On clones. Tet-On c-Myb-A4 and 443-A2 show a high level of background expression, c-Myb-B3 and v-Myb^{AMV}-D6 are examples of clones with low induction levels, c-Myb-C6 and c-Myb$^{1.443}$-D5 are examples of clones that express proteins of the wrong size (double bands, the upper band is c-Myb$^{1-443}$). The clones c-Myb-D2, c-Myb$^{1-443}$-B5, c-Myb$^{1-443}$-D3, v-Myb^{AMV}-A4 and v-Myb^{AMV}-C4 were the ones with the desired qualities of low background and high expression upon induction (marked with *).

Of the 54 Tet-On clones tested two had failed to survive the freezing, 22 lacked expression, 9 had protein products of the wrong size (mainly truncations), and 13 had high background expression (leaking promoters). The remaining eight clones were considered to show sufficient qualities as to be used for further experiments, based on the criteria of low expression of the inserted genes in the uninduced state, and high expression after 16 hours induction. One clone was prioritized for each construct, whilst the other potential clones were kept as back-up should there be problems occurring with the first clone, or if it was decided to compare different clones in later experiments. The selected clones were as follows (figure 6):

- v-Myb^{AMV}-A4: Displayed the desired qualities of high expression upon induction and minimal signs of leakage. v-Myb^{AMV}-D6 had a somewhat low level of expression and

v-Myb^{AMV}-C4 revealed some signs of leakage, but both were kept as potential alternatives.

- c-Myb$^{1-443}$-D3: As v-Myb^{AMV}-A4, a very good candidate. c-Myb$^{1-443}$-B5 was very nearly equally qualified, but c-Myb$^{1-443}$-D3 was prioritized because it had used a shorter time on the selection process.
- c-Myb-D2: Appeared to be the best of the c-Myb clones. Two other clones were kept as alternatives; c-Myb-B3, that exhibited rather lower expression upon induction than that observed with c-Myb-D2, and c-Myb-B1 (not shown), a clone that showed good expression upon induction, but also had some degree of leakage in the uninduced state.

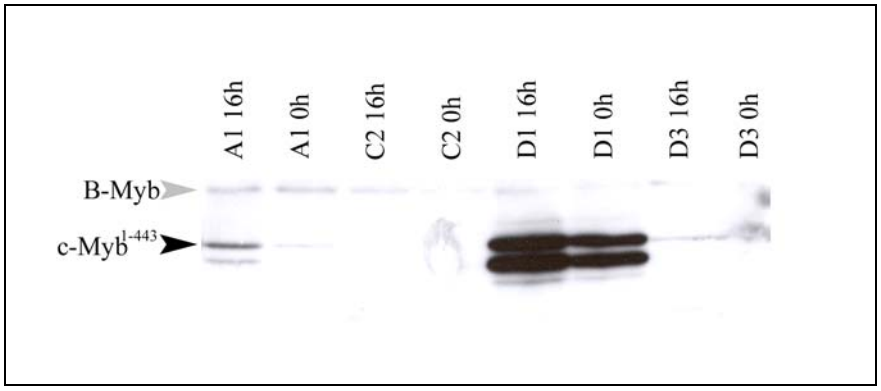


Figure 7 Western blot of selected c-Myb$^{1-443}$ Tet-Off clones. c-Myb$^{1-443}$-D1 shows a very high background expression, the other clones very low induction levels.

Of the Tet-Off clones, only 12 were tested, the reason being the rather poor results observed for these 12. Five of the clones had not even survived the freezing process, leaving only seven remaining clones to be analyzed by Western. Three of these seven clones revealed complete lack of expression of c-Myb, the remaining four clones had such high levels of leakage that there was hardly any change to be observed between the samples with or without induction (figure 7). Remnants of Dox in the medium might have helped explain some of these results, but this should have been avoided by the precautions that had been taken. The cells were given an additional medium change four hours after the first to remove any Dox that could have been attached to the extracellular matrix of the cells [162], and the FBS used for all these experiments was of the Tet system approved version, guaranteed to be free of any tetracycline derivatives. These results for the tested Tet-Off clones, in combination with the high mortality and odd morphology observed for this cell line alongside the more cumbersome form of induction compared to that of the Tet-On system made us decide to put aside the Tet-Off clones, and proceed only with the selected Tet-On clones, where we had found potential

clones for all three c-Myb constructs, providing cell lines with inducible expression of v-Myb^{AMV}, c-Myb$^{1-443}$ and c-Myb.

3.2.4 Characterization of the established cell lines; Induction curves

The Western blots with the 16 hour induction used in the clonal selection described in the previous chapter clearly indicated that this induction time was sufficient for generating rather high expression levels of the integrated *c-myb* genes. However, it would be valuable for future experiments to analyze how the induction level of the cells changes over time, so as to determine proper induction times needed for each experiment. For instance in the case of microarray experiments, it would be preferable with as short an induction time as possible, so as to determine primary rather than secondary target genes, yet the expression level would have to be sufficiently high so as to activate these target genes. An ideal induction time would then be one that was long enough to give a sufficiently high expression level of c-Myb to activate primary target genes, but still short enough to avoid activation of secondary target genes.

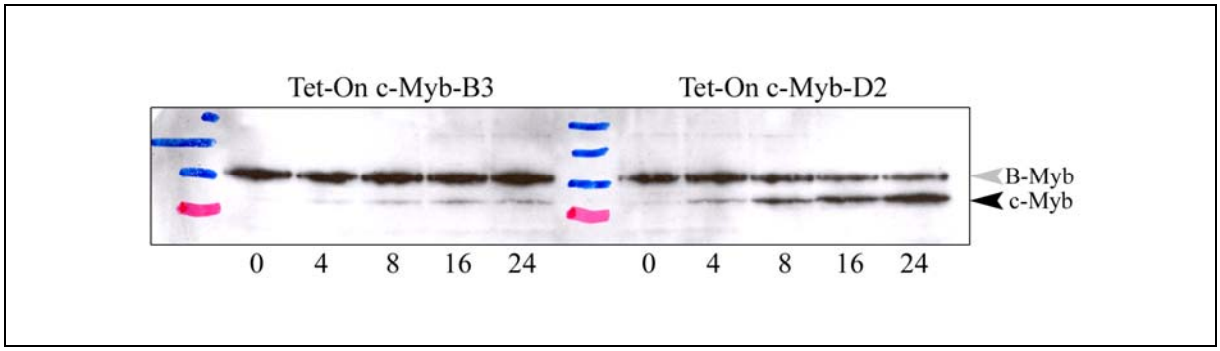


Figure 8 Western blot of lysates from the Tet-On clones c-Myb-B3 and Tet-On c-Myb-D2 with 0, 4, 8, 16 and 24 hours induction.

To determine how the level of c-Myb in the cells was altered as a result of the time passed after addition of Dox to the growth medium, Western blots were run on lysates from clones with 0, 4, 8, 16 and 24 hours induction. Results for two of the selected Tet-On c-Myb clones, c-Myb-B3 and c-Myb-D2, are shown in figure 8. This blot clearly illustrates the difference in expression levels between the two clones. The expression level in c-Myb-B3 was so low that it was a serious cause for concern regarding the usefulness of this clone, thereby confirming our choice of c-Myb-D2 as our primary c-Myb candidate for further experiments. c-Myb-D2

displays increasing protein levels of c-Myb at all time points, whereas c-Myb-B3 does not show any significant increase between the 16 and 24 hour time points.

Based on these blots, it was determined that a four hour induction probably would be insufficient for such purposes as microarray analysis, as the level of c-Myb expression would be too low at this point, whereas the eight hour induction, at least for the stronger clones such as c-Myb-D2, did appear to produce enough c-Myb protein to potentially activate target genes. The shortest induction time to be used for those experiments was therefore determined to be eight hours.

3.2.5 c-Myb activity in selected clones

While the Western blots described in the previous sections determined that the clones did indeed express c-Myb proteins of the expected size upon induction, they did not reveal anything about the activity of these transcription factors. However, luciferase assays with a c-Myb-responsive promoter construct could be used for this purpose (section 2.4.6). Should the clones following induction of c-Myb expression give an increase in luciferase activity, it would indicate that the clones expressed active c-Myb proteins that would also be able to regulate the expression of endogenous target genes. Should they be unable to activate luciferase activity, the reason for this had to be determined to assess whether the problem was caused by the clone in question, the cell line or the pTRE2hyg plasmid used for the generation of the clones.

There are several different luciferase reporter constructs that can be used for determining the activity of c-Myb proteins, based on both synthetic c-Myb-responsive promoters and promoters of known c-Myb target genes. A reporter construct from the first category was first tried out, one based on the promoter of the c-Myb target gene *TRHR* [125]. In previous experiments performed in our lab using other cell lines than HEK293 (mainly CV-1), this reporter construct had given very good results in luciferase assays, but that was not the case with the Tet-On clones. The luciferase activity remained virtually unaltered upon induction of c-Myb expression for the v-Myb^{AMV}-A4, c-Myb$^{1-443}$-D3 and c-Myb-D2 clones, keeping at the same level as that observed for the Tet-On parental cell line. The fact that all three clones had failed to increase luciferase activity following addition of Dox indicated that the separate clones were unlikely to be at fault, or the pTRE2hyg constructs. To rule out the possibility

that the induction had failed the lysates from the luciferase assay were analyzed by Western blotting. The blot showed induction levels quite similar to that normally observed for the clones in previous experiments, indicating that this was probably not the cause for the lack of luciferase activity (results not shown).

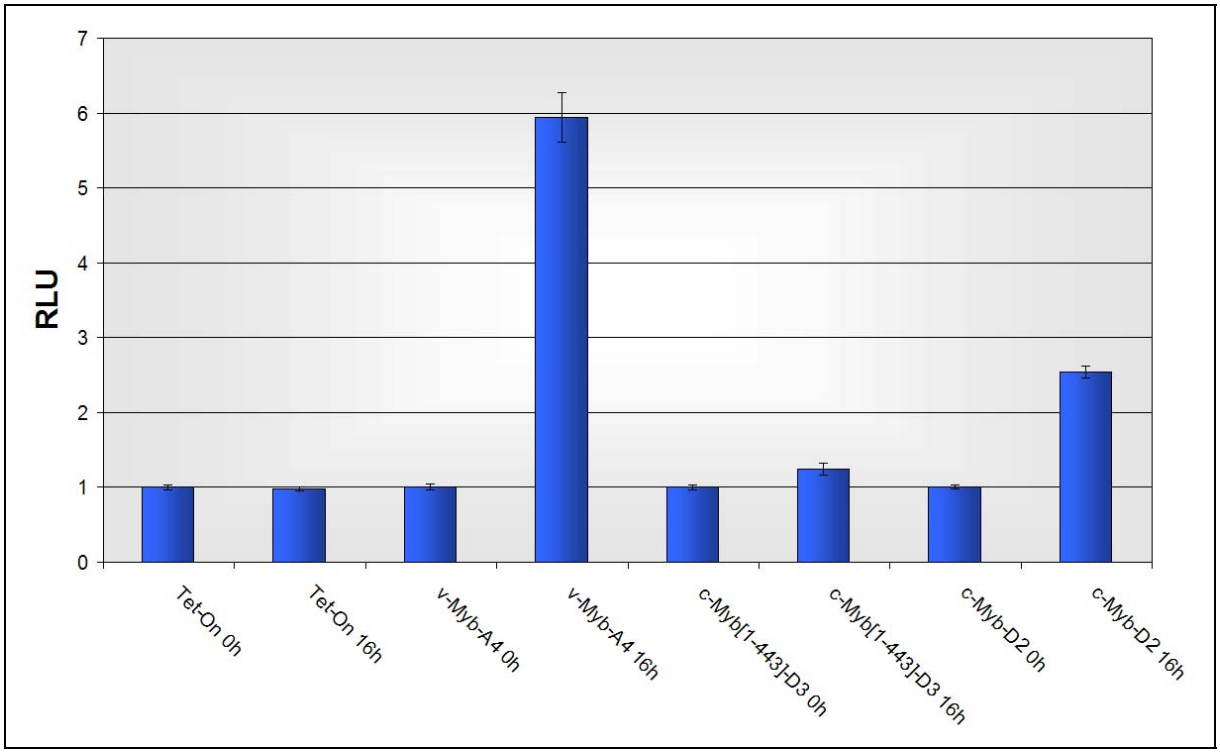


Figure 9 Luciferase assay for the determination of Myb activity in selected Tet-On clones. The cells were transfected with 1 µg 3xGG reporter plasmid, three parallels of each sample. The average luciferase readouts for the uninduced cells were set to 1 for each clone/cell line. RLU = relative luciferase units.

c-Myb is not a very strong activator, and requires the assistance of cofactors for the activation of several of its target genes. It might be that the reason for the failure to induce expression of luciferase when using the TRHR reporter construct in the HEK293 Tet-On cells was simply that these cells did not express the cofactors necessary to activate this reporter. It was therefore decided to test a different reporter construct: pGL2tk-3xGG. The 3xGG reporter is a synthetic Myb responsive promoter, with three repeats of a MRE [163]. With this reporter construct as a replacement for the TRHR reporter construct, the luciferase assays were repeated for the Tet-On clones v-Myb^{AMV}-A4, c-Myb$^{1-443}$-D3 and c-Myb-D2, in addition to parental HEK293 cells, three parallels per sample. This experiment provided results that were more consistent with the expected values, with a six-fold increase in luciferase activity for v-Myb^{AMV}-A4 and a 2.5 fold increase for c-Myb-D2 (figure 9). However, the results for c-Myb$^{1-443}$-D3 were far from what was expected, which would have been an increase in

luciferase activity close to that observed for v-Myb^{AMV}-A4. There was hardly any increase at all in luciferase activity upon addition of Dox to the c-Myb$^{1-443}$-D3 clone. To rule out the possibility that lack of induction was to blame for this, the lysates were analyzed by Western blotting. The blot showed no abnormalities in induction level compared to previous blots (results not shown), so there had to be a different explanation for the luciferase assay results.

