DEVELOPMENT OF A CELL ASSAY TO STUDY POLYCOMB GROUP GENES

By

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B.Sc. Biology, Université Laval, 1999

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

In

THE FACULTY OF GRADUATE STUDIES

Department of Zoology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 2003

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ABSTRACT

Silencing is a form of transcriptional repression in which specialized structures of DNA or chromatin are inherited epigenetically. In Drosophila, the Polycomb group (PcG) genes are the best model system for studying silencing. Mutations in PcG genes cause posterior transformation in embryos and adults because homeotic genes are derepressed. PcG proteins act as members of oligomeric complexes. In Drosophila, PcG proteins mediate silencing of homeotic genes through complex, modular regulator elements ranging form 1 kb to 5 kb named PcG Response Elements (PREs). In transgenic flies, PREs maintain embryonic silencing of reporter constructs containing endogenous homeotic promoters and (para)segment-specific enhancers. This assay was used to identify PREs for many homeotic genes. PcG genes are required for maintenance rather than initiation and without the PRE, the transgene exhibits correct initiation of repression, but early in embryogenesis, the transgene becomes derepressed. All known Drosophila PcG genes have mammalian homologs and it appears that PcG function is conserved in mice and flies. Unfortunately, no one has identified a mammalian PRE in any system. Making transgenic mice is expensive and time-consuming, and no group has undertaken a systematic search for mammalian PREs. There is one published report of a group identifying a response element required for maintenance in mammals (Milne *et al.* 2002). Their approach tested different region of the *Hox c8* locus in a reporter assay system and depended on the used of immortalized mouse embryonic fibroblasts (MEF). Based on the same principles, this thesis was aimed at identifying a murine PRE using immortalized fibroblast and a system of reporter vectors. The main goal of this thesis was to establish three MEF mutant cell lines for the PcG genes: *rae28* the homolog of *Polyhomeotic*; *M33* the homolog of *Polycomb*, or *Asxl1*, the homolog of *Additional sex combs.* The secondary goal was to use these cells to test a putative mammalian PRE. This thesis reports the successful establishment of immortalized MEF mutant cell lines for *Asxl1, M33* and *rae28.* Our data suggest that MEF cell line of different genotypes may not be useful for studying the expression of endogenous genes. However, they may be useful for short-term studies of transgenes. One of these lines, *rae28-/-* was tested with a putative PRE from *rae28* itself. Our preliminary results suggest that a PRE exists upstream of *rae28*, but the overall low transfection efficiency of the MEF, and the resulting high variability in expression of the reporter, prevents definitive conclusions. The strengths and weaknesses of this system are discussed and suggestion are made for ways to improve these experiments in the future.

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ACKNOWLEDGMENTS

First, I would like to thank my supervisor Dr. Hugh Brock for accepting me in is laboratory and providing me with the opportunity to learn English and work in a great laboratory. I am grateful for the constant support you have put into forwarding my career. You helped me greatly in the writing of this thesis and I want to thank you for your patience and generosity.

I would like to thank all the individuals who greatly helped me with constructive criticism and stimulating discussions. The work presented in this thesis was, in large part, performed at the Terry-Fox Laboratory. I would like to thank Dr. Keith Humphries for his hospitality and for the great time had working in his laboratory. I would principally like to thank Dr. Nicolas Pineault, Dr. Jennifer Antonchuk, Dr. Andrew Thompson, Patty Rosten and Dr. Cheryl Helgason for sharing with me their precious knowledge.

My sincere thanks to all the member of the Brock Lab: Bob Argiropoulos, Ester O'Dor, Cynthia Fisher, Jacob Hodgson, Yung-Jun Wang, Sebastien Bloyer, Joyce Tan. It was always pleasant working with you. Above all I would like to thank Sebastien for the collaborative RT-PCR we did.

Finally I would like to dedicate this work to my mom, my sister and the rest of my family for their constant support and love.

LIST OF ABBREVIATIONS

-/-	Homozygous Mutant
+/+	Wild-type
Asx	*Additional sex combs*
ATP	Adenosine Triphosphate
bp	Base pair
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
COOH	Carboxyl terminal end
DMEM	Dulbecco's Modifiied Eagle's Medium
DNA	Deoxyribonucleic acid
EBNA	Epstein Barr Virus Nuclear Antigen
Esc/E(z)	Extra sex combs- Enhancer-of-zeste
FACS	Fluorescent activated cell sorter
FCS	Fetal calf serum
FRT	Flp Recombination Target
GAF	GAGA factor
GFP	Green fluorescent protein
GTFs	General transcription factors
IRES	Internal ribosome entry site
kb	Kilobase-pair
MEF	Mouse embryonic fibroblasts
MIG	MSCV-IRES-GFP vector

MIR	MSCV-IRES-RFP (dsRed) vector
Mll	Mixed Lineage Leukemia
mRNA	Messenger RNA
MSCV	Murine Stem Cell Virus
NH_2	Amino terminal end
Orip	Epstein-Barr Virus origins of replication
PBS	phosphate buffered saline
PcG	Polycomb group
PCR	Polymerase chain reaction
ph	Polyhomeotic
Pho	Pleiohomeotic
PRC1	Polycomb repressive complex 1
PRE	Polycomb response element
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse transcriptase
SV40	Simian Virus 40
Tag	Large Tumor antigen
TBP	TATA-binding protein
TBX2	*T-box factor 2*
TRE	Trithorax Group response elements
trxG	Trithorax group

CHAPTER 1 General Introduction

Maintenance and Epigenesis

Most somatic cells in an organism have the same DNA sequence, but a wide variety of intra- and extracellular stimuli change gene expression patterns in individual cells. In development, and throughout the life of an organism, cells must pass on their gene expression patterns to their daughter cells. Gene expression patterns are initiated by the binding of transcription factors to regulatory regions that act to activate or repress gene activity. Yet the expression of transcription factors is usually transient, the proteins have short half lives, and they are stripped from DNA prior to replication. On formal grounds, this suggests that initiation of gene expression is separable from maintenance of gene expression from mother cell to daughter cells. So how is the gene expression pattern maintained in daughter cells? It cannot be DNA sequence alone, since all cells have the same DNA sequence. This suggests that an epigenetic mechanism is important in maintenance.

In recent years it has become evident that chromatin plays a major role in maintenance. Failure to maintain the established gene expression patterns can lead to developmental defects or cancer (reviewed in Jacobson and Pillus 1999; Klochendler-Yeivin and Yaniv 2001; Muyrers-Chen and Paro 2001; Jones and Baylin 2002; Neely and Workman 2002). Examples of epigenetic maintenance include X-inactivation in mammals, parental imprinting, position-effect variegation in Drosophila, and mating-type silencing and telomeric position-effects in yeast (Henikoff 1994; Karpen 1994; Barlow 1995; Loo and Rine 1995; Lee and Jaenisch 1997). Overall, it appears that changes in chromatin structure, modifications to histones, and recruitment of specific proteins all play a role in maintenance. The epigenetic

mark that is passed from mother cell to daughter cells is now thought to be specific patterns of histone modification, principally methylation, but also including acetylation and phosphorylations (Jenuwein and Allis 2001). This has come to be called the "histone code" hypothesis (Strahl and Allis 2000).

Silencing and the Polycomb group

Silencing is a form of transcriptional repression in which specialized structures of DNA or chromatin are inherited epigenetically. In Drosophila, the Polycomb group (PcG) genes are the best model system for studying silencing. PcG genes were identified originally because they encode chromatin proteins required to maintain silencing of homeotic and other loci (Jürgens 1985; McKeon and Brock 1991; Simon *et al.* 1992; McKeon *et al.* 1994; Pelegri and Lehmann 1994). Mutations in PcG genes cause posterior transformation in embryos and adults because homeotic genes are derepressed. A key insight came from observations that embryos lacking maternal and zygotic PcG mRNA exhibit wild-type initiation of homeotic genes, but that the initial expression pattern was not maintained. This shows that PcG genes are required for maintenance rather than initiation (Struhl and Akam 1985; Jones and Gelbart 1990; Soto *et al.* 1995).

In Drosophila at least 15 PcG genes have been identified (Simon 1995; Yamamoto *et al.* 1997). Based on deletion analysis it is estimated that as many as 30-40 PcG genes may exist (Jürgens 1985; Landecker *et al.* 1994). Ten PcG genes have been cloned. All known PcG genes encode chromatin proteins and they bind to many targets on the chromosomes. Many PcG proteins have domains that are conserved between flies and mammals, and that are shared with known chromatin proteins (Paro and Hogness 1991; Alkema *et al.* 1995; Kyba

and Brock 1998; Satijn and Otte 1999). PcG proteins act as members of oligomeric complexes. The two best studied are the Polycomb repressive complex 1 (PRC1) (Shao *et al.* 1999) and the Extra sex combs/Enhancer-of-zeste (Esc/E(z)) complexes (Cao *et al.* 2002; Czermin *et al.* 2002; Kuzmichev *et al.* 2002; Muller *et al.* 2002). The Esc/E(z) complex acts early in embryogenesis and is thought to set the stage for the PRC1 complex which set the long-term memory. Both complexes are conserved between flies and mammals, although redundant copies of PcG genes are present in mammals and the existence of complexes with varied composition has been proposed (Satijn and Otte 1999).

It is not known how PcG proteins silence their target loci. At least 6 potential mechanisms of PcG-dependent silencing have been proposed. These models are not mutually exclusive. The most popular model is that PcG proteins alter chromatin structure to resemble heterochromatin, thus preventing access of transcription factors, or of general transcription factors (GTFs) (Alberts and Sternglanz 1990; Gaunt and Singh 1990; Paro 1990). A model rapidly gaining favour is that PcG proteins alter the histone code (Jenuwein and Allis 2001; Simon and Tamkun 2002) by chemically modifying the histones. The Esc/E(z) complex is a histone methyltransferase (Cao *et al.* 2002; Czermin *et al.* 2002; Kuzmichev *et al.* 2002; Muller *et al.* 2002), and there are reports that histone deacetylases associate with PcG proteins (van der Vlag and Otte 1999; Tie *et al.* 2001; Chang *et al.* 2002). A third model is that PcG proteins antagonize the proteins required to maintain activation. These proteins, termed the trithorax group (trxG) are members of ATP-dependent chromatin remodelling complexes, or are histone modifying enzymes (Brock and van Lohuizen 2001; Simon and Tamkun 2002). A fourth model is that PcG proteins antagonize GTFs (Bienz 1992), a view supported by recent observations that PcG complexes contain TATA-binding protein (TBP) and TBP-associated

factors, and bind to promoters (Orlando *et al.* 1998; Breiling *et al.* 2001; Saurin *et al.* 2001). A fifth model is that interactions between PcG proteins, bound on PcG binding sites placed along the DNA, results in looping of the DNA preventing interaction of the enhancer and promoter (Pirrotta 1995), and a sixth model is that PcG proteins localize target genes into transcriptionally inactive nuclear compartments (Schlossherr *et al.* 1994).

Targets of PcG regulation

In Drosophila, the best understood targets of PcG silencing are the homeotic genes. As noted above, PcG mutants exhibit homeotic transformations that arise from misexpression of homeotic genes (Struhl and Akam 1985; Jones and Gelbart 1990; McKeon and Brock 1991; Simon *et al.* 1992; Soto *et al.* 1995). Histochemical studies show that antibodies to individual PcG proteins bind to polytene chromosomes at about 100 targets, including the homeotic loci, consistent with the idea that PcG proteins regulate homeotic genes. In many cases (Polycomb, Polyhomeotic, Polycomblike, Sex combs on midleg) there is complete overlap of binding sites (Zink and Paro 1989; DeCamillis *et al.* 1992; Lonie *et al.* 1994; Bornemann *et al.* 1998), whereas there is less overlap with Additional sex combs, Extra sex combs, Enhancer of Zeste, and Posterior sex combs (Rastelli *et al.* 1993; Carrington and Jones 1996; Sinclair *et al.* 1998; Tie *et al.* 1998). In total, PcG proteins bind about 150 discrete targets on polytene chromosomes. The identity of most of these targets is unknown. Genetic studies (McKeon *et al.* 1994; Pelegri and Lehmann 1994; Randsholt *et al.* 2000) suggest that segmentation genes like *hunchback, even-skipped, hedgehog, patched* and *engrailed* are PcG targets. Recently, chromatin immunoprecipitation (ChIP) experiments have confirmed that PcG proteins bind to *engrailed* and *invected* (a paralog of *engrailed*)

(Strutt and Paro 1997) and to *hedgehog* (Maurange and Paro 2002). One unexpected observation is that PcG proteins appear to bind to PcG loci themselves (Zink and Paro 1989; DeCamillis *et al.* 1992). This is unexpected because in flies PcG expression is ubiquitous (DeCamillis and Brock 1994), and because PcG proteins are thought to silence their targets. Nevertheless, genetic analysis shows that *polyhomeotic (ph)* autoregulates itself, and its expression is sensitive to mutations in *Posterior sex combs* (Fauvarque *et al.* 1995). Unpublished studies by Bloyer, Cavalli, Brock and Dura (personal communication) confirm that PcG proteins bind to regulatory regions of *ph*. These regions will be described more fully in the next chapter.

PcG Response Elements (PREs)

In Drosophila, PcG proteins mediate silencing of homeotic genes through PcG Polycomb Response Elements (PREs). PREs are complex, modular regulator elements from 1-5 kb long in homeotic loci (Simon *et al.* 1990; Chan *et al.* 1994; Gindhart and Kaufman 1995; Muller *et al.* 1999; Tillib *et al.* 1999; Horard *et al.* 2000; Hodgson *et al.* 2001). Like enhancers, PREs are orientation-independent, can act 5' or 3' to the promoter, and can act at distance of up to 50 kb (Pirrotta 1997). In transgenic flies, PREs maintain embryonic silencing of reporter constructs containing endogenous homeotic promoters and (para)segment-specific enhancers. This assay was used to identify PREs for many homeotic genes. Without the PRE, the transgene exhibits correct initiation of repression, but early in embryogenesis, the transgene becomes derepressed, and is expressed in every segment (Simon *et al.* 1990; Chan *et al.* 1994; Chiang *et al.* 1995; Gindhart and Kaufman 1995; Kapoun and Kaufman 1995; Hagstrom *et al.* 1997; Mihaly *et al.* 1997; Shimell *et al.* 2000). Silencing

by transgenes is abrogated in PcG mutants, linking silencing, the PRE, and PcG genes. Different PREs are sensitive to mutations in different PcG genes, suggesting that different PcG proteins act at different PREs (Fauvarque and Dura 1993; Kassis 1994; Gindhart and Kaufman 1995). In Drosophila, PREs are intermingled with trxG response elements (TREs), suggesting that maintenance of activation and repression are coordinated jointly (Tillib *et al.* 1999). For this reason, it has been suggested that PREs and TREs be renamed maintenance elements (Brock and van Lohuizen 2001). However, for this thesis, we will refer to PREs.