Generation of stable transfectants always bring along the possibility of clonal effects, depending on the insertion site of the plasmid. Should that be the cause of the unexpected luciferase assay results for c-Myb$^{1-443}$-D3, then another c-Myb$^{1-443}$ clone or a colony of transiently transfected parental Tet-On cells would still give results as expected in a luciferase assay. Should the fault rather lie with the pTRE2hyg-hcM[1-443]-HA construct, then even transiently transfected cells would behave as c-Myb$^{1-443}$-D3. Therefore HEK293 Tet-On cells were transiently transfected with the pTRE2hyg-hcM[1-443]-HA and the luciferase assay repeated as described in section 2.4.6, three parallels per sample, and 16 hours induction. The results did not differ markedly from those observed for c-Myb$^{1-443}$-D3: There appeared to be no difference in luciferase activity between induced and uninduced cells. This would indicate that there would be no use in replacing the c-Myb$^{1-443}$-D3 clone with another c-Myb$^{1-443}$ clone, as the problem would be expected to be the same for all these clones. One might think that the PCR mutation in the pTRE2hyg-hcM[1-443]-HA could be to blame, but this problem had not occurred for anyone in the lab using the pCIneo-hcM[1-443]-HA construct, which includes the same mutation. Due to time constraints, the matter was left unresolved. It was simply decided to put aside the c-Myb$^{1-443}$ clones, and go ahead with only the v-Myb^{AMV} and c-Myb clones.

In the light of these results, it was determined that the v-Myb^{AMV}-A4 and c-Myb-D2 clones were qualified for use in further experiments, as they both appeared to express active c-Myb proteins, whereas all plans for experiments with the c-Myb$^{1-443}$ clones were put aside due to the lack of c-Myb activity in these cells.

3.3 Microarray analysis of cell lines with induced expression of c-Myb and v-Myb^{AMV}

When the work on this thesis was begun in 2002, the microarray technology was still a rather new method, with all the difficulties that usually entails. When the scanner and hybridization station that were to be used for the work on this thesis arrived at our lab the autumn of 2002, we were under the assumption that microarray analysis was a simple, sturdy method, well documented and should have been safe sailing when it came to generating consistent, good data sets. It proved to be everything but.

In the course of the time from the beginning of 2003 until late January 2005 several different microarray methods were tried and tested: cDNA arrays vs. oligoarrays, direct vs. indirect labeling, manual hybridizations vs. automated hybridization stations. As the technology was so new, and changed so fast, the recommendations from the NMC and others on how to perform microarray experiments kept changing rapidly. While we were still in the process of optimizing a procedure, a new procedure that was assumed to be superior to the old one became available, and so we altered our method once again rather than try to make the old one work. In general, there were not performed a sufficient number of hybridizations on any of the procedures that we chose to abandon to present any results here as the statistical data are too poor. Instead, only the final microarray results for the Affymetrix and oligoarray analyses are presented in this chapter, whilst our and others general experiences with the different microarray techniques are discussed in section 4.3.

This chapter describes the RNA isolations and following microarray results for the two stably transfected Tet-On clones v-Myb^{AMV}-A4 and c-Myb-D2 that were isolated and tested for both expression and activity of their respective v-Myb^{AMV} and c-Myb proteins in the previous sections.

3.3.1 RNA isolation from c-Myb-D2 and v-Myb^{AMV}-A4

Microarray analyses require rather large pools of RNA of very high quality. Though 10 µg of total RNA is sufficient for a single labeling reaction with the FairPlay indirect labeling method (section 2.6.2), two labeling reactions for a single sample are required for each slide to be hybridized, and with the need for dye-swap replicates, the minimum amount of RNA

necessary from a single pool of RNA isolations for a sample is 40 µg. Add to the equation that it is not unusual for reactions to fail, hybridizations to go wrong and that one should always perform several technical replicates in order to strengthen the statistic material, then it becomes apparent that the amount of RNA isolated from a single sample/pool of cells should preferably be at least 160 µg. In addition the samples need to be completely free of any contaminants such as chromosomal DNA and proteins, and it is also crucial that no RNA degradation is allowed to take place.

Cells were seeded out in 15 cm plates at a density so as to be 50-80 % confluent upon harvesting, four plates for each clone, allowing for two plates without induction, one plate with 8 hours induction, and one plate with 24 hours induction. RNA was isolated from the cells with the Trizol method (section 2.5.1), then subjected to DNase treatment and purification (section 2.5.2). The Bioanalyzer instrument was used to control the RNA quality of the samples, to ensure that no degradation had taken place (section 2.5.3). Spectrophotometric analysis of the samples was also done to measure both concentration and quality of the RNA (section 2.2.7). A typical yield of RNA from a 15 cm plate of cells was 200-300 µg from the Trizol isolation, with a final yield of 150-200 µg after all purification and quantification steps were done.

For the earlier RNA isolations used for the Affymetrix microarray (section 3.3.2), the induction level of c-Myb had to be determined from the RNA sample by the use of real-time RT-PCR. For all the following RNA isolations, as a precaution, cells were seeded out in six well plates and induced in parallel with the cells for the RNA isolations, so that the induction level could be assessed with the use of Western blotting. One could also have isolated protein from the Trizol lysates, but this isolation method is somewhat more laborious, and in order to properly compare the results with those of previous blots, it was preferable to keep to the exact same procedure as before. In general, the results for these blots were in keeping with those observed for previous blots of v-Myb^{AMV}-A4 and c-Myb-D2, indicating that the cell lines had not lost expression of the integrated c-Myb genes.

Thus, with the RNA quality, quantity and level of induction of the integrated c-Myb genes all found to be satisfactory, the isolated RNA samples were ready to be used for microarray analysis.

3.3.2 Affymetrix microarray analysis of c-Myb-D2

Based on the experience from other microarray projects and our own first attempts at using this method, we realized that a full in-house microarray analysis would go far beyond the time limit of this thesis. It was therefore decided to try out the Affymetrix microarray system for a sample of c-Myb-D2 with 24 hour induction versus a sample without induction.

The Human Genome Focus Array used for this experiment represents over 8 500 human sequences from the NCBI RefSeq database, with every sequence represented by eleven features (probes) on each array. Based on the number of features observed for each gene/sequence in the scan following the hybridization with labeled mRNA, each gene was determined to be Present or Absent, which indicates whether it was expressed at a sufficient level for observation or not. Of the 232 genes found to be Present in both samples and increased, decreased, marginally increased or marginally decreased according to Affymetrix statistics, only one was found to be up-regulated above the threshold of two-fold change, and it was expressed at a rather low level. The gene in question was that for the lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1), a receptor found mainly on epithelial cells and assumed to be involved in atherosclerosis (reviewed in [164]). It has never been coupled to c-Myb in any way, and the results from this one microarray experiment were not convincing enough to start doing so, as it was found to be up-regulated just two-fold. The low level of expression also makes the readings somewhat more uncertain, indicating that the induction of c-Myb expression in these cells had not had any statistically significant effect on the total gene expression pattern in the HEK293 cells in this experiment.

The reason for the lack of statistically significant change in gene expression in the HEK293 cells upon induced expression of c-Myb was first assumed to be that the cells had lost their responsiveness to Dox, and no longer expressed c-Myb. According to the user manual for the Tet-system, loss of regulation can also be a result of switching off or methylation of the viral promoter. To assess whether the cells had indeed ceased to express c-Myb upon addition of Dox to the medium, the induction level of c-Myb in the RNA samples used for the Affymetrix microarray analysis was determined with the use of real-time RT-PCR. According to those results, the expression of c-Myb was about four-fold up-regulated after 24 hours induction (results not shown). This was about the same fold induction observed for the HEK293 Tet-Off cells transiently transfected with pTRE2hyg-AMV-HA with only 16 hours induction (figure

4). According to the supplier stably transfected cells should show significantly higher fold induction than transiently transfected cells, so to be on the safe side the passage of the c-Myb-D2 clone used for this experiment was discarded, and one of the earliest passages (P2) was taken into use for the further experiments. As an extra precaution, as mentioned in section 3.3.1, Western blots of cells seeded out in parallel with the cells for the RNA isolations were also performed to ensure that the clones did not show loss of regulation.

The Affymetrix microarray analysis was not considered a success, as the results were almost equivalent to no results at all. This was taken as an indication that the level of expression of c-Myb was too low in the passage of the c-Myb-D2 clone used to induce any significant change in the total gene expression of the HEK293 cells. An earlier passage of the c-Myb-D2 clone was therefore taken into use for further RNA isolations and following microarray experiments, and precautions were taken to ensure that no RNA isolated from clones with loss of regulation was used for microarray experiments. The Affymetrix microarray analysis was never repeated, mainly due to cost considerations in combination with the weak results.

3.3.3 Oligoarray microarray analysis of v-Myb^{AMV}-A4 and c-Myb-D2

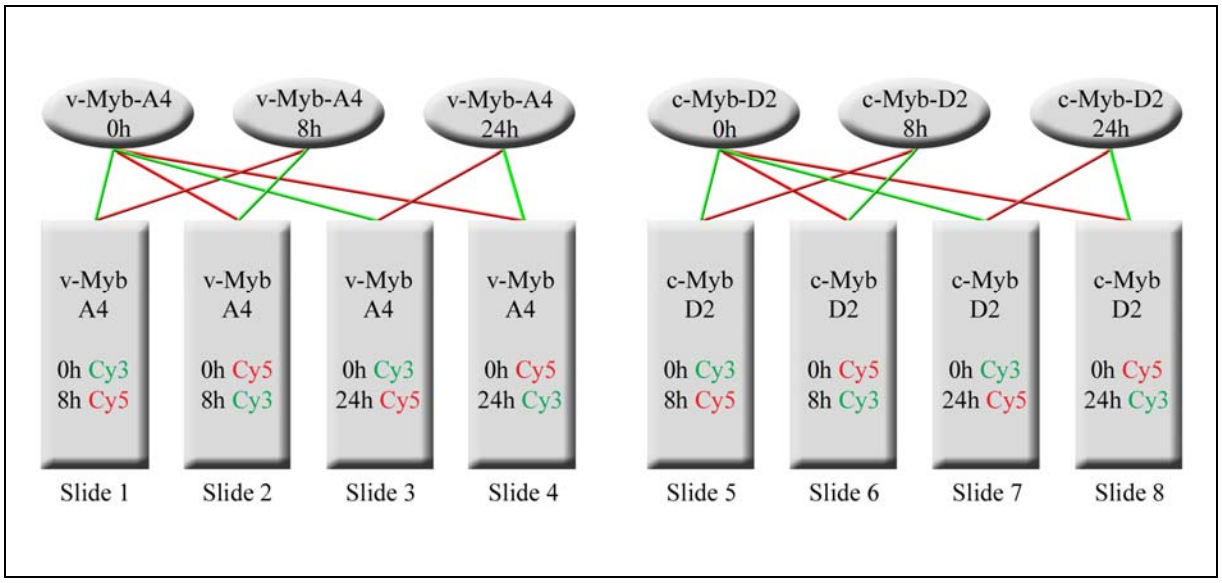


Figure 10 Overview of the oligoarray slides hybridized. The ovals symbolize the RNA samples with their respective induction times, the colored lines the dye used for labeling (red: Cy5, green: Cy3).

RNA samples from v-Myb^{AMV}-A4 and c-Myb-D2 with 0, 8 and 24 hours induction times were used for the oligoarray microarray analysis. The samples were labeled with Cy3 or Cy5, and were then hybridized to the slides as described in section 2.6.2-2.6.3. Figure 10 gives an

overview of the hybridizations performed, with a total of eight slides used. The scanned images were first analyzed in GenePix, where a GPR file containing the identity and analysis data for each spot was generated and several spots were flagged due to salt streaks, dust particles and similar factors that would otherwise cause problems for the data analysis. The GPR file was then transferred to BASE, where the data analysis was performed as described in section 2.6.4. Table 5 gives an overview of the number of spots and reporters remaining after each filtering and normalization step.

BASE filtering	**Tet-On v-Myb^{AMV}-A4**		**Tet-On c-Myb-D2**	
	Spots	**Reporters**	**Spots**	**Reporters**
Original data set	92928	21463	92928	21463
Spot and reporter filtering	39900	16111	46720	15882
Lowess normalization	37384	15105	40387	14216

Table 5 Overview of the number of spots and reporters remaining after each filtering and normalization step performed in BASE for the microarray analysis of the Tet-On cell lines v-Myb^{AMV}-A4 and c-Myb-D2. The numbers correspond to all four slides hybridized for each cell line.

The resulting MA-plots (ratio plotted against intensity of the spots, in log-values) for each dye-swap pair of hybridizations are shown in figure 11 and 12. Genes that were found to be up-regulated at least twofold ($M \geq 1$) in both parallel assays (dye-swap hybridizations) are marked as red dots in the MA plots, genes found to be down-regulated at least two-fold ($M \leq -1$) in both assays are marked as green dots. The corresponding data for differentially expressed genes for the assay sets of v-Myb^{AMV}-A4 are found in tables 6-9, for c-Myb-D2 the results are listed in table 10.

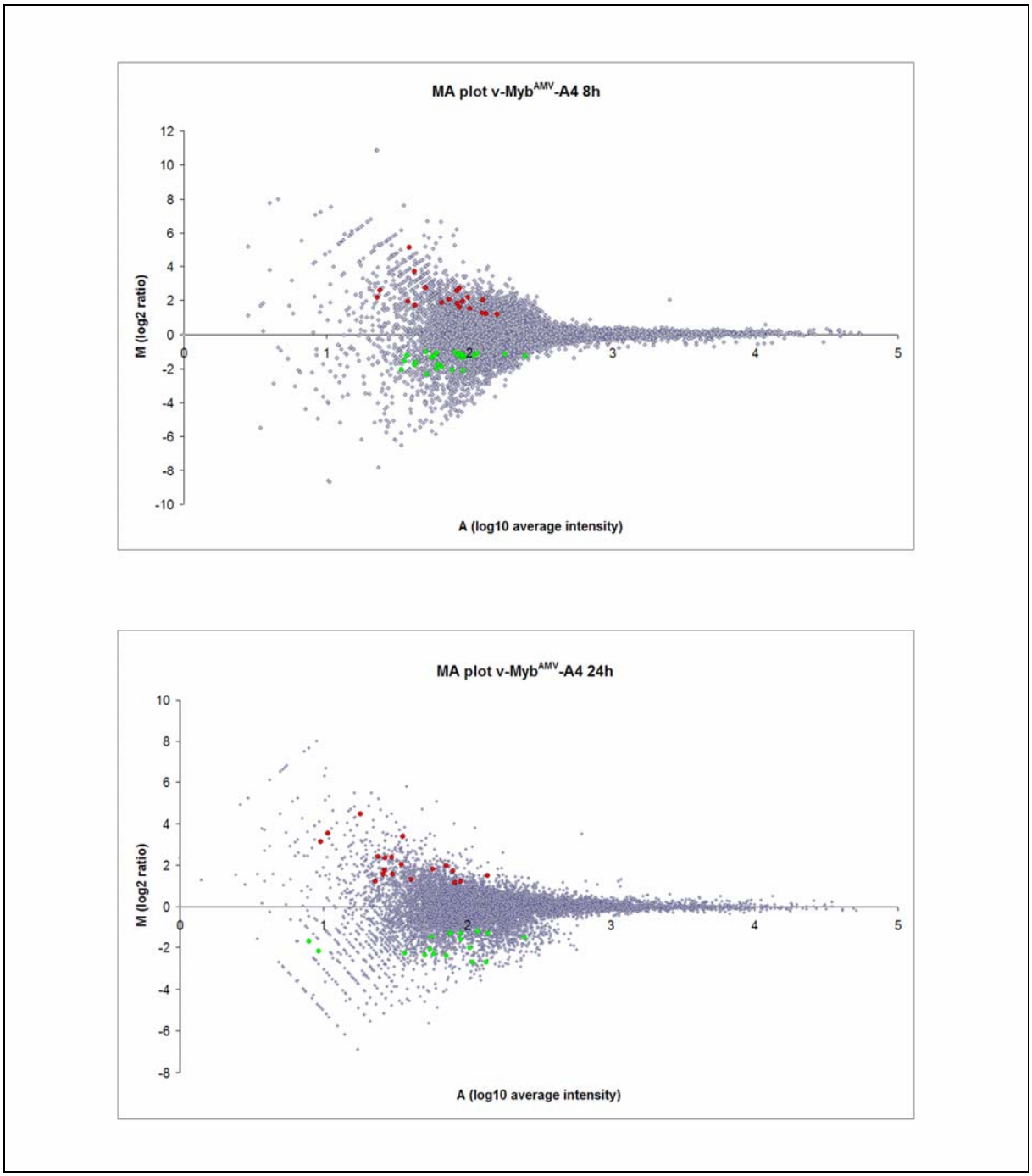


Figure 11 MA plots for v-Myb^{AMV}-A4. Genes found to be up-regulated at least twofold in both arrays ($M \geq 1$) are marked as red dots, genes down-regulated at least twofold in both arrays ($M \leq -1$) in both arrays are marked as green dots.

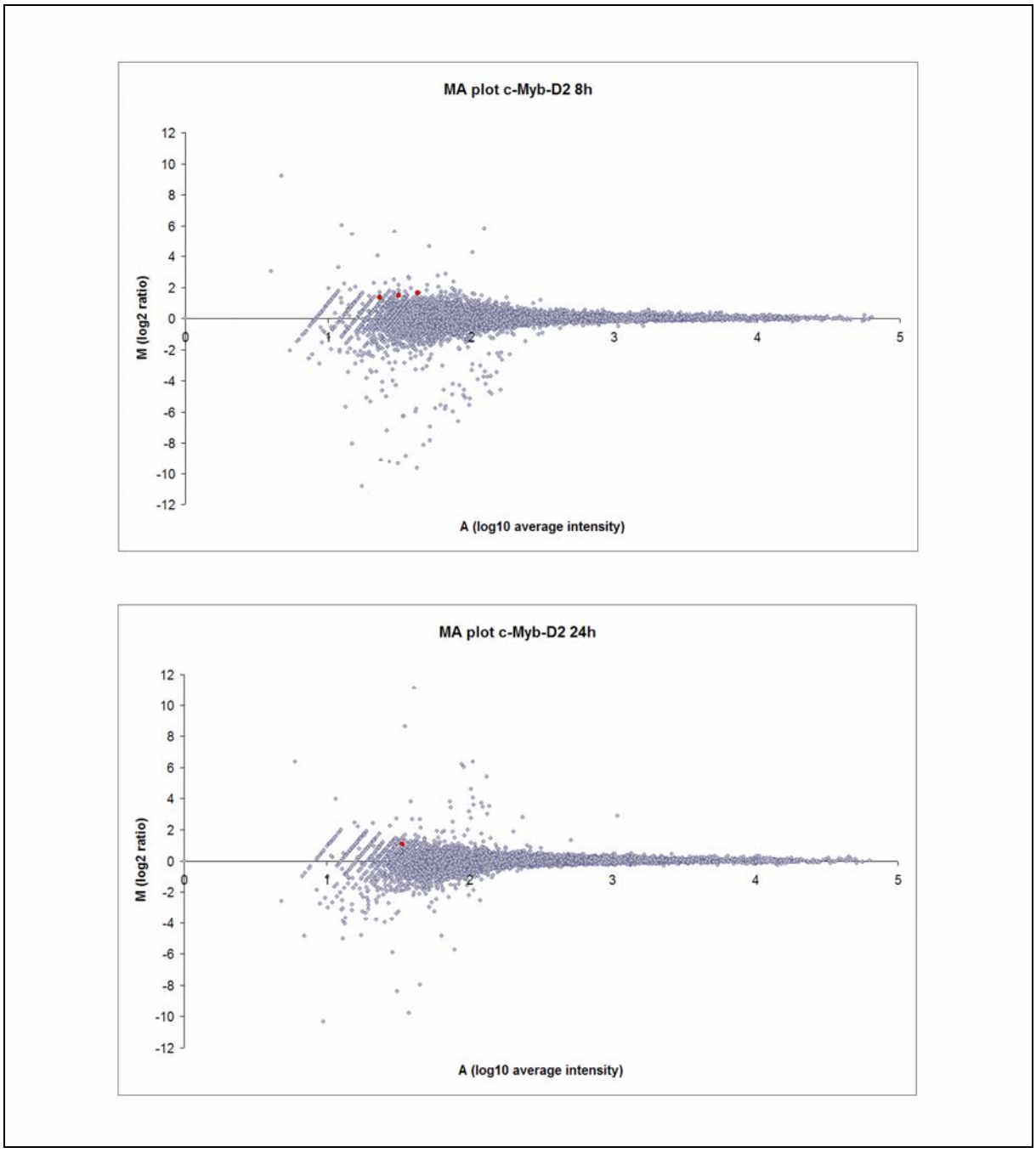


Figure 12 MA plots for c-Myb-D2. Genes found to be up-regulated at least twofold in both arrays (M≥1) are marked as red dots. No genes were found to be down-regulated at least two-fold in both arrays.

Gene name	Gene symbol	M (2)	A (2)	M (1)	A (1)	M of 8h	A of 8h
GA binding protein transcription factor, alpha subunit 60kDa	GABPA	-1.56	1.65	-1.02	2.41	-1.29	2.03
Nuclear transcription factor Y, alpha	NFYA	-1.04	1.30	-1.05	2.10	-1.05	1.70
Benzodiazepine receptor (peripheral)	BZRP	-1.06	1.47	-1.00	2.33	-1.03	1.90
Dystrophia myotonica-protein kinase	DMPK	-1.99	1.48	-2.64	1.92	-2.31	1.70
RAB11 family interacting protein 4 (class II)	RAB11FIP4	-1.12	1.80	-1.16	2.31	-1.14	2.06
Mature T-cell proliferation 1	MTCP1	-1.26	1.60	-2.78	1.94	-2.02	1.77
PREDICTED: similar to RIKEN cDNA 9530066K23 [Source:RefSeq_peptide_predicted;Acc:XP_371777]		-1.61	1.10	-1.11	2.37	-1.36	1.73
Ribosomal protein L28	RPL28	-1.56	1.57	-2.59	2.19	-2.07	1.88
Mitochondrial tumor suppressor 1	MTUS1	-1.04	1.78	-3.22	2.14	-2.13	1.96
Nucleosome assembly protein 1-like 5	NAP1L5	-1.02	1.12	-2.52	2.11	-1.77	1.62
Tripartite motif-containing 62	TRIM62	-1.01	1.64	-1.46	2.20	-1.24	1.92
WIRE protein	WIRE	-1.04	1.67	-1.66	2.22	-1.35	1.95
KIAA1463 protein	KIAA1463	-1.38	1.96	-1.20	1.55	-1.29	1.75
B-cell CLL/lymphoma 6, member B (zinc finger protein)	-	-1.54	2.34	-1.02	2.44	-1.28	2.39
Zinc finger protein 692	FLJ20531	-1.21	1.77	-1.02	2.09	-1.11	1.93
Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	MAP3K7IP1	-1.05	1.97	-2.20	1.28	-1.63	1.62
Chromosome 14 open reading frame 126	C14orf126	-1.40	1.76	-1.21	2.17	-1.31	1.97
Adenosine A2b receptor	ADORA2B	-3.01	0.90	-1.10	2.15	-2.05	1.52
Cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	CELSR2	-1.03	1.82	-1.58	2.09	-1.30	1.95
Pygopus homolog 2 (Drosophila)	PYGO2	-1.11	1.39	-1.07	2.15	-1.09	1.77
Mediator of RNA polymerase II transcription, subunit 28 homolog (yeast)	MED28	-1.86	1.12	-1.21	1.97	-1.54	1.55
Homo sapiens cDNA FLJ12364 fis, clone MAMMA1002384	N/A	-1.27	2.04	-1.24	1.86	-1.26	1.95
V-myb myeloblastosis viral oncogene homolog (avian)-like 1	MYBL1	-1.24	0.93	-1.27	2.19	-1.25	1.56
HEJ1, [Source:Uniprot/SPTREMBL;Acc:Q96RF1]		-1.33	2.35	-1.02	2.15	-1.18	2.25
Kelch-like 24 (Drosophila)	DRE1	-2.00	1.36	-1.79	2.26	-1.90	1.81
FKSG39 (FKSG39)		-2.17	1.56	-1.29	1.99	-1.73	1.78

Table 6 Genes found to be down-regulated at least two-fold in both microarray hybridizations of the RNA isolated from the v-Myb^{AMV}-A4 cell line after 8 hours induction. Numbering in brackets next to the M and A values correspond to the slide number from figure 11.

Gene name	Gene symbol	M (2)	A (2)	M (1)	A (1)	M of 8h	A of 8h
Heterogeneous nuclear ribonucleoprotein L	HNRPL	1.13	1.91	1.28	2.32	1.21	2.12
Mannose-P-dolichol utilization defect 1	MPDU1	1.27	2.03	1.25	2.15	1.26	2.09
Kruppel-like factor 11	KLF11	2.12	0.69	2.02	3.03	2.07	1.86
Zinc finger, MYND-type containing 15	ZMYND15	3.88	1.57	1.22	2.25	2.55	1.91
cancer susceptibility candidate 2	-	1.61	1.61	1.56	2.26	1.59	1.93
Hypothetical protein BC009732	LOC133308	1.84	1.68	1.87	1.93	1.85	1.81
Immunoglobulin lambda chain, mAb 667	Igk-V8	2.16	1.09	1.70	2.05	1.93	1.57
Diacylglycerol kinase, delta 130kDa	DGKD	3.06	1.63	1.27	2.34	2.16	1.99
EGF-like repeats and discoidin I-like domains 3	EDIL3	2.35	1.89	1.67	2.30	2.01	2.09
Obscurin-like 1	KIAA0657	2.11	1.67	1.54	2.17	1.82	1.92
Cullin-associated and neddylation-dissociated 1	TIP120A	1.69	1.69	1.35	2.32	1.52	2.00
KIAA1211 protein	KIAA1211	4.02	1.60	1.35	2.26	2.69	1.93
Tripartite motif-containing 43	TRIM43	3.31	0.78	1.03	1.93	2.17	1.36
MRNA; cDNA DKFZp434L1016 (from clone DKFZp434L1016)		9.15	0.94	1.05	2.22	5.10	1.58
Chromatin modifying protein 4A	C14orf123	2.66	1.60	1.24	2.31	1.95	1.95
Crystallin, zeta (quinone reductase)-like 1	CRYZL1	1.26	1.94	1.12	2.44	1.19	2.19
Glypican 6	GPC6	2.00	1.78	3.45	1.60	2.73	1.69
Hypothetical protein BC015395	LOC130940	1.19	1.50	2.25	1.74	1.72	1.62
Similar to hypothetical protein SB153 isoform 1		1.91	0.98	3.27	1.77	2.59	1.37
Stringency oligo ctrl6-8, Match 80%		4.80	1.14	2.57	2.09	3.69	1.61

Table 7 Genes found to be up-regulated at least two-fold in both microarray hybridizations of the RNA isolated from the v-Myb^{AMV}-A4 cell line after 8 hours induction.