How PcG proteins are recruited to PREs remains unclear. Only one PcG protein, Pleiohomeotic (Pho), binds DNA directly (Brown *et al.* 1998), but Pho sites themselves are not found in all PREs (Mihaly *et al.* 1998), and Pho sites are not sufficient to create a PRE (Brown *et al.* 1998; Fritsch *et al.* 1999; Tillib *et al.* 1999; Shimell *et al.* 2000). It is suggested that Pho recruits Polycomblike to PREs (Mohd-Sarip *et al.* 2002). Binding sites for the GAGA factor (GAF) are found in most PREs, and GAF has been found in PcG complexes, suggesting that GAF may recruit PcG proteins to PREs (Hagstrom *et al.* 1997; Strutt *et al.* 1997; Horard *et al.* 2000; Hodgson *et al.* 2001; Faucheux *et al.* 2003). Another protein that binds the GAGA sequence, Pipsqueak, may also recruit PcG proteins to PREs (Hodgson *et al.* 2001; Huang *et al.* 2002). Zeste has also been proposed to be required for PcG function (Hur *et al.* 2002), and is found in PRC1 (Saurin *et al.* 2001). Detailed analysis of the *engrailed* PRE suggests that multiple proteins act to recruit PcG proteins (Americo *et al.* 2002).

Mammalian PcG genes

All known Drosophila PcG genes have mammalian homologs (Gould 1997; Schumacher and Magnuson 1997; Stankunas *et al.* 1998; van Lohuizen 1999; Brock and van

Lohuizen 2001). As in Drosophila, PcG proteins are members of large, multimeric complexes (Satijn and Otte 1999). Mice in which PcG genes have been mutated exhibit various hematopoietic defects (Raaphorst *et al.* 2001), and have homeotic transformations in the anterior-posterior axis (van der Lugt *et al.* 1994; Alkema *et al.* 1995; Akasaka *et al.* 1996; Schumacher *et al.* 1996; Core *et al.* 1997; Takihara *et al.* 1997; Katoh-Fukui *et al.* 1998). The hematopoietic defects may be correlated with derepression of *Hox* genes, as *Hox* over-expression has dramatic effects in hematopoiesis and leukemia in mammals (Sauvageau *et al.* 1995; Sauvageau *et al.* 1997; Thorsteinsdottir *et al.* 1997; van Oostveen *et al.* 1999). In mammals PcG genes repress targets required for cell-cycle regulation like *p16/INK4a* (Jacobs *et al.* 1999) and *c-myc* (Tetsu *et al.* 1998). Strikingly, *M33,* the mouse homolog of *Polycomb* can rescue *Polycomb* mutant phenotypes in flies, suggesting that functions of PcG proteins are strongly conserved (Muller *et al.* 1995). Unlike in flies, mammalian PcG proteins are not expressed ubiquitously (Gunster *et al.* 2001). In fact, expression varies greatly among tissues and even among specific cell types within a particular tissue. Overall, it appears that PcG function is conserved in mice and flies, but PcG proteins are likely to have acquired additional functions in mice.

Identification of mammalian PREs

No one has identified a mammalian PRE in any system. The obvious place to look is in the Hox genes of mice. If regulation of Hox genes in mice parallels that in flies, one would expect that mice expressing transgenes with *Hox* regulatory DNA would show normal initiation of spatially-regulated expression, but that unless a PRE was present, would fail to maintain regulated expression. Interestingly, despite the fact that many labs have made such

transgenes, no one has reported a problem with maintenance, even though the *Hox* regulatory sequences chosen for analysis were quite small (see Tuggle *et al.* 1990; Behringer *et al.* 1993; Gould *et al.* 1997 for a small sample). One could propose that mammalian Hox genes are regulated differently than Drosophila Hox genes. But this explanation can be ruled out because analysis of Hox expression in PcG and trxG mutations in mice shows that initiation of Hox expression is normal, but then is not maintained (Yu *et al.* 1998; Tomotsune *et al.* 2000), suggesting that maintenance is necessary for Hox regulation. It may be that murine PREs are located very close to promoters, so that all transgenes contain PREs. While this possibility has not been tested directly, for at least *Hox c8*, it is known that the DNA element required for maintenance of activation is located about 8 kb 3' to the gene, consistent with the distant location of maintenance elements in Drosophila (Bradshaw *et al.* 1996).

In any case, making transgenic mice is expensive and time-consuming, and no group has undertaken a systematic search for mammalian PREs. Several laboratories have used Drosophila to assay mammalian sequences for maintenance of homeotic gene expression, but successful results have not been published.

There is one published report of a group identifying a response element required for maintenance in mammals (Milne *et al.* 2002). The approach they used depended on the use of immortalized mouse embryonic fibroblasts (MEF). If mouse embryos are trypsinized, and cells are plated on plastic dishes, fibroblasts attach to the plastic. Primary fibroblasts have a very limited lifespan (about 5 passages), but can be immortalized by transfection with large T antigen. In this case, MEF were obtained from wild-type and *Mll* mutant mice. *Mll* is a homolog of *trithorax*, and is required for maintenance of homeotic gene activation (Yu *et al.* 1998). When expression of *Hox* genes was compared in wild-type and *Mll-/-* MEF, the same

Hox genes that were *Mll*-dependent in embryos were also *Mll*-dependent in MEF, suggesting that MEF were a good model system for analysis of *Hox* gene expression (R. Hanson and J. Hess, personal communication). Milne et al. (2002) constructed reporter gene vectors regulated by the *Hox c8* basal promoter, plus different putative regulatory DNA from the locus, and screened for DNA sequences that activated the reporter in *Mll*+/+ but not *Mll*-/- MEF. When a putative sequence was found, it was then rechecked in *Mll*-/- fibroblasts that had been transfected with an expression vector for *Mll*, to confirm that the change in reporter activity was *Mll*-dependent, and not an indirect effect of the *Mll* mutation. Interestingly, Mll appears to act directly on the *Hox c8* promoter, rather than through a TRE (Milne *et al.* 2002).

The goals of this thesis

Based on the above experiments we hypothesized that there would be other regulatory DNA sequences that would act as PREs for different PcG genes. Thus we wished to develop a similar assay system for PREs in mammals. Understanding epigenetic gene regulation will have important implications for human biology and diseases. Unfortunately, our progress in this field has been limited by a lack of known target binding site for mammalian PcG homologs. The main goal of this thesis was to establish MEF mutant for three different PcG genes: *rae28,* the homolog of *ph*; *M33,* the homolog of *Polycomb*, and *Asxl1*, the homolog of *Additional sex combs.* The secondary goal was to use these cells to test a putative mammalian PRE. The reasons for these choices will be introduced in the next chapter. This thesis reports the successful establishment of three immortalized MEF cell lines mutant for *Asxl1, M33* or *rae28.* One of these lines, *rae28*-/- was tested with a putative PRE from *rae28* itself. Our preliminary results suggest that a PRE exists upstream of *rae28*, but the overall low

transfection efficiency of the MEF, and the resulting high variability in expression of the reporter, prevents definitive conclusions. The strengths and weaknesses of this system are discussed, and suggestions are presented for ways to improve these experiments in the future.

CHAPTER 2 Materials and methods

Molecular biology

Preparation of DNA, restriction enzyme digestion, bacterial transformation, agarose gel electrophoresis were performed according to standard procedures (Sambrook *et al.* 1989). Enzymes were purchased from New England Biolabs (Pickering, ON, Canada) or Invitrogen (Burlington, ON, Canada). Large-scale DNA preparations (Maxi-prep kit, Qiagen, Mississauga, ON, Canada)) were performed according to the manufacturer's directions.

Cell lines

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC) unless specified otherwise. The $GP^{+}E86$ (Markowitz *et al.* 1988) cells containing the MSCV-IRES-GFP (E86-MIG) and MSCV- rae28 cDNA-IRES-GFP (E86-MIG-rae28) provirus constitutively producing ecotropic virus were obtained from Dr. Yoshihiro Takihara (Department of Developmental Biology and Medicine, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan) and were produced in Dr. Keith Humphries' Laboratory (Terry-Fox Laboratory, BC Cancer Research Centre, Vancouver, Canada). All cell lines were maintained in tissue culture medium; Dulbecco's modified essential medium (DMEM) (StemCell Technologies, Vancouver, BC, Canada) with 10% fetal calf serum (FCS) (Invitrogen) and 100 UI penicillin/ 100μg streptomycin (Invitrogen) per ml. Established cell lines were cultivated and frozen according to standard procedures (Freshney 2000).