Gene name	Gene symbol	M (4)	A (4)	M (3)	A (3)	M of 24h	A of 24h
Stathmin-like 4	STMN4	-3.53	1.44	-1.93	2.64	-2.73	2.04
Trans-golgi network protein 2	TGOLN2	-2.98	1.22	-1.54	1.91	-2.26	1.56
Family with sequence similarity 89, member B	MTVR1	-1.47	1.99	-1.13	1.77	-1.30	1.88
BM88 antigen	BM88	-1.39	1.99	-1.67	2.81	-1.53	2.40
Zinc finger homeobox 1b	ZFHX1B	-3.29	1.40	-1.43	2.01	-2.36	1.71
Chromatin modifying protein 5	SNF7DC2	-4.15	1.76	-1.27	2.51	-2.71	2.13
Rap guanine nucleotide exchange factor (GEF) 5	RAPGEF5	-1.48	0.80	-2.87	1.13	-2.18	0.97
Butyrophilin, subfamily 3, member A1	BTN3A1	-1.91	0.74	-1.50	1.06	-1.70	0.90
Gelsolin (amyloidosis, Finnish type)	GSN	-1.36	1.93	-1.04	2.22	-1.20	2.07
Mastermind-like 2 (Drosophila)	MAML2	-1.32	1.89	-1.28	2.40	-1.30	2.14
Protein kinase, AMP-activated, gamma 3 non-catalytic subunit	PRKAG3	-2.60	1.51	-1.44	2.52	-2.02	2.02
Cholinergic receptor, nicotinic, alpha polypeptide 3	CHRNA3	-2.86	1.20	-1.31	2.29	-2.08	1.74
Eyes absent homolog 1 (Drosophila)	EYA1	-1.56	1.68	-1.03	2.23	-1.29	1.95
Opioid receptor, kappa 1	OPRK1	-3.21	1.26	-1.43	2.28	-2.32	1.77
Hypothetical protein FLJ22955	FLJ22955	-1.14	1.58	-1.49	2.19	-1.31	1.89
Hypothetical protein FLJ30990	FLJ30990	-1.75	1.68	-1.20	1.82	-1.47	1.75
Chromosome 14 open reading frame 145	C14orf145	-1.98	1.59	-1.22	2.32	-1.60	1.96
Hypothetical LOC339483	LOC339483	-3.29	1.45	-1.54	2.26	-2.41	1.86

Table 8 Genes found to be down-regulated at least two-fold in both microarray hybridizations of the RNA isolated from the v-Myb^{AMV}-A4 cell line after 24 hours induction.

Gene name	Gene symbol	M (4)	A (4)	M (3)	A (3)	M of 24h	A of 24h
Steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	SRD5A1	2.15	1.33	1.35	1.52	1.75	1.42
Keratin 9 (epidermolytic palmoplantar keratoderma)	KRT9	1.92	2.49	1.06	1.79	1.49	2.14
Protein kinase D1	PRKD1	4.13	0.65	2.16	1.31	3.14	0.98
Potassium channel tetramerisation domain containing 2	KCTD2	1.29	1.55	1.82	1.28	1.55	1.41
Chromobox homolog 4 (Pc class homolog, Drosophila)	CBX4	2.28	1.79	1.34	1.74	1.81	1.76
Neurofascin homolog (chicken)	NFASC	1.97	1.30	1.15	1.65	1.56	1.48
Olfactomedin-like 3	OLFML3	2.30	1.79	6.65	0.72	4.48	1.26
Leucine proline-enriched proteoglycan (leprecan) 1	LEPRE1	1.05	2.08	1.29	1.75	1.17	1.91
Steroid 5 alpha-reductase 2-like	SRD5A2L	2.73	1.63	2.04	1.33	2.39	1.48
Reticulon 4 receptor	RTN4R	1.36	1.17	1.08	1.56	1.22	1.36
limkain b1 isoform 2 [Source:RefSeq_peptide;Acc:NP_061954]		1.24	2.22	5.57	0.88	3.40	1.55
CDNA: FLJ22382 fis, clone HRC07514		1.32	1.55	1.33	1.67	1.32	1.61
Musashi homolog 1 (Drosophila)	MSI1	2.51	1.68	1.42	2.03	1.97	1.86
Zinc finger protein 669	FLJ12606	2.18	1.97	1.24	1.84	1.71	1.90
Zinc finger, AN1-type domain 2B	LOC130617	1.16	1.94	1.30	1.97	1.23	1.95
SATB family member 2	SATB2	2.86	1.22	2.00	1.54	2.43	1.38
Tripartite motif-containing 45	TRIM45	2.18	1.64	1.88	1.45	2.03	1.54
PHD finger protein 20	PHF20	1.69	1.26	3.01	1.60	2.35	1.43
Randomly generated negative control	-	4.58	0.72	2.54	1.34	3.56	1.03

Table 9 Genes found to be up-regulated at least two-fold in both microarray hybridizations of the RNA isolated from the v-Myb^{AMV}-A4 cell line after 24 hours induction.

Gene name	Gene symbol	M (6)	A (6)	M (5)	A (5)	M of 8h	A of 8h
Bone morphogenetic protein 5	BMP5	1.87	1.54	1.04	1.19	1.45	1.36
Transient receptor potential cation channel, subfamily M, member 4	TRPM4	1.24	2.05	1.20	1.20	1.22	1.63
Cysteine/histidine-rich 1	CYHR1	1.02	1.62	1.09	1.37	1.06	1.49

Gene name	Gene symbol	M (8)	A (8)	M (7)	A (7)	M of 24h	A of 24h
KIAA1160 protein	KIAA1160	1.10	1.68	1.02	1.38	1.06	1.53

Table 10 Genes found to be up-regulated at least two-fold in both microarray hybridizations of the RNA isolated from the c-Myb-D2 cell line after 8 and 24 hours induction. No genes were found to be down-regulated in accordance to the criteria used.

As is evident in the MA plots, there were not many genes that were found to be up- or down-regulated in the cells in a statistically significant manner. There appears to be several more changes in the v-Myb^{AMV} data sets than in those for c-Myb, as would be expected, since v-Myb^{AMV} is a stronger transcriptional activator than c-Myb is, but the MA-plots also reveal that the noise level is higher for v-Myb^{AMV}, making these data more uncertain, especially for the genes that show a low intensity/expression level. Still, the criteria by which the up-regulated and down-regulated genes were measured were rather strict, and several potential genes were discarded due to the fact that they were only present in one of the data sets, since it would be impossible to discern dye-specific artifacts from true target genes without corresponding spot values for both of the dye-swap parallels. Several dye-specific artifacts were observed in the data sets, confirming the importance of such dye-swap hybridizations and our strict criteria for singling out potential target genes for c-Myb and v-Myb^{AMV}.

Gene symbol	**M(8)**	**A(8)**	**M(7)**	**A (7)**	**M (6)**	**A (6)**	**M (5)**	**A (5)**
WT1	-	-	1.07	1.38	-0.60	1.33	-0.23	1.98
GBX2	-	-	-	-	-	-	0.16	1.69
CD34	-	-	-4.85	1.80	-	-	-4.94	1.94
CD4	-	-	-	-	1.76	1.82	0.06	1.95
MAT2A	0.05	3.23	-0.15	3.25	-0.08	3.24	-0.03	3.34
TRHR	-0.12	2.69	0.03	2.75	0.09	2.73	-0.17	2.87

Table 11 Microarray results for some previously validated c-Myb target genes in the hybridizations performed with samples from the Tet-On c-Myb-D2 cell line. M and A represent the log2 ratio and log10 intensity levels for the spots on the array, respectively, the numbers in brackets correspond to the slide number, as described in figure 10. All M-values are reported as log2 ratio for induced cells over un-induced cells. Notice in particular the M-values for *CD34*, a reporter with typical dye-specific expression pattern.

Also the data for several other known c-Myb target genes, such as *WT1*, *GBX2*, *CD34*, *CD4*, *MAT2A* and *TRHR* (see references in table 1) were studied in the c-Myb-D2 data sets (slides five to eight), and in those cases where the spots for the gene were present (sufficiently high intensity, and not flagged), there was hardly any change in expression levels (table 11). *CD34* appears to be very strongly down-regulated in the data sets for slide five and seven, but there is a known problem with this reporter that it always appears to be down-regulated (green) (information from the BASE website). It is not present in the data sets for slide six and eight. The data presented in table 11 also indicate one of the major problems regarding our selection criteria, with the absence of several reporters in the data set from some of the slide hybridizations. This is also quite evident from table 5, where the number of spots remaining after the filtering process is but a fraction of the spots in the original data set.

Possibly we may have found more potential target genes for c-Myb and v-Myb^{AMV} had several more hybridizations been performed, which would also have strengthened the statistical basis, but the time limitations of this thesis did not allow for further experiments. In general, the expression of c-Myb and v-Myb^{AMV} in the HEK293 cells appeared to have minor effects on the gene expression profile of the cells, as had been observed with the Affymetrix microarray analysis of c-Myb-D2.

4. Discussion

The main aim of this study was the generation and verification of cell lines with inducible expression of the hematopoietic transcription factor c-Myb, its viral counterpart v-Myb^{AMV} or the C-terminally truncated c-Myb$^{1-443}$, with the secondary aim of employing these cell lines in microarray experiments for the determination of potential target genes for these transcription factors. Our hope was that by comparison of the effects each of these transcription factors had on the global gene expression patterns of the cells in which they were expressed, we might come one step closer to understanding some of the underlying mechanisms for oncogenic activation of c-Myb as well as its normal biological function.

The work on this thesis mainly became a matter of establishing new methods for the group, both regarding the generation of double-stable cell lines and the microarray experiments. This discussion will therefore reflect largely on these experiences, dwelling on technical aspects concerning the Tet gene regulation systems and the microarray experiments, in addition to the issues regarding the c-Myb$^{1-443}$ construct and our findings from the preliminary microarray experiments performed on the selected cell lines.

4.1 Choice of cell line and experiences with the Tet-On and Tet-Off systems

The main criterion for the parental cell line chosen for the generation of the stable cell lines with inducible expression of c-Myb was that it did not display endogenous expression of this transcription factor. Further, the cell line should preferentially have adherent growth properties to allow for easier selection of stably transfected clones. These criteria excluded most hematopoietic cell lines, yet we still wished to use a cell line in which c-Myb would be in a cell environment as close to its native one as possible. The choice of Clontech's Tet-systems as our gene regulation system in combination with the desire to test out both the Tet-On and Tet-Off system limited the number of available cell lines further, resulting in the choice of the HEK293 Tet-On and Tet-Off cell lines. HEK293 is a well established human cell line that appeared to possess all the desired qualities for our purposes, though later findings indicated that we might have profited from a different choice of cell line. Howe *et al.* reported as early as in 1995 that the HEK293 cell line generally displayed decreased responsiveness in tetracycline regulated gene expression systems compared to GH3 cells

[165], findings that were in keeping with our observations that the stably transfected clones generally did not exhibit very large changes in expression level of c-Myb upon induction, though we did manage to find a few clones that did respond well to Dox. The problem has been reported to be of particular significance in the Tet-On system, and Clontech has recently marketed a HEK293 cell line with a new version of the Tet-system designed to overcome these drawbacks; the Tet-On Advanced Inducible Gene Expression System [166]. This system is optimized for transgenic animals in particular, where the previous Tet-On system was hampered by varying levels of Dox in different organs, and in certain cell lines where the rtTA was unstable, resulting in background expression of the Tet-regulated gene. The main difference between the new Tet-On system and the one used in this thesis lies in the new rtTA's increased sensitivity to Dox and its decreased affinity for the TREs in the uninduced state.

The odd morphology we observed for the Tet-Off cells in particular is also a known feature of HEK293 cells; their morphology often changes following transfection. Clontech has also reported that the Tet-On and Tet-Off cells are only semi-adherent, not adherent as the parental cell line HEK293 is. This was particularly obvious with the Tet-Off cell line used in this work, in which the cells did not maintain the monolayer epithelial growth pattern expected of HEK293 cells, but rather formed dense clumps of cells. The cells were also generally smaller than those of the Tet-On cell line. This odd morphology, in combination with the high mortality rate following storage, was the main reason why we decided not to proceed further with the Tet-Off clones. The prolonged non-optimal storage conditions (-80 °C rather than in liquid nitrogen vapor) may have been largely to blame for the mortality rate, since HEK293 are considered to be rather sensitive cells in this regard (information from Clontech website). The morphology and mortality issues for the Tet-Off cells thus cemented our preference for the Tet-On system, which was also more convenient to use in regard to the induction procedure and time intervals for addition of antibiotics to the growth medium, and from which we succeeded in our goal of generating double-stable cell lines with inducible expression of c-Myb or v-Myb^{AMV}.

4.2 Issues regarding the c-Myb$^{1-443}$ construct and clones

The lack of luciferase activity in the reporter assays for the pTRE2hyg-hcM[1-443]-HA construct and c-Myb$^{1-443}$ is a matter that can only be described as baffling. Western blots performed indicated that a protein of the expected size was expressed upon induction, and was recognized by the 5e11 antibody specific to c-Myb DBD. A Western blot was also performed with an α-HA antibody (not shown), indicating that the HA-tag was intact as well, ruling out the possibility of frameshift mutations. More importantly, the pCIneo-hcM[1-443]-HA construct and constructs derived from it had been used in reporter assays in several other cell lines with results as expected, yielding an increase in luciferase activity quite similar to that observed for v-Myb^{AMV} constructs (Marit Ledsaak and Ole Stian Bockelie, personal communication). These results indicate that the PCR-generated mutation in the construct is probably not to blame for the lack of activity of c-Myb$^{1-443}$ in HEK293 cells. A full sequencing of the entire construct might be the right action to take to find the reason behind these puzzling results, or perform a luciferase assay with the HEK293 Tet-On cells using the pCIneo-hcM[1-443]-HA construct in order to determine whether there are cell type specific factors at work inactivating c-Myb$^{1-443}$ specifically, but not c-Myb or v-Myb^{AMV}.