Mouse embryonic fibroblasts (MEF)

M33 +/+ and *M33* -/- MEF were obtained from day 14.5 fetal livers from wild-type or *M33* mutant mice (Katoh-Fukui *et al.* 1998)(a gift from Dr. Toru Higashinakagawa, Department of Biology, Waseda University, Shinjuku, Tokyo, Japan). *rae28* +/+ and *rae28* -/- MEF were obtained from day 14.5 embryos wild-type or *rae28* mutant mice (Takihara *et al.* 1997) (a gift of Dr. Yoshihiro Takihara). *Asxl1*+/+ and *Asxl1*-/- MEF were obtained from day 12.5 embryos wild-type or *Asxl1* mutant mice (C. Fisher and H. Brock, in preparation). Pregnant females were sacrificed by cervical dislocation and the uteri removed. Each decidua was transferred to a sterile petri dish. The muscle layer of the uterus was removed and the Reichert's membrane cut to allow the separation from of the placenta from the yolk sac surrounding the embryo. The embryos were freed from the yolk sac, decapitated and eviscerated before being minced with sterile scissors. The tissues were disrupted using a 16 gauge blunt-end needle (StemCell Technologies) and a 12cc syringe in a small volume of PBS (StemCell Technologies). For embryos of day 13.5 or older, 1 ml of collagenase (Invitrogen) was added to each embryo and incubated for 1 hr at 37 °C with frequent mixing before disrupting the embryonic cells with the blunt needle. The cells were then resuspended in 40 ml of PBS, centrifuged and then plated in 10 cm tissue culture dishes (BD Falcon™, Oakville, Canada) in tissue culture medium. After allowing cells to attach to the culture dish, unattached cells were removed by aspiration, and the primary fibroblasts were grown to confluence. DNA from the liver tissue was purified using the DNAzol (Invitrogen) standard protocol and used to genotype the embryos by polymerase chain reaction (PCR) as described in the next section. MEF obtained from wild-type and -/- embryos were expanded to have enough to freeze three vials containing ~1X10^6 cells. The freezing media used for the MEF

contains 45% DMEM, 45% FCS and 10% Dimethylsulfoxide (DMSO) (Sigma, Oakville, Canada).

Retroviral infection of MEF for immortalization

I. TBX2 immortalizing vector

The LZRS-delta-BamH1-*TBX2*-ires-eGFP vector (see Figure 3-2) was a generous gift from Dr. M. van Lohuizen from the Netherlands Cancer Institute, Amsterdam, The Netherlands (Jacobs *et al.* 2000). This is a modification of the Moloney murine leukemia virus-based retroviral vector, LZRS, system (Kinsella and Nolan 1996) producing a retrovirus vector capable of expressing TBX2 and GFP in infected cells (Jacobs *et al.* 2000). The LZRS system uses two elements from the Epstein-Barr virus the Epstein-Barr Virus origins of replication (*Orip*) and the Epstein Barr Virus Nuclear Antigen 1 (EBNA-1), to confer stable episomal maintenance capabilities under puromycin selection. Cells infected with the retrovirus cannot be selected for but GFP expression serves as a convenient marker.

II. Production of high-titre TBX2-immortalizing retrovirus

Production of high-titre helper-free retrovirus was carried out by standard procedures, to obtain high-titre supernatants from transfected Phoenix™ Ecotropic packaging cell lines (Kinsella and Nolan 1996). Phoenix™ cells were plated to achieve ~70% confluence in a 10cm tissue culture plate the day of the transfection. 10 μg of circular LZRS-delta-BamH1-*TBX2*-ires-eGFP plasmid was transfected according to the calcium phosphate protocol of the Cellphect transfection Kit (Amersham Biosciences, Baie d'Urfe, Canada). At 24 hr post-transfection, medium was replaced with 8 ml of fresh medium. At 48 hr post-transfection,

supernatant containing the retroviral vector was collected for titer or frozen at -80 °C to await titering at a later time point. All cells were trypsinized with 1,5 ml of trypsin-EDTA (Invitrogen) and placed into two 10 cm tissue culture plates containing fresh medium and the antibiotic puromycin at concentration of 600 μg/ml and 1000 μg/ml (Sigma). Cells were maintained in the selective medium until three confluent plates were obtained. Subsequently cells were ready to be frozen or used for infection. Cells were maintained in the selective medium until 48 hr prior to any given collection of retroviral vector. At 48 hr prior to collection for retroviral vector, cells were overlaid with puromycin-free medium. Then 24 hr before collecting the *TBX2*-immortalizing virus, medium was again replaced with 8 ml of fresh puromycin-free medium. Collected viral supernatant that was not used immediately for titer determination or MEF immortalization was frozen on dry ice in 4 ml aliquots before being transferred to a -80 °C freezer to await use at a later time. All viral supernatants were filtered through 45 μm low protein binding Gelman Acrodisc® filters (Fisher Scientific, Nepean, Canada) before being frozen or used for infection.

III. Titer determination

Frozen or fresh vector stocks collected from producer cell lines were serially diluted with fresh DMEM to obtain the 1/1, 1/3, 1/10 and 1/33 final ratio (retroviral supernatant/final volume) in a total volume of 500 μl. Diluted and undiluted vector stocks were overlaid onto $1.5X10^5$ NIH-3T3 cells that had been plated the day before in 6-well plates. Polybrene (hexadimethrine bromide, Sigma) was then added to the vector-containing medium to a final concentration of 5 μg/ml to promote viral entry. At 4 hr post-infection, 2.5 ml of prewarmed fresh medium was added. To determine the number of cells per well at the time of infection,

cells from two wells were harvested and counted using a hemocytometer. At 48 hr post-infection, cells were harvested and the percentage of GFP expressing cells was determined by FACS. Titer (transducing units/ml) was calculated as: 2X(% of GFP positive cells)(NIH-3T3 per well at infection time)(dilution factor).

IV. MEF infection and immortalization with TBX2-immortalizing vector

MEF were plated in 10 cm tissue culture plates at less than 50% confluence. 8 ml of fresh retroviral *TBX2*-immortalizing supernatant was directly transferred from the high-titer producer Phoenix™ cells to the MEF after being filtered through a 45 μm low protein binding Acrodisc® filter (Gelman). Polybrene (hexadimethrine bromide, Sigma) was then added to the vector-containing medium to a final concentration of 5 μg/ml. 4 hr post-infection, medium was replaced with 10 ml of fresh medium. Cells were passaged 4 days after infection and the percentage of GFP positive cells was determined by FACS. Subsequently cells were kept in culture until stable cell lines were established. Genotypes of established cell lines were confirmed by PCR and large numbers of samples were then frozen.

V. Genotyping of the embryos and MEF by PCR

Genomic DNA was isolated from confluent 6 cm tissue culture plates or from the embryo liver tissues using the reagent DNAzol (Invitrogen) following the recommendations provided by the manufacturer. DNA pellets were resuspended for at least 24 hr at 4 °C in 40 μl of Ultrapure™ Water. Each PCR sample used 1/4000 of the DNA obtained, dNTPs at 0.2 mM, 0.2 μM of each primers (see Table 2-1 for primer details), PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 2 mM or 1.5 mM $MgCl_2$ for *Asxl1* -/- and *Asxl1* +/+ or *rae28* -/-,

rae28 +/+, *M33* -/- and *M33* +/+ respectively and 2 U or 1.25 U Platinum® Taq DNA polymerase for Asxl1 -/- and Asxl1 +/+ or *rae28* -/-, *rae28* +/+, *M33* -/- and *M33* +/+ respectively. PCR conditions were: for *Asxl1* +/+ and *Asxl1* -/-, 30 sec at 94 °C, 30 sec at 62 °C and 30 sec at 72 °C for 40 cycles; for *rae28* +/+ and *rae28* -/-, 30 sec at 94 °C, 30 sec at 55 °C and 40 sec at 72 °C for 40 cycles; for *M33* +/+ and *M33* -/- 30 sec at 94 °C, 30 sec at 62 °C and 30 sec at 72 °C for 40 cycles. All reagents used were from Invitrogen.