4.3 Microarray analysis – Technical considerations and challenges

When the work on this thesis was begun in 2002, microarray technology was truly the method of the moment. For a time it almost seemed as if every lab that could possibly afford it were purchasing scanners and hybridization equipment, and our lab was one of them. We were at the time under the impression that the microarray technology was pretty well established, based partly on the optimism still reigning in the majority of the scientific community, but mostly on our communication with the NMC, where they had successfully been producing in-house spotted slides and performing hybridizations for some time. Still, it was about this time that critics of the technology and the way in which it was being used voiced their opinions loudly enough to be heard. The initial optimism in the second half of the nineties had begun to dim somewhat as it became apparent that the microarray technology was not quite the straight-forward analysis method for determination of target genes that it was first assumed to be. When used correctly it is an immensely powerful tool, but it is also a method that places strict demands on experimental design and reproducibility in order to contribute with

meaningful results. Statistical considerations have now become a major factor due to the huge amounts of data generated. A 99 % confidence interval may seem good in other contexts, but when the number of reporters studied is 23 000, this will result in a statistical 230 false positives/negatives.

General aspects concerning microarray technology have been extensively reviewed over the past five years, and a full discussion of these aspects is beyond the scope of this thesis. Rather I would like to point out a few of our personal experiences from the challenging but rewarding process of establishing a procedure for microarray analyses in our lab, some of the experiences reported from the NMC and their collaborators and draw in a few relevant publications.

The first aspect to consider when planning microarray experiments is experimental design. What sample should be used as reference, what samples should be hybridized together? What is the biological relevance of the comparison, what are the sources of variation? How much sample RNA is available, how many hybridizations can be performed, and what are the limiting factors? Once these factors have been established, one can begin to consider what experimental design to use. In our case we had a quite simple experimental design, as our main objective was just to compare gene expression in cells with or without expression of c-Myb or v-Myb^{AMV}. Should we however have wished to study further the differences between c-Myb and v-Myb^{AMV} expressing cells, we might have benefited from the use of a different experimental design, one where there was a common denominator in all the hybridizations. Had we used RNA from the parental HEK293 Tet-On cell line rather than the un-induced c-Myb-D2 and v-Myb^{AMV}-A4 clonal cell lines, we would have achieved a better statistical basis for direct comparison of these two double-stable clones, but the statistical data for potential target genes for each clone would have suffered from increased noise levels due to the larger biological differences between reference and sample RNA. As it were, we designed the experiments based on our consideration of what was more important of direct comparison of c-Myb and v-Myb^{AMV} or target genes for each transcription factor, and our choice fell on the target genes. General considerations for experimental design are discussed in [167-169].

The questions regarding experimental design also relate to the discussion concerning technical replicates versus biological replicates. A technical replicate will in this regard be two separate hybridizations of the same RNA samples, whereas a biological replicate will be two separate

hybridizations of RNA samples from two individuals, cell lines or animals. Ideally, one should perform both, which was also one of reasons why we kept multiple potential clones for each construct in this work. Based on our observations and the reports from the NMC regarding reporters such as *CD34*, technical replicates in general and dye-swap replicates in particular are important when performing microarray experiments with in-house spotted arrays, as there are a fair number of spots/reporters that are dye-specific and these can only be revealed by use of such replicates. In addition there is the element of salt streaks and other factors that result in the removal of several reporters from the data set as we observed (table 5), reporters that may have been quite interesting for the study. However, if one is using commercial microarray platforms such as Affymetrix, the technical variation from experiment to experiment is reported to be negligible [170] while the cost per hybridization is higher, so in this case it would be a better choice to prioritize biological replicates. In either case one cannot draw any conclusions based on a single microarray hybridization, so replicates are an absolute necessity, but whether they should preferentially be technical or biological replicates is highly dependent on the microarray platform used.

In this work we tried out both in-house spotted arrays (cDNA-arrays and oligo-arrays) and the Affymetrix platform. The main drawback with the Affymetrix platform is the cost, which is many times that of in-house spotted arrays. When one can perform perhaps ten hybridizations with in-house spotted arrays for the same price as a single Affymetrix hybridization pair (Affymetrix uses one array per sample), it comes down to simple cost considerations for most laboratories. The first in-house spotted microarrays we tried out in this work were 15k cDNA arrays based on the ResGen 40k I.M.A.G.E clone set. These arrays went out of production at the NMC during the course of this work, mainly due to the introduction of the cheaper and better 21k oligo-arrays. Reports of uneven hybridizations and some reporters that did not represent the correct gene were key factors in the choice made by the NMC to discontinue the production of the cDNA arrays. Several articles have presented results from comparisons of arrays manufactured with oligos or cDNA fragments, and generally the long oligo-arrays (50-80 bp) have come out favorably [171, 172], and have now nearly completely taken over for the cDNA arrays.

For labeling of the samples to be hybridized to these in-house spotted arrays, we briefly tried out a direct labeling method for incorporation of Cy3 and Cy5 before ending up with the FairPlay protocol for indirect labeling. The few samples we did manage to hybridize after

labeling with the direct method illustrated the main weakness of this method: Our samples had high incorporation of Cy3, but hardly any Cy5, assumed to be caused by the difficulties experienced by the reverse transcriptase due to the larger size of the Cy5 molecules. Which method is superior has been a matter of some debate, as the results are difficult to compare directly, since the labeling method appears to have a rather significant effect on the results. It has become increasingly clear over the past years that different microarray platforms may yield strikingly different results, depending on choice of labeling method, array type and hybridization conditions [173, 174]. In addition there is the matter of automated hybridization stations versus manual hybridizations. We tested both methods in this work, and the results were in keeping with the information presented by the manufacturers of the hybridization stations and the experiences of the staff at NMC: Automated hybridization stations result in lower background, but also weaker spots. We chose the use of automated hybridization stations mainly due to the more homogenous distribution of the hybridization solution, as these stations pump the solution back and forth across the slide in the hybridization chamber, resulting in lower across-slide variation than observed for the manual hybridizations where the solution remains still across the slide as capillary forces hold the cover slide in place.

A few environmental considerations became apparent to us during this work, mainly that microarray experiments should not be performed in July or August due to higher ground-levels of ozone in combination with heat and humidity. The Cy-dyes are very sensitive to oxidation and sunlight and the slides to increased humidity, all factors that contribute to making the summer months an unfortunate time of year for microarray experiments. Reducing the level of ozone in the microarray laboratory is reported to significantly increase the reproducibility of microarray experiments where Cy-dyes are used for labeling [175].

All in all, microarray experiments come down to statistics and minimizing sources of variation while keeping in mind the goal of the study and the experimental design. Following such a process of methodological trial and error regarding a technology that is constantly evolving has been a challenge, but a rewarding one at that.

4.4 Microarray results for v-Myb^{AMV} and c-Myb

Due to the time limitations of this thesis, the number of microarray hybridizations we could perform became too few to be able to draw any conclusions concerning potential target genes for c-Myb or v-Myb^{AMV}. We did however make a few interesting observations regarding differences in the gene expression profiles of the cells in which these two versions of c-Myb were expressed. It would appear that v-Myb^{AMV} both represses and activates more genes than c-Myb does, not surprising in view of its lack of negative regulation. The noise level of the v-Myb^{AMV} hybridizations is also higher than those for c-Myb, as is evident by the larger spread of the spots with low intensity in the v-Myb^{AMV} MA plot.

Expression of c-Myb in the HEK293 cell line did not appear to induce differential expression of many genes either in the Affymetrix or the oligo-array hybridizations. In addition, the KIAA1160 protein reporter found to be the only reporter up-regulated more than two-fold in the data set from the 24 hour induction has been reported to probably not represent a gene (information from the BASE website). LOX-1 (*OLR1*) was found to be up-regulated two-fold in the Affymetrix experiment, but was not present in the oligo-array data set for c-Myb-D2 24 hour induction. However, it was present in the 8 hour induction data set, where it was found to be up-regulated in the data corresponding to slide six, but did not display any altered expression in the data set for slide five (data not shown). It is also interesting to note the results for *TRHR* in the data sets in light of the lack of response observed in the luciferase assays with the reporter construct based on the *TRHR* promoter, indicating that c-Myb is not able to activate neither the reporter construct nor resident chromosomal gene of *TRHR* in HEK293 cells. The MA plots show that there are several spots that are up- or down-regulated in the data set that have not been found to be statistically significant in accordance to our stringent criteria, but altering these criteria would also result in an increase in false positives and negatives, making it difficult to discern Cy-dye specific artifacts or noise from true c-Myb targets. Performing more hybridizations is probably the best way to determine whether c-Myb really is able to significantly alter the expression of potential target genes in HEK293 cells, as the present data set is insufficient for this purpose.

For v-Myb^{AMV} the microarray experiments resulted in a far longer list of potential candidates than for c-Myb, but this list also includes several unknown reporters and some reporters that are clearly not potential target genes, such as the randomly generated negative control and the

stringency oligo found in the data sets in table 7 and 9. There is also a distinct lack of correlation between the data sets from the 8 and 24 hours induction samples, as there are no genes that are found in both data sets. As for the data sets for the c-Myb samples, these are just preliminary results, and repeated experiments may result in quite different lists of potential target genes. Still, it is interesting to note the occurrence of genes like *MYBL1* (A-Myb), *MTUS1*, *MTCP1* and *GSN*, and hopefully further experiments will help to determine which, if any, genes on these lists are true v-Myb^{AMV} target genes.

During the time course of this thesis, three articles were published where a microarray approach had been utilized to study the transcription factors c-Myb, A-Myb, B-Myb and v-Myb^{AMV} [141-143]. These articles highlight several important aspects regarding the Myb proteins:

- There is little correlation between the genes regulated by c-Myb, A-Myb and B-Myb, respectively.
- There is little correlation between the genes regulated by c-Myb and v-Myb^{AMV}.
- There is hardly any correlation between the genes regulated by the respective Myb proteins in different cell lines.

It would appear that the Myb transcription factors function in a context specific manner that is highly dependent on the availability of co-activators, cooperating transcription factors and proteins mediating post-translational modifications (discussed in [143]). These results also put into question the results from previous reporter assays, as it would appear that these are not representative for the function of Myb proteins in their native cellular environment. In light of these findings, it will be interesting to see if further microarray experiments in our model system will display the same results as was observed in these experiments with adenovirus transfected cell lines and use of the Affymetrix microarray platform. Our preliminary results do indeed indicate that v-Myb^{AMV} is not just a more active version of c-Myb, but a transcription factor with a unique set of activities, as was observed by Liu *et al*. [141].

4.5 c-Myb – A context-specific and repressed activator of transcription

In the past few years it has become increasingly evident that c-Myb is a transcription factor that is highly dependent on post-translational modifications and interaction partners to fulfill its potential as a transcriptional activator. Many of the modifications and interaction partners

function to repress c-Myb activity, while others are vital for activation of certain target genes (discussed in chapter 1.3). In light of the recent articles on microarray experiments with c-Myb [141-143], it is quite interesting to note how many of the previously published articles regarding c-Myb function have reported conflicting results depending on the model system used, as mentioned in the introduction of this thesis and observed in our own experiments with the luciferase assays with the TRHR reporter construct, a construct that has worked well in other cell lines, but not in HEK293. It is also quite interesting to note the large difference in the number of genes found by Liu *et al.* to be differentially regulated by c-Myb and v-Myb^{AMV} in MCF7 cells compared to primary human monocytes that are more closely related to the myeloid cells transformed by v-Myb^{AMV} [141]. Based on their results, it would seem as if these transcription factors are far more active in a cellular environment that is more akin to their native environment than in other human cell lines, and regulate different sets of genes in these cells, raising the question of whether c-Myb is mainly present in a repressed form in cell lines in which it does not naturally belong. Whether this repressed form is the result of post-translational modifications and/or interaction partners or lack of these remains an open question, and will probably be the subject of many studies to come.

4.6 Concluding remarks and future prospects

The main goal of this thesis was to establish a model system for the study of c-Myb and its viral counterpart v-Myb^{AMV} as well as a truncated version of c-Myb, designated c-Myb$^{1-443}$. We succeeded in establishing two cell lines with inducible expression of c-Myb or v-Myb^{AMV}, but the cell lines with inducible expression of c-Myb$^{1-443}$ were put aside until the reasons behind the bewildering observations made during the verification process have been determined. These two cell lines, c-Myb-D2 and v-Myb^{AMV}-A4, will be a valuable asset for the laboratory group in further studies regarding determination of target genes for c-Myb and v-Myb^{AMV} as well as other studies that may contribute to the understanding of mechanisms underlying oncogenic activation of c-Myb.

The preliminary microarray experiments performed in this work will need to be repeated and verified with other methods such as ChIP, real-time RT-PCR or Western in order for us to be able to draw any conclusions regarding target genes for c-Myb and v-Myb^{AMV}, but a few interesting observations were made, mainly that c-Myb appears to be a rather weak activator

of transcription in the HEK293 cells, whereas v-Myb^{AMV} both activates and represses more genes than c-Myb. In addition it is interesting to note that several genes reported to be c-Myb targets in other model systems are not differentially expressed in this system, findings that are in keeping with recent publications that report c-Myb to be a highly context-specific transcription factor with differing activities in diverse cell lines.