Analysis of Gene Expression in PcG MEF

Total RNAs were isolated from $4x10^6$ cells using the Spin Protocol of the RNeasy® mini kit (Qiagen) according to the manufacturer's direction. Cells were grown in a monolayer, trypsinized and collected as a cell pellet prior to lysis. Each sample was homogenized by passing the lysate 5 times through a 20-gauge needle fitted to an RNase-free syringe. Prior to cDNA amplification, remaining DNA was removed using the Amplification Grade DNase from Invitrogen according to the manufacturer's direction. 8 µl of the resulting RNA was used for the cDNA amplification using the Superscript kit (Invitrogen) following the manufacturer's protocol. For each cell line, a control without reverse transcriptase was also produced. The resulting cDNA were stored at –80 °C. PCR amplification of the transcript of interest was performed using the primers described in Table 2-2 and the PCR Master kit (Roche, Laval, Quebec). PCR conditions were 30 sec at 94 °C, 30 sec at 60 °C and 30 sec at 72 °C for 27-35 cycles.

Primer construction

Required primers were designed or verified using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and identity with murine sequence was determined with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Table 2-1 Genotyping PCR primers

Primers	Orientation	Sequence 5' -> 3'	Length	Tm	+/+ pb	-/- bp[a]	Source/reference
mAsxKO2-TFor	For	CCTCTGCATCCTATAATGATGGC	23		(TFor→TRev) 253	(TFor→ PGK-PR-AS) 426	From Cynthia Fisher
mAsxKO2-TRev	Rev	CACATCAGCGGAGTCCTCTGG	21				
PGK-PR-AS	Rev	CCGCTTCCATTGCTCAGCGGT	21				
M33-A	For	GTAGCCAAGCCAGAGCTGAA	21		(TFor→Tre) 200	(TFor→ PGK-PR-AS) 325	GenBank : X62537
M33-B	Rev	AGAGGCCTCTTTGGTGTGG	19				
PGK-PR-AS	Rev	CCGCTTCCATTGCTCAGCGGT	21				
rae28-1	For	GGAGCTAGCCTGTCGACTG	19		(TFor→TRev) 225	(TFor→ PGK-PR-AS) 325	Dr. Yoshihiro Takihara
rae28-2	Rev	CTGCCTTGATGTTCCTGG	18				
rae28-3 (neo^r)	Rev	GCGCTGACAGCCGGAACACG	20				

[a]A longer amplification fragment is also possible but is not present at the short extension time used.

Table 2-2 Primers for RT-PCR

Primers	Orientation	Sequence 5' -> 3'	Length	Tm	Product bp	Source/reference
B-actin-F	For	TGGGAATGGGTCAGAAGGACTC	22		228	Tom Milne (Mice)
B-acin-R	Rev	GGGTCATCTTTTCACGGTTGGC	21			
HoxA3-F	For (1076)	CACCGAGCGCCAGATCAAGA	20	64	326	Dr. Yoshihiro Takihara
HoxA3-R	Rev (1402)	CATAGCTGCCATTGCCCTGC	20	64		
HoxA4-F	Rev	GTGTGGGCTGTGAGTTTGTGCTT	23		345	(Kostic and Capecchi 1994)
HoxA4-R	For	AAGGGCAAGGAGCCGGTGGTGTA	23			
HoxA5-F	For (98)	GCGAGCCACAAATCAAGCAC	20	62	439	Dr. Yoshihiro Takihara
HoxA5-R	Rev (537)	TCTCTGCTGCTGATGTGGGT	20	62		
HoxA7-F	For (133-152)	TAGCAAATATACGGCGGGGG	20	58	332	(Kessel *et al.* 1987)
HoxA7-R	Rev (465-444)	ATCCAGGGGTAGATGCGGAAAC	22			
HoxA9-F	For	GAAGGCACACACACACAAGG	20		~300	Tom Milne (Mice)
HoxA9-R	Rev	CCCAGCACAGAAGTTTGAGTCAC	23			
HoxB3-F	For (3191)	GGAACCTGGACTACAACGGG	20	64	512	Dr. Yoshihiro Takihara
HoxB3-R	Rev (3703)	CCTGTCTTGGCTCCCTCTAC	20	64		
HoxB4-F	For (932)	CTATCTACCCTACCCTCACT	20	60	375	Dr. Yoshihiro Takihara
HoxB4-R	Rev (1207)	TGAAAACCAGGCGCAGCAGA	20	62		
HoxB7-F	For (343-362)	AGCAGAGGGACTCGGACTTG	20	56	588	GenBank :Y00436
HoxB7-R	Rev (931-911)	AACATAGAAAAGGGGAGGCAC	21	56		
HoxC4-F	For (318-339)	GGAGGAGAATGTCTGCTGCATG	22	59.7	533	GenBank : X63507
HoxC4-R	Rev (850-831)	TCCAATTCCAGCGTCTGGTG	20	59.7		
HoxC5-F	For (64-88)	CAAACTTGTGGGAACTATGGATCGG	25	59.5	437	GenBank : NM_008271
HoxC5-R	Rev (501-479)	GGTCTGGTAGCGCGTGTAACTGG	23	59.5		

Table 2-2 Continued

HoxC6-F	For (441-462)	GTTCTGAGCAGGGCAGGACTGC	22	59.8	102	GenBank : X16511
HoxC6-R	Rev (543-523)	GCTCCGTAACCGACCCCACTG	21	60.2		
HoxC8-F	For	AGCATGAGCTCCTACTTCG	19		396	Tom Milne, from Rob Hanson (Mice)
HoxC8-R	Rev	CTGATTTAAGTGGCCTTGTCC	21			
HoxC9-F	For (20-44)	GGTCCCTGATGAGATATGATTCGTC	25	56.7	486	(Erselius *et al.* 1990)
HoxC9-R	Rev (506-484)	CAGGACGGAAAATCGCTACAGTC	23	56.7		
Rae 28-2-F	For (3190-3209)	GTGCTACATGGTGACAGCTT	20		251	(Lessard *et al.* 1998) (3' UTR of nt 3190-3440 of Acc # U63386)
Rae 28-2-R	Rev (3440-3421)	AGCTAGGAAAGCTGACCTCT	20			
Rae28-3-F	For (1040-1059)	GGCAGAAGCAGATGGGAGTG	20	55	352	(Koga *et al.* 2002)
Rae28-3-R	Rev (1392-1373)	GAGGGGCAGTGAGGGTTGTT	20	55		
mel18-F	For	AGTTCCTCCGCAACAAAATG	20	55		
mel18-R	Rev	GGAGCGCCATTAACAGTCAT	20	55		
scmh1-F	For	AGCCACCATCACCTTCCCACAACTT	25	55		
scmh1-R	Rev	ACTGCCAGGCTTGGGACCTCTCTTC	25	55		
mph2-F	For	CACTGGCATCTCCAGGTTTT	20	55		
mph2-R	Rev	GAGGTATGGGGAAGGGGTTA	20	55		
M33-F	For (1346-1365)	AGCTGACTTGCAAGGCAACG	20		322	(Lessard *et al.* 1998)(Downstream chromodomain nt 1346-1667 of Acc #X62537)
M33-R	Rev (1667-1648)	GACTCCTTCACGGTGACAGT	20			
Asxl-1-F	For (43-60)	GAAGGACCCGCGTGGAGC	18	62	340	From Cynthia Fisher data (mAsx cDNA seq.)
Asxl-3-R	Rev (383-366)	TCCATCCACTGTAGCTGC	18	56		
Mll-7-F	For (10990-11013)	CTGAGGTCCACCTAAGGAAGTCAG	24	58	582	Dr. Jay Hess's lab
Mll-7-R	Rev (11571-11549)	CACAGTTGCAGGGTAGCTTGTTG	23	58		

Rescue of RAE28 expression in *rae28*-/- fibroblasts

Ecotropic Producer cells $GP^{+}E86$ (Markowitz *et al.* 1988) containing the MSCV-rae28cDNA-IRES-GFP (E86-MIG-rae28) vector and constituvely producing the MIG-*rae28* virus were obtained from Dr. Yoshihiro Takihara (produced in Dr. Keith Humphrie's Laboratory). Once integrated in the genome it is able to express RAE28 and GFP as a selection marker. Producer cells were grown to confluence and the medium was replaced by 8 ml of fresh medium 24 hours prior to infection. Viral supernatant was collected, filtered through 45 μm low protein binding Acrodisc® filters (Gelman) and used to infect *rae28*-/- fibroblast plated at a confluency of less than 40%. Polybrene (hexadimethrine bromide, Sigma) was then added to the vector-containing medium to a final concentration of 5 μg/ml. 4 hours later, the infection was repeated to ensure a maximum number of cells would be infected. 4 hours past the second infection, the viral supernatant was replaced by 10ml of fresh medium. 96 hours later the cells were ready to be transfected with the PRE assay reporters or to be used for the RT-PCR analysis of gene expression. Infection efficiency was measured by detecting GFP expression by FACS.

MEF transfection optimization

Cells were plated to reach a confluence of 60% in 6-well tissue culture plates on the day of transfection. Effectene™ (QIAGEN) transfection reagent was used following the recommendations provided by the manufacturer to transfect the MEF with MSCV-IRES-RFP (MIR). MSCV-IRES-RFP (MIR) was generated by Dr. Jennifer Antonchuk in Dr. Keith Humphries' laboratory by replacing the GFP fragment of the GFP vector (Antonchuk *et al.* 2001) digested *NcoI/ClaI* (blunted) with the *NcoI/xbaI* fragment of pDsRed (Clontech,

Mississauga, Canada). Cells transfected with the vector produce the DsRed protein that is detectable by fluorescence in the FL2 channel on a FACScan™ or FACSCalibur™ (Becton Dickinson) equipped with a 488-nm argon laser. Transfection efficiency was measured by FACS two days after transfection. Different ratios of DNA/Effectene™ (following the manufacturer's recommendation) were tested using the quantities of DNA indicated. The conditions resulting in the highest DsRed fluorescence were determined as being the optimum condition to transfect the MEF.

MEF dose response curve to Hygromycin B

Immortalised MEF were plated at 80% confluence in 24-well plates. Cellular viability was evaluated after 12 days of culture in medium containing between 50 μg/ml to 1200 μg/ml of Hygromycin B in μg/ml. Cells were trypsinized and 500 μl of the resuspended cells were diluted 500 μl with a 0.4% trypan blue solution. The number of stained cells was than counted using a hemocytomer. The percentage of unstained cells represent the percentage of viable cells.

Luciferase reporter assay

I. DNA preparation and transfections

Three independent transfections were performed for each vector, in each cell line for each of the luciferase assays performed. All luciferase reporter vectors were linearized with KpnI and pMSCVhyg (Clontech) was linearized using XhoI. Proteins were extracted with two phenol/chloroform steps and one chloroform step before been precipitated and resuspended in sterile water. 30,000 MEF were plated in each well of a 6 well plate 24 hours

prior to transfection. 750 ng of reporter vector (described in the Results section), and 50 ng of the vector pMSCVhyg were transfected into the MEF using a 1:50 ratio of DNA to Effectene™ (QIAGEN) according to the manufacturer's protocol and the transfection optimization previously described. The transfection medium was left on the cells for 6 hours, the cells were washed, and fresh medium was added. 72 hours post transfection, selection media containing 100 µl/ml of hygromycin was added to the cells. The selection media was replaced after 6 days. 10 days post transfection, mock-transfected plates were stained for 10 minutes with a solution of 0.2% crystal violet stain in 10% phosphate-buffered formalin and washed until clear to evaluate the number of colonies present. None were observed, wherea small colonies were already visible in the cells co-transfected with the reporter and the pMSCVhyg carrying the hygromycin resistance gene. When macroscopic colonies were visible, each well was trypsinized and replated to allow a more uniform growth, and hygromycin selection was replaced by fresh medium.

II. Luciferase Assays

Luciferase Assays were performed using the Luciferase Assay System with Reporter Lysis Buffer (Promega, Madison, USA). Cells were grown to confluence and cell lysate was collected according to the manufacturer's directions. All samples were immediately frozen on dry ice and transferred at –80 °C for storage. When ready to perform the luciferase assay, all cell extracts were warmed to room temperature, vortexed for 15 seconds and centrifuged at 12,000 x g in a microcentrifuge for 15 seconds. 10 µl of room temperature supernatants were mixed with 50 µl of room temperature Luciferase Assay Reagent in Durex™ Borosilicate Glass tubes and light intensity was quantified with a luminometer for 10 seconds.

Background light was quantified by replacing the cell extracts with 10 µl or Reporter Lysis Buffer included with the kit. Luciferase activity was normalized for each sample by mixing 50 µl of the cell extract with 150 µl of water and reading OD260 on a spectrophotometer. The blank was 50 µl of Lysis Buffer and 150 µl of water.

Analysis by Fluorescent Activated Cell Sorter (FACS)

Cells were trypsinized and washed in PBS (Stemcell Technologies) containing 2% FCS (Invitrogen). The cell pellet was resuspended in 400 µl FACS buffer (PBS, 2% FCS) containing 1 µg/ml propidium iodide (PI) (Sigma). Fluorescence was detected by FACScan™ or FACSCalibur™ (Becton Dickinson, Mississauga, Canada) equipped with a 488-nm argon laser. The FL1 emission channel was used to monitor GFP fluorescence; the FL3 channel was used to identify PI red fluorescence to exclude dead cells and the FL2 channel was used to detect DsRed fluorescence. Non-infected/transfected cells were used as negative control for GFP/DsRed fluorescence. The analysis was done on CellQuest plus™ Software (Becton Dickinson).

Photography

MEF in 10 cm tissue culture plate were photographed with a Handheld Canon S40 digital camera through the eyepiece of a Leitz (Wetzlar, Germany) DIAVERT inverted microscope using the Phaco 10/0.25, 170/- objective and appropriate filter. Magnification used was the same for all cell type.

CHAPTER 3 Experimental Results

Introduction

MEF were established from mice mutated in three PcG genes: *Asxl1*, *M33,* and *rae28*. *Additional sex comb-like 1* (*Asxl1*) is the homolog of *Additional sex combs (Asx)* in Drosophila. *Asx* is unusual because it is required for maintenance of both activation and repression. *Asx* mutants exhibit both the posterior transformations typical of PcG mutations, but also exhibit anterior homeotic transformations typical of trxG mutations (Sinclair *et al.* 1992). In addition, *Asx* mutations enhance the phenotype of both PcG and trxG mutations (Milne *et al.* 1999). Our laboratory has established a mouse knock-out model of *Asxl1*. Mutant mice die perinatally, and exhibit posterior and anterior transformations in the antero-posterior axis, suggesting that *Asxl1*, like *Asx* is needed for maintenance of both repression and activation. *Asxl1-/-* mice exhibit hematopoietic defects, and defects in eye development (C. Fisher, K. Humphries, and H. Brock, personal communication). Asx physically interacts with Trx, the homolog of MLL, which is mutated in many aggressive pediatric leukemias (Milne *et al.* 1999; Ayton and Cleary 2001), and this interaction is conserved in ASXL1 and MLL in humans as determined by biochemical assays (E. O'Dor, H. Brock personal communication). Because the role of *Mll* in regulation of *Hox c8* has been well-studied (Milne *et al.* 2002), we wanted to make *Asxl1-/-* MEF so that it would be possible to study the role of *Asxl1* in regulation of *Hox c8*, and to determine if Asxl1 and Mll interact in vivo.