The context-specific aspects regarding c-Myb may indicate that a more biologically relevant model system for the study of this transcription factor would be knock-down of c-Myb in cell lines with endogenous expression of c-Myb. Such a model system is already established in our lab, based on siRNA knock-down of c-Myb in the K562 cell line. Further experiments with both this siRNA/K562 system and the model system established in the work on this thesis will hopefully contribute to an increased understanding of the hematopoietic transcription factor c-Myb and its biological function.

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Appendix

Appendix I: Materials and solutions

Commercial kits and solutions

Product	Supplier
Cy3, NHS-ester leaving group	Amersham
Cy5, NHS-ester leaving group	Amersham
ECL Plus kit	Amersham
FairPlayII Microarray Labeling Kit	Stratagene
FuGENE 6 Transfection Reagent	Roche
Genopure Plasmid Maxi Kit	Roche
GFX Micro Plasmid Prep Kit	Amersham
GFX PCR DNA and Gel Band Purification kit	Amersham
Lipofectamine	Invitrogen
Luciferase assay system	Promega
Micro BCA Protein Assay Reagent Kit	Pierce
Passive lysis buffer	Promega
RNA 6000 Nano LabChip kit	Agilent Technologies
RNeasy Mini Kit	Qiagen
SYBR Green Dye	Roche
Trizol reagent	Invitrogen
First strand cDNA synthesis kit for RT-PCR	Roche
FastStart DNA master SYBR green I	Roche

Antibiotics

Product	Supplier
Ampicillin	Sigma
Doxycyclin	Clontech
Hygromycin B	Invitrogen
Penicillin/streptomycin	Invitrogen

Antibodies

Antibody	Host	Supplier/reference
5e11, α-c-Myb	Mouse	[161]
α-mouse IgG, HRP conjugated	Goat	Zymed
α-HA, polyclonal	Rabbit	Sigma-Aldrich
α-rabbit IgG, HRP conjugated	Goat	Zymed

Enzymes

Restriction enzyme	Buffer	Supplier
*Eco*RI	NEBuffer *Eco*RI	New England Biolabs
*Eco*RV	NEBuffer3 + BSA	New England Biolabs
*Kpn*I	Buffer *Kpn*I	MBI Fermentas
*Nhe*I	NEBuffer2 + BSA	New England Biolabs
*Not*I	NEBuffer3 + BSA	New England Biolabs
*Psh*AI	NEBuffer4 + BSA	New England Biolabs
*Sal*I	NEBuffer3 + BSA	New England Biolabs
*Xho*I	NEBuffer2 + BSA	New England Biolabs
*Xmn*I	NEBuffer2 + BSA	New England Biolabs

Other enzymes	Buffer	Supplier
Vent DNA polymerase	ThermoPol buffer	New England Biolabs
Taq DNA polymerase	ThermoPol buffer	New England Biolabs
T4 DNA ligase	T4 ligase buffer	MBI Fermentas

Miscellaneous

Product	Supplier
21K human oligonucleotide array v.2.3	NMC
6mm paper	Whatman
Cloning discs, 5mm	Sigma-Aldrich
DMEM	Invitrogen
Hybond P membrane	GE Healthcare
L-Glutamine	Invitrogen
Opti-MEM	Invitrogen
PBS	Invitrogen
Tet System Approved FBS	Clontech
Trypsine/EDTA	Invitrogen

Appendix II: Recipes

Buffers and solutions

All solutions used for microarray experiments were made with "Molecular biology grade water" from Eppendorf. For stock solutions and most buffers, double-distilled milliQ water was used, whereas normal distilled water was used for electrophoresis buffers and washing solutions such as TBS-T and SDS-PAGE electrophoresis buffer.

APS
5.0 g APS
Dissolved in dH_2O to 50 ml.

Sterile filtered and stored in aliquots at -20 ºC.

0.5 M EDTA
93.06 g $Na_2EDTA \times 2H_2O$ (MW 372.24 g/mol)
Dissolved in dH_2O to about 400 ml.
pH was adjusted to above 7 in order to dissolve the EDTA.
After everything was dissolved the pH was adjusted to 8.0 with NaOH.
Volume was adjusted to 500 ml with dH_2O.

Autoclaved.

Freezing medium for mammalian cells
25 ml FBS
2.8 ml DMSO

Stored at -20 ºC.

1 M HCl
2.6 ml 35 % HCl
22.4 ml H_2O

Total volume 25 ml.

Loading buffer agarose gel
6 ml 85 % glycerol
1 ml 0.5 M EDTA
3 ml dH_2O
A few grains of bromophenol blue

Stored in aliquots at -20 ºC.

Minus(÷)-buffer
500 ml Plus(+)-buffer
2.62 g e-Amino-n-Caproic Acid (Sigma, A-7824)

2.5 M NaAc pH 4.5
10.25 g NaAc
Dissolved in glacial acetic acid to pH 4.5.
H_2O to 50 ml.

Sterile filtered.

5M NaCl
73.05 g NaCl
H_2O to 250 ml.

Autoclaved.

0.1 M NaOH
10 M NaOH was diluted 1:100 to desired volume shortly before use.

10 M NaOH
40 g NaOH pellets (MW 40 g/mol)
H_2O to 100 ml

Autoclaved.

Lower buffer (1.5 M Tris-HCl pH 8.8)
90.83 g Tris base (MW 121.1 g/mol)
dH_2O to 400 ml
pH was adjusted to 8.8 with concentrated HCl.
Volume was adjusted to 500 ml with dH_2O.

Autoclaved.

Plus(+)-buffer
3 g Tris
20 % methanol
dH_2O to 1 l

10x Ponceau S
5 ml Ponceau S
10 ml acetic acid
dH_2O to 100 ml

Pre-hybridization solution
1 g BSA fraction V dissolved in 70 ml H_2O
17.5 ml 20xSSC
1 ml 10 % SDS
H_2O to 100 ml

Made fresh for every day to be used, but may be stored a short period in the fridge.

RIPA buffer
150 mM NaCl
1.0 % NP-40
0.5 % Sodium deoxycholate
0.1 % SDS
50 mM Tris-HCl pH 8.0

10 % SDS
5.0 g SDS
dH_2O to 50 ml.

3x SDS loading buffer
6.0 ml 10 % SDS
1.0 ml β-Mercaptoethanol
5.0 ml 85 % Glycerol
0.5 ml 0.5 M Tris-HCl pH 6.8
A few grains of bromophenol blue.

Stored in fridge.

10x SDS-PAGE electrophoresis buffer
14.4 g Glycine
3.06 g Tris base
10 ml 10 % SDS
dH_2O to 1000 ml.

20x SSC buffer
175.3 g NaCl
88.2 g Sodium citrate
800 ml dH_2O
pH was adjusted to 7.0 with a few drops of 10 M NaOH.
dH_2O to 1000 ml.

Stripping buffer
100 mM β-Mercaptoetanol
2% SDS
62.5 mM Tris-HCl pH 6.7

50x TAE buffer
121 g Tris base (Tris[hydroxymetyl]aminometan)
50 ml 0.5 M EDTA
28.5 ml glacial acetic acid
Volume was adjusted to 500 ml with dH_2O.

Autoclaved.

10x TBS-T
50 ml 1M Tris-HCl pH 8.0
150 ml 5M NaCl
2.5 ml Tween 20
dH_2O to 500 ml

TBS-T with milk powder
50 ml 10x TBS-T
25 g dry milk powder
dH_2O to 500 ml

TE buffer
10 ml 1 M Tris-HCl, pH 8.0
2 ml 0.5 M EDTA
Volume was adjusted to 1000 ml with dH_2O.

Autoclaved.

1M Tris-HCl, pH 8.0
121.1 g Tris base
Dissolved in dH_2O to about 800 ml.
pH was adjusted to 8.0 with concentrated HCl (approximately 45 ml).

Volume was adjusted to 1000 ml with dH_2O.

Autoclaved.

Upper buffer (0.5 M Tris-HCl pH 6.8)
30.28 g Tris base
Dissolved in dH_2O to 400 ml.
pH was adjusted to 6.8 with concentrated HCl.
Volume was adjusted to 500 ml with dH_2O.

Autoclaved.

Gels

0.9 % agarose gel
0.54 g agarose
60 ml TAE buffer
Was heated in a microwave until all agarose was dissolved, then cooled down to about 50 °C before addition of 1-2 drops of EtBr.

10 % SDS-PAGE gel (small)
Separating gel (10 %)
4 ml 40 % acrylamide C=2,7
4 ml Lower buffer
8 ml dH_2O
160 µl 10 % SDS
50 µl APS
10 µl TEMED

Stacking gel (5 %)
1.25 ml 40 % acrylamide C=2.7
2.5 ml Upper buffer
6.25 ml dH_2O
100 µl 10 % SDS
30 µl APS
10 µl TEMED

Medium for bacteria

LB-medium
10 g Trypton
5 g Yeast extract
10 g NaCl
H_2O to 1000 ml
pH was adjusted to 7.2 with 10M NaOH.

Autoclaved.

Antibiotics were added if needed once the medium had cooled below 50 ºC.
- Ampicillin, 100 mg/ml (1 ml 100 mg/ml to 1 l medium)
- Chloramphenicol, 25 mg/ml (1 ml 25 mg/ml to 1 l medium)

LB plates
400 ml LB-medium (without antibiotics)
6.0 g agar

Autoclaved and cooled down to about 50 ºC.

Antibiotics were added if needed (see recipe for LB-medium).
Poured into petri dishes, about 20 ml per dish.

Medium for mammalian cell cultures

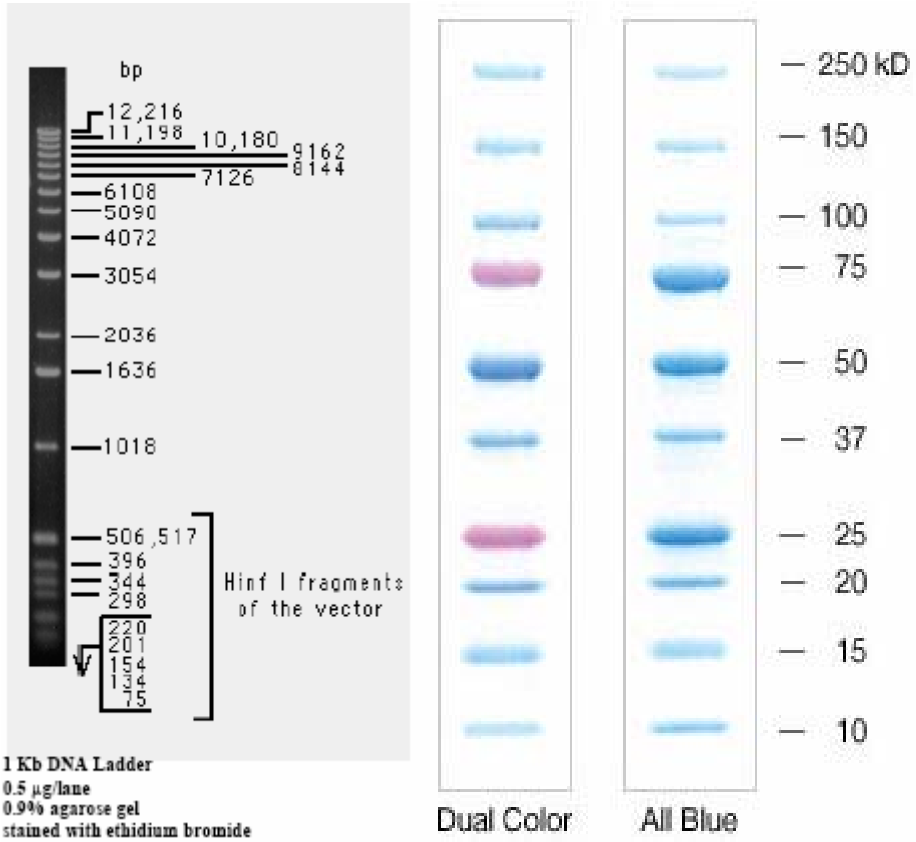
HEK293 Tet-On
500 ml Dulbecco's modified eagle medium (DMEM)
50 ml FBS
5 ml penicillin/streptomycin

For non-transfected Tet-On cells geneticin was added to a final concentration of 100 μg/ml. Double stable cell lines were grown in medium with 100 μg/ml geneticin and 50 μg/ml hygromycin added.

HEK293 Tet-Off
500 ml Dulbecco's modified eagle medium (DMEM)
50 ml FBS
5 ml penicillin/streptomycin

Geneticin and hygromycin as for the Tet-On cells, but to the medium of double-stable cell lines Dox was added to a final concentration of 1 μg/ml every 48 hours.

Appendix III: Mw standards



"1 Kb DNA Ladder" from Invitrogen/Life Technologies. Used for agarose gel electrophoresis.	"Precision Plus Protein Standards" from Bio-Rad. Used for SDS-PAGE.

Appendix IV: Sequences

Primers used for PCR and sequencing

C158: Forward primer for hcM-[1-443]-HA PCR-product
5'-gactcagtgtcgaccATGGCCCGAAGACCCCGGCACAGC-3'

C177: Reverse primer for hcM-[1-443]-HA PCR-product
5'-GAGTTTTCACAGTCTGGTCTCTATG-3'

S082: Forward primer for sequencing of pCIneo-hcM-[1-443]-HA
5'-GACATCCACTTTGCCTTTCTCTCC-3'

S068: Reverse primer for sequencing of pCIneo-hcM-[1-443]-HA
5'-GAGTTTTCACAGTCTGGTCTCTATG-3'

S078: Forward primer for sequencing of pTRE2hyg-hcM-[1-443]-HA
5'-ccacgctgttttgacctccatagaag-3'

S079: Reverse primer for sequencing of pTRE2hyg-hcM-[1-443]-HA
5'-tcccattctaaacaacaccctgaaaa-3'

Primers used for real-time RT-PCR

v-Myb^{AMV}
Forward (**L035**): 5'-TGgcgtcccctAATCG-3'
Reverse (**L036**): 5'-GGTTCTTGACAGCGTTAT-3'

c-Myb
Forward (**L001**): 5'-AAATACGGTCCCCTGAAGATGCTA-3'
Reverse (**L002**): 5'-GTCTGCGTGAACAGTTGGGTATTC-3'

β-actin
Forward (**L003**): 5'-GGGAAATCGTGCGTGACATT-3'
Reverse (**L004**): 5'-CAGGAAGGAAGGCTGGAAGA-3'

Sequence for human c-Myb

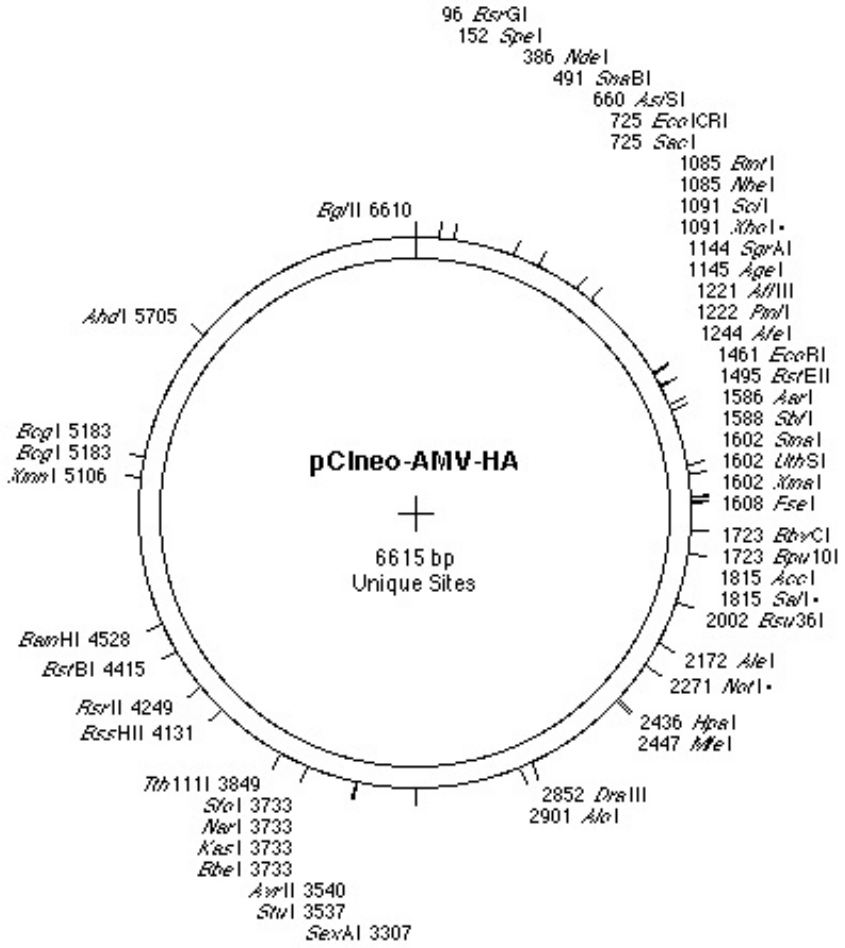
Amino acid sequence of human c-Myb, deviations observed in c-Myb$^{1-443}$ marked in yellow:

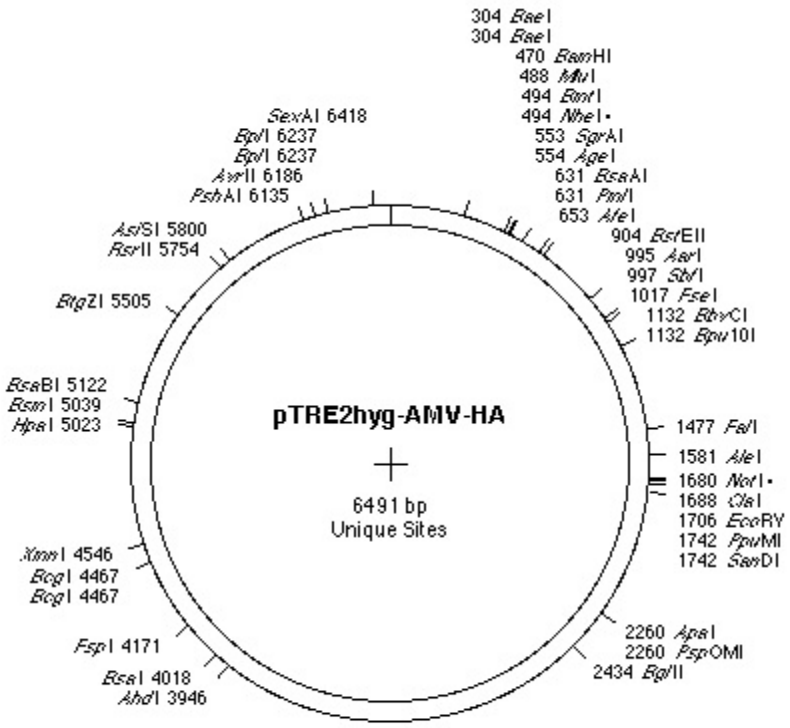
MARRPRHSIY SSDEDDEDFE MCDHDYDGLL PKSGKRHLGK TRWTREEDEK
LKKLVEQNGT DDWKVIANYL PNRTDVQCQH RWQKVLNPEL IKGPWTKEED
QRVIELVQKY GPKRWSVIAK HLKGRIGKQC RERWHNHLNP EVKKTSWTEE
EDRIIYQAHK RLGNRWAEIA KLLPGRTDNA IKNHWNSTMR RKVEQEGYLQ
ESSKASQPAV ATSFQKNSHL MGFAQAPPTA QLPATGQPTV NNDYSYYHIS
EAQNVSSHVP YPVALHVNIV NVPQPAAAAI QRHYNDEDPE KEKRIKELEL
LLMSTENELK GQQVLPTQNH TCSYPGWHST TIADHTRPHG DSAPVSCLGE

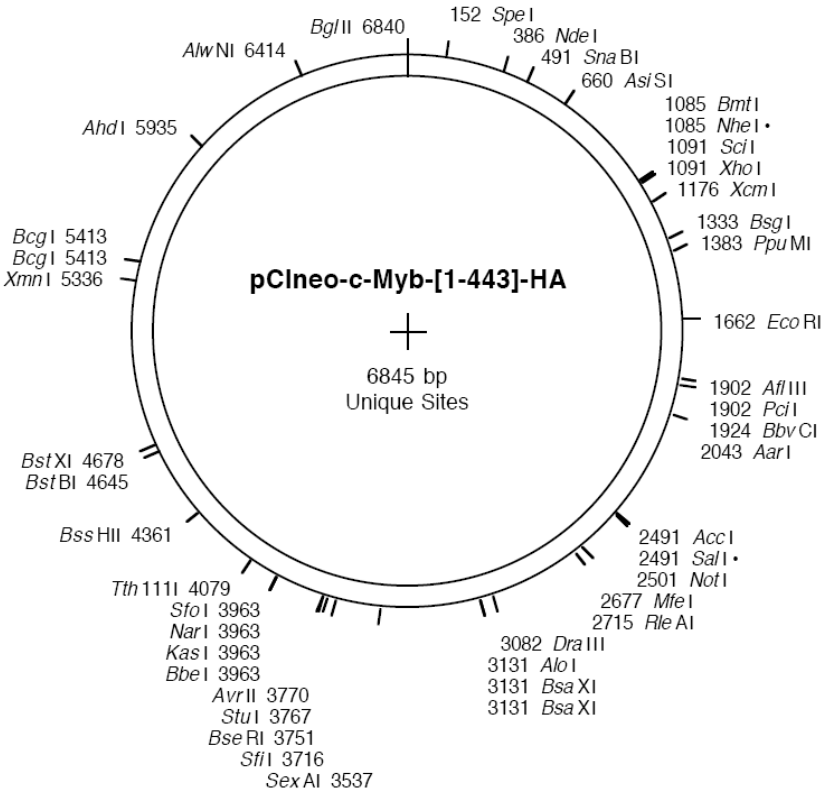
HHSTPSLPAD PGSLPEESAS PARCMIVHQG TILDNVKNLL EFAETLQFID
SFLNTSSNHE NSDLEMPSLT STPLIGHKLT VTTPFHRDQT VKTQKENTVF
RTPAIKRSIL ESSPRTPTPF KHALAAQEIK YGPLKMLPQT PSHLVEDLQD
VIKQESDESG IVAEFQENGP PLLKKIKQEV ESPTDKSGNF FCSHHWEGDS
LNTQLFTQTS PVADAPNILT SSVLMAPASE DEDNVLKAFT VPKNRSLASP
LQPCSSTWEP ASCGKMEEQM TSSSQARKYV NAFSARTLVM

Appendix V: Plasmid maps

This section lists the plasmids constructed in the work on this thesis as well as the plasmids and vectors from which they were derived. In the plasmid map presented here for pCIneo-hcM[1-443]-HA the plasmid has been given the incorrect name of pCIneo-c-Myb-[1-443]-HA. All maps with the exception of those for the vectors were created in Strider.




304 BsaI
304 BsaI
470 BamHI
488 MluI
494 EcoRI
494 NheI•
553 SgrAI
554 AgeI
631 EcoNI
631 PmlI
653 AleI
904 BsrGI
995 AarI
997 SbfI
1017 FseI
1132 EheCI
1132 EpuVI0I
1477 FseI
1581 AleI
1680 NotI•
1688 XbaI
1706 EcoRV
1742 PpuMI
1742 SanDI
2260 ApaI
2260 PspOMI
2434 BglII
Fsp I 4171
BsaI 4018
AhdI 3946
XmnI 4546
BcgI 4467
BcgI 4467
BsaBI 5122
BsmI 5039
HpaI 5023
BtgZI 5505
AsiSI 5800
RsrII 5754
SexAI 6418
EpuVI 6237
EpuVI 6237
AvrII 6186
PshAI 6135
pTRE2hyg-AMV-HA
6491 bp
Unique Sites