M33 is the murine homolog of *Polycomb*. Its function is conserved in mice and flies (Muller *et al.* 1995), and M33 with Rae28, is a member of the PRC1 complex of mammals (Levine *et al.* 2002). *M33-/-* mice exhibit posterior homeotic transformations, sex reversal,

and hematopoietic defects (Core *et al.* 1997; Katoh-Fukui *et al.* 1998). Mice heterozygous for the *M33* mutation were generously donated to us by Dr. T. Higashinakagawa.

Mice knockout for *rae28* exhibit posterior transformations in the anteroposterior axis, hematopoietic defects, and defects consistent with defects in cervical neural crest cells (Takihara *et al.* 1997; Tomotsune *et al.* 2000; Tokimasa *et al.* 2001; Ohta *et al.* 2002; Shirai *et al.* 2002). This gene is also called *mph1* or HPH1, but in this thesis, we will use the original name of the locus, *rae28,* so-called because it was identified first as a retinoic acid early response gene (Nomura *et al.* 1994). The regulatory region of *rae28* has been identified and characterized (Motaleb *et al.* 1999).

In experiments carried out by Dr. Leonie Ringrose in the laboratory of Jean-Maurice Dura, Institut de Génétique Humaine, Marseilles, a detailed comparison of the regulatory regions of *ph* and *rae28* was undertaken in an effort to identify conserved regulatory regions (personal communication). The analysis below is her work, and is summarized here so that the logic of the next experiments is apparent. We thank her for permission to describe her unpublished work. We also refer to unpublished work by Sebastien Bloyer, then a doctoral student in the laboratory of Jean-Maurice Dura, and now a post-doctoral fellow in our laboratory.

As noted in Chapter 1, it is surprising that PcG proteins bind to PcG loci, because PcG genes are expresses ubiquitously, and yet PcG proteins are supposed to repress. The *ph* locus is duplicated, and the *ph-proximal* and *ph-distal* transcription units are regulated independently (Hodgson *et al.* 1997). Sebastien Bloyer has identified a 3 kb region that is the minimal element required for regulation of *ph* by PcG genes in functional assays, indicated in

Figure 3-1 as fragment ph418. One of the functionally identified characteristics of ph418 is that it is sensitive to PcG mutations in vivo, defining this fragment as a PRE. He also identified a similar region upstream of the *ph-distal* transcript.

Dr. Ringrose compared the sequences of these regions and identified two regions of high similarity. Within these similar regions are binding sites for GAGA factor (GAF), and for Pleiohomeotic (PHO), two known PRE-binding proteins. The location of these conserved regions is shown in Figure 3-1B. She also compared the sequences of the Drosophila *ph* regulatory regions with the 3.8 kb immediately upstream of the transcription start of *rae28*, and identified two small regions of sequence conservation that are present in the mammalian and Drosophila sequences. These also consist of GAF and PHO recognition sequences. This apparent sequence conservation raises the possibility that the 3.8 kb upstream of *rae28* might be a PRE. Thus we needed a system where we could test the function of this putative regulatory region as a PRE. Using the same approach as Milne *et al*, (2002) we decided to use a reporter system and MEF cell lines mutant for different PcG genes.

The traditional way to immortalize primary cells is to grow many cells, and select for a spontaneous mutation that immortalizes the cells (Todaro and Green 1963; Rittling 1996). This method requires many embryos, and it is difficult to compare different cell lines, because the mechanism of immortalization is likely to be different. The Large T-antigen (Tag) of SV40 has also been used to immortalize primary cells, and MEF in particular (Milne *et al.* 2002). Tag is required for the induction and maintenance of malignant transformation of nonpermissive cells by the SV40 virus. Tag reduces the levels of cell cycle-dependent kinase inhibitors in contact-inhibited cells. Tag also interferes with cell cycle regulation because it interacts with pRb and p53 proteins. Cells expressing Tag divide faster than normal cells,

exhibit drastically changed phenotypes, and often become polyploid, probably because the cell cycle checkpoints that block progression in response to DNA damage are missing (Fanning and Knippers 1992; Saenz-Robles *et al.* 2001; Sullivan and Pipas 2002).

For the experiments reported here, a newly described gene, *TBX2* was used to immortalize MEF. *TBX2* was detected in a screen for genes that allowed for the bypass of senescence of primary fibroblasts. *TBX2* encodes a mammalian T-box transcription factor that downregulates the *INK4a-ARF* locus. The p19ARF protein, one of the two tumor suppressors encoded by the *INK4a-ARF* locus, activates p53. TBX2 expression does not confer a growth advantage to immortalized cells, and cells that suffer DNA damage undergo apoptosis (Jacobs *et al.* 2000). This means that cells immortalized with TBX2 are much less abnormal than cells immortalized with Tag.

In the next section, experimental data on the immortalization of PcG mutant MEF is described and attempts to use these cells to test for putative PRE activity in *rae28* regulatory DNA is reported.