152 SpeI
386 NdeI
491 SnaBI
660 AsiSI
1085 BmtI
1085 NheI•
1091 SciI
1091 XhoI
1176 XcmI
1333 BsgI
1383 PpuMI
1662 EcoRI
1902 AflIII
1902 PciI
1924 BbvCI
2043 AarI
2491 AccI
2491 SalI•
2501 NotI
2677 MfeI
2715 RleAI
3082 DraIII
3131 AloI
3131 BsaXI
3131 BsaXI
Tth111I 4079
SfoI 3963
NarI 3963
KasI 3963
BbeI 3963
AvrII 3770
StuI 3767
BseRI 3751
SfiI 3716
SexAI 3537
BssHII 4361
BstXI 4678
BstBI 4645
BcgI 5413
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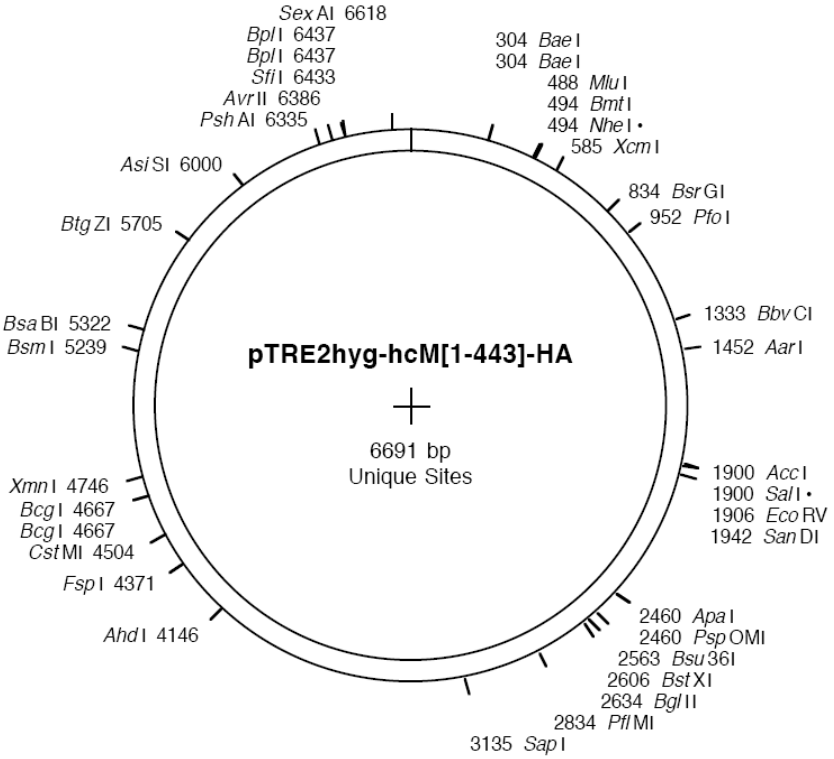
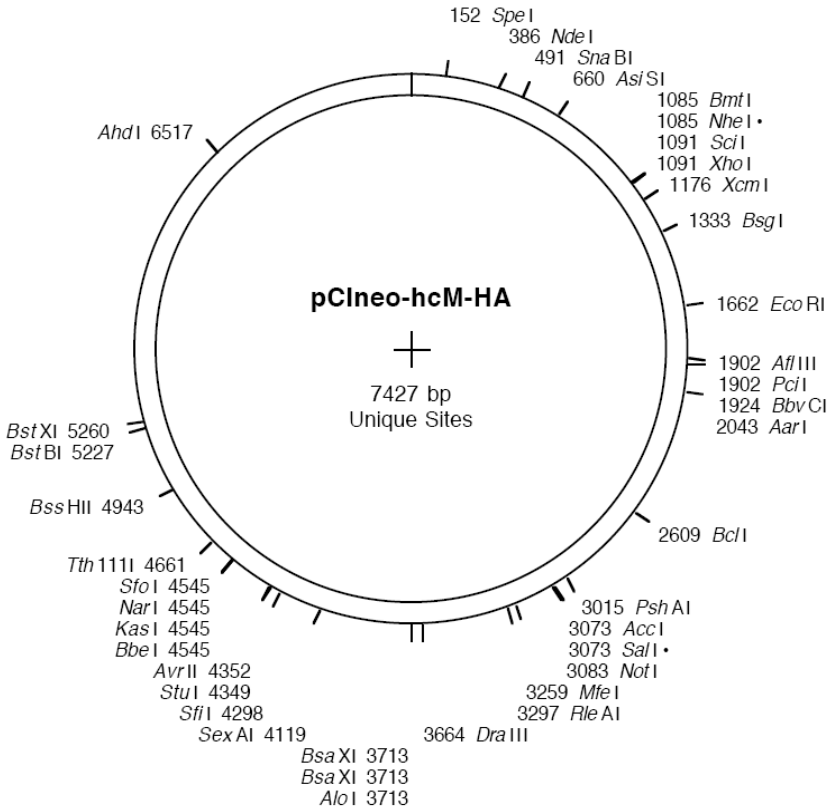
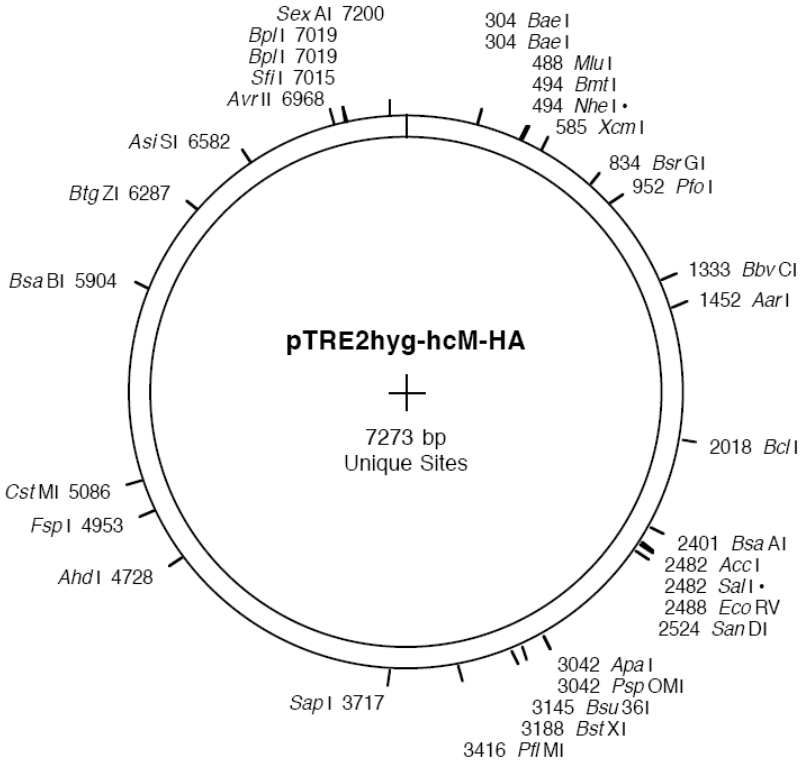
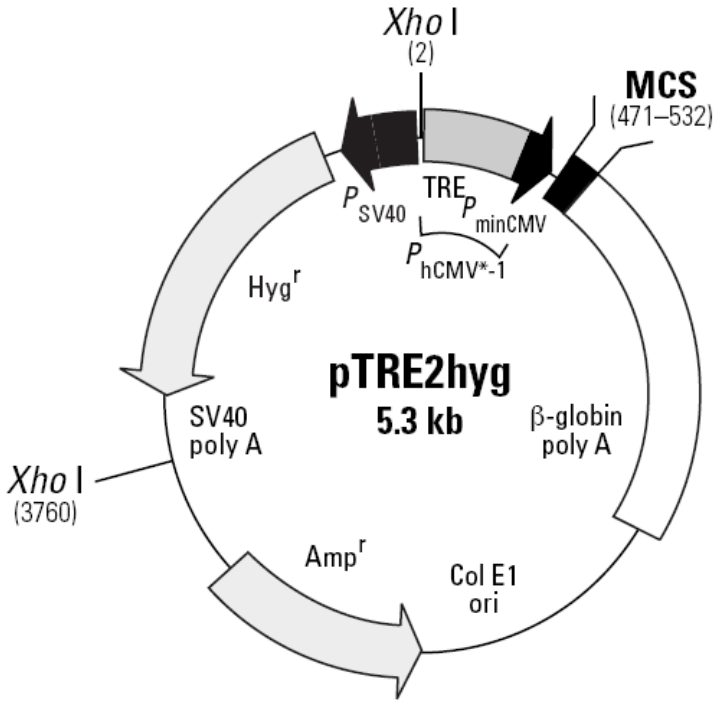
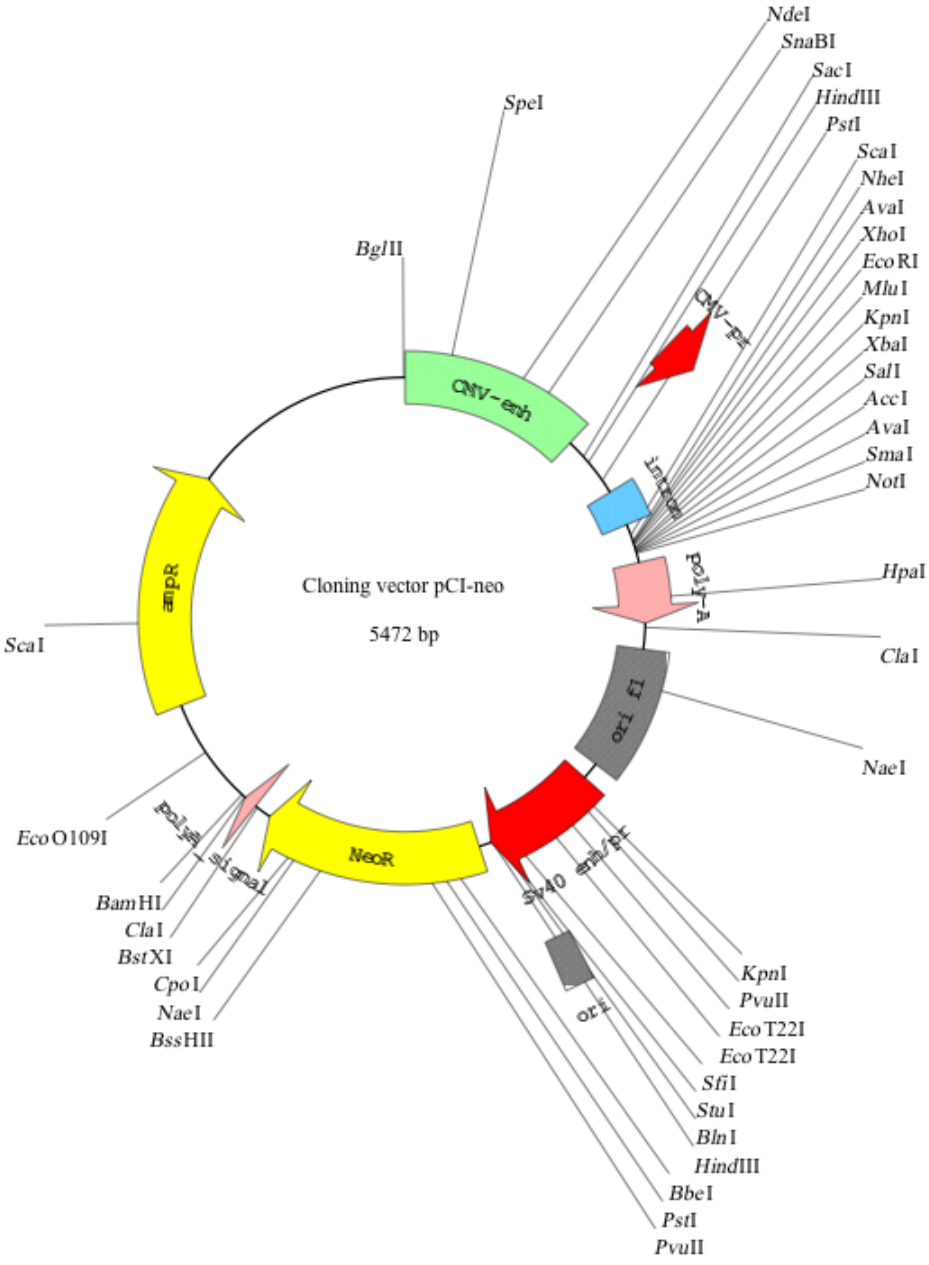
XmnI 5336
AhdI 5935
AlwNI 6414
BglII 6840
pCIneo-c-Myb-[1-443]-HA
6845 bp
Unique Sites


pTRE2hyg-hcM[1-443]-HA
6691 bp
Unique Sites
Sex AI 6618
Bpl I 6437
Bpl I 6437
Sfi I 6433
Avr II 6386
Psh AI 6335
Asi SI 6000
Btg ZI 5705
Bsa BI 5322
Bsm I 5239
Xmn I 4746
Bcg I 4667
Bcg I 4667
Cst MI 4504
Fsp I 4371
Ahd I 4146
304 Bae I
304 Bae I
488 Mlu I
494 Bmt I
494 Nhe I •
585 Xcm I
834 Bsr GI
952 Pfo I
1333 Bbv CI
1452 Aar I
1900 Acc I
1900 Sal I •
1906 Eco RV
1942 San DI
2460 Apa I
2460 Psp OMI
2563 Bsu 36I
2606 Bst XI
2634 Bgl II
2834 Pfl MI
3135 Sap I


pCIneo-hcM-HA
7427 bp
Unique Sites
Ahd I 6517
Bst XI 5260
Bst BI 5227
Bss HII 4943
Tth 111I 4661
Sfo I 4545
Nar I 4545
Kas I 4545
Bbe I 4545
Avr II 4352
Stu I 4349
Sfi I 4298
Sex AI 4119
Bsa XI 3713
Bsa XI 3713
Alo I 3713
152 Spe I
386 Nde I
491 Sna BI
660 Asi SI
1085 Bmt I
1085 Nhe I •
1091 Sci I
1091 Xho I
1176 Xcm I
1333 Bsg I
1662 Eco RI
1902 Afl III
1902 Pci I
1924 Bbv CI
2043 Aar I
2609 Bcl I
3015 Psh AI
3073 Acc I
3073 Sal I •
3083 Not I
3259 Mfe I
3297 Rle AI
3664 Dra III


SexAI 7200
BplI 7019
BplI 7019
SfiI 7015
AvrII 6968
AsiSI 6582
BtgZI 6287
BsaBI 5904
CstMI 5086
FspI 4953
AhdI 4728
SapI 3717
304 BaeI
304 BaeI
488 MluI
494 BmtI
494 NheI •
585 XcmI
834 BsrGI
952 PfoI
1333 BbvCI
1452 AarI
2018 BclI
2401 BsaAI
2482 AccI
2482 SalI •
2488 EcoRV
2524 SanDI
3042 ApaI
3042 PspOMI
3145 Bsu36I
3188 BstXI
3416 PflMI
pTRE2hyg-hcM-HA
7273 bp
Unique Sites


XhoI
(2)
MCS
(471–532)
P SV40
TRE
P minCMV
P hCMV*-1
Hygr
pTRE2hyg
5.3 kb
SV40
poly A
β-globin
poly A
XhoI
(3760)
Ampr
Col E1
ori


NdeI
SnaBI
SacI
HindIII
PstI
ScaI
NheI
AvaI
XhoI
EcoRI
MluI
KpnI
XbaI
SalI
AccI
AvaI
SmaI
NotI
SpeI
BglII
CMV-pr
CMV-enh
intron
poly A
HpaI
ClaI
ori f1
NaeI
ampR
ScaI
Cloning vector pCI-neo
5472 bp
EcoO109I
poly A signal
NeoR
BamHI
ClaI
BstXI
CpoI
NaeI
BssHII
SV40 enh/pr
SV40 ori
KpnI
PvuII
EcoT22I
EcoT22I
SfiI
StuI
BlnI
HindIII
BbeI
PstI
PvuII

Appendix VI: Web page addresses

Affymetrix	www.affymetrix.com
Amersham	www.amersham.com
Applied Biosystems	https://www2.appliedbiosystems.com/
ATCC cell database	www.atcc.org
BASE (UiO)	http://alba.uio.no/base
BASE homepage	http://base.thep.lu.se
BD Biosciences	www.bdbiosciences.com
Clontech	www.clontech.com
Eppendorf	www.eppendorf.com
Invitrogen	www.invitrogen.com
MACF	http://core.rr-research.no/index.php?section=8
MBI Fermentas	www.fermentas.com
NanoDrop	www.nanodrop.com/
New England Biolabs	www.neb.com
NMC	www.microarray.no
OMIA	http://omia.angis.org.au/
OMIM	www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM&itool=toolbar
PubMed	www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed
Qiagen	www.qiagen.com/
Roche	www.roche.com
Sigma-Aldrich	www.sigmaaldrich.com
Stratagene	www.stratagene.com
SYM	www.rikshospitalet.no/sym