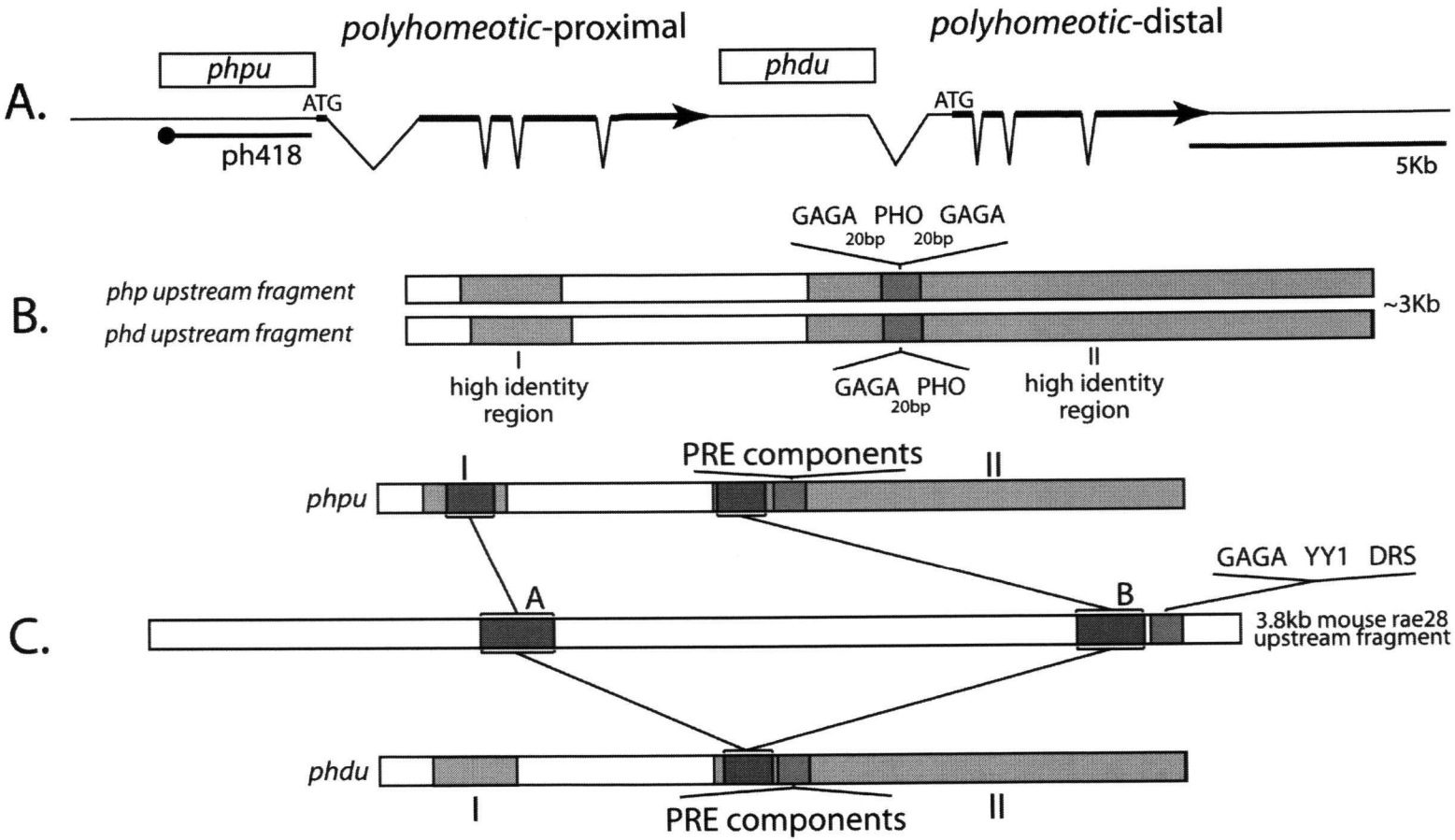

Figure 3-1 Comparison of *ph* locus and *rae28* putative PRE

(A) Schematic representation of the *ph* locus. Conserved regions are found upstream of the two transcription units of *php* (*phpu*) and *phd* (*phdu*) and are represented by two boxes. The position of the *ph*418 fragment is also indicated (B) The two conserved regions upstream of the *php* and *phd* contain two regions of high similarity and known PRE components. (C) Comparison of *phpu* and *phdu* with the 3.8kb fragment upstream of the mouse *rae28.* Two regions highly conserved between the mouse and fly fragments are indicated by the letter A and B on the mouse fragment

Results and Discussion

I. Obtaining mouse embryonic fibroblasts.

To obtain mouse embryonic fibroblasts (MEF) from PcG mutant embryos, male and female mice heterozygous for the PcG mutations *rae28, M33,* or *Asxl1* were crossed. After checking for successful mating, twelve (*Axl1*) or fourteen days (*rae28, M33*) later, pregnant mothers were sacrificed, embryos were dissected from the uteri and genotyped as described in the Materials and Methods (Chapter 2). Meanwhile, embryonic tissues were minced, disrupted by passage through a needle, and if necessary, treated with collagenase (see Materials and Methods), rinsed with phosphate buffered saline (PBS), and then plated in 10 cm plastic culture dishes in Dulbecco's Modifiied Eagle's Medium (DMEM) containing 10% fetal calf serum and antibiotics. After allowing cells to attach to the culture dish, unattached cells were removed by aspiration, and the primary fibroblasts were grown to confluence. Three vials containing approximately 10^6 cells each were frozen down to use for immortalization experiments for each pair of wild-type and mutant MEF. In each case, fibroblasts were prepared from sibling homozygous mutant and wild-type embryos, to control for possible differences in age, or degree of back-crossing, for each PcG mutation examined. The consequence is that for each mutant cell line, an appropriate wild type cell line had to be established using a wild type sibling embryo. Mutant MEF were always compared to the appropriate control.

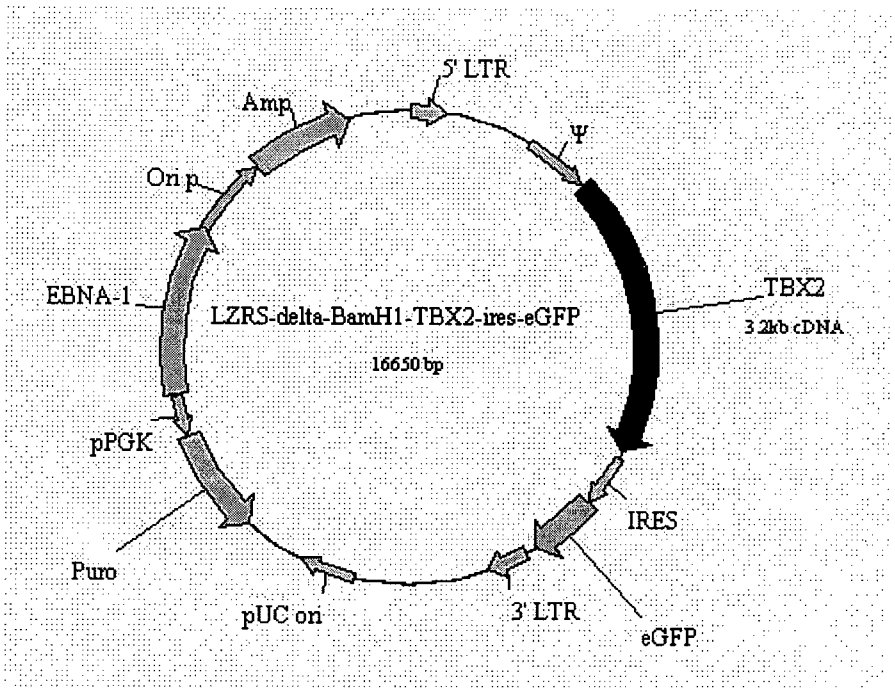

Figure 3-2 LZRS-delta-BamH1-*TBX2*-ires-eGFP vector

The LZRS-delta-BamH1-*TBX2*-ires-eGFP vector uses a modified version of the LZRS system developed by (Kinsella and Nolan 1996) to produce a retrovirus vector capable of expressing TBX2 and GFP in infected cells. The retroviruses are produced using Phoenix™ producer cells. The LZRS system uses two elements from the Epstein-Barr virus *Orip* and EBNA-1, to confer stable episomal maintenance capabilities under puromycin selection. Cells infected with the retrovirus express green fluorescent protein (GFP) as a convenient marker.

II. Production of TBX2 immortalizing retrovirus

As discussed in the Introduction, primary fibroblasts have a limited lifespan in culture (approximately 5 passages). To obtain immortalized MEF, TBX2 was expressed, which immortalizes cells, but has fewer side effects than the expression of large T antigen. We obtained a retroviral expression vector for *TBX2*, LZRS-delta-BamH1-*TBX2*-ires-eGFP (Figure 3-2) as described in the Materials and Methods, as a generous gift from Dr. M. van Lohuizen. This is a modification of the LZRS Virus system (Kinsella and Nolan 1996), that contains an enhanced green fluorescent protein (GFP). Briefly, LZRS-delta-BamH1-*TBX2*-

ires-eGFP was introduced into an ecotropic retroviral packaging cell line (Phoenix^{TM}) by calcium phosphate transfection. Using procedures detailed in the Materials and Methods, supernatants containing the retrovirus were obtained from the cells after transfection, or after puromycin selection for cells containing the retrovirus. Culture medium containing the virus was filtered, and titred immediately, or frozen for later titering as described in the Materials and Methods. As shown in Figure 3-3, unselected cells give very low titres (less than 10^4 transducing units/ml), whereas cells selected on 0.6 mg/ml and 1 mg/ml puromycin yielded titres of almost 10^6 transducing units/ml.

Wild-type and PcG mutant primary MEF were plated at low density, and infected with the TBX2-immortalizing virus using Polybrene as described in the Materials and Methods. After changing the medium, the cultures were grown to confluence, and then passaged at a dilution of 1/10. In separate control experiments, uninfected primary MEF never grew for more than 5 passages in total (See Table 3-1). Because of the time needed to amplify the primary MEF to obtain enough cells for infection with the TBX2-immortalizing virus, uninfected cells stopped dividing very shortly after the experimental cells were infected (data not shown). Table 2-1 shows the minimum number of passages for each immortalized line constructed.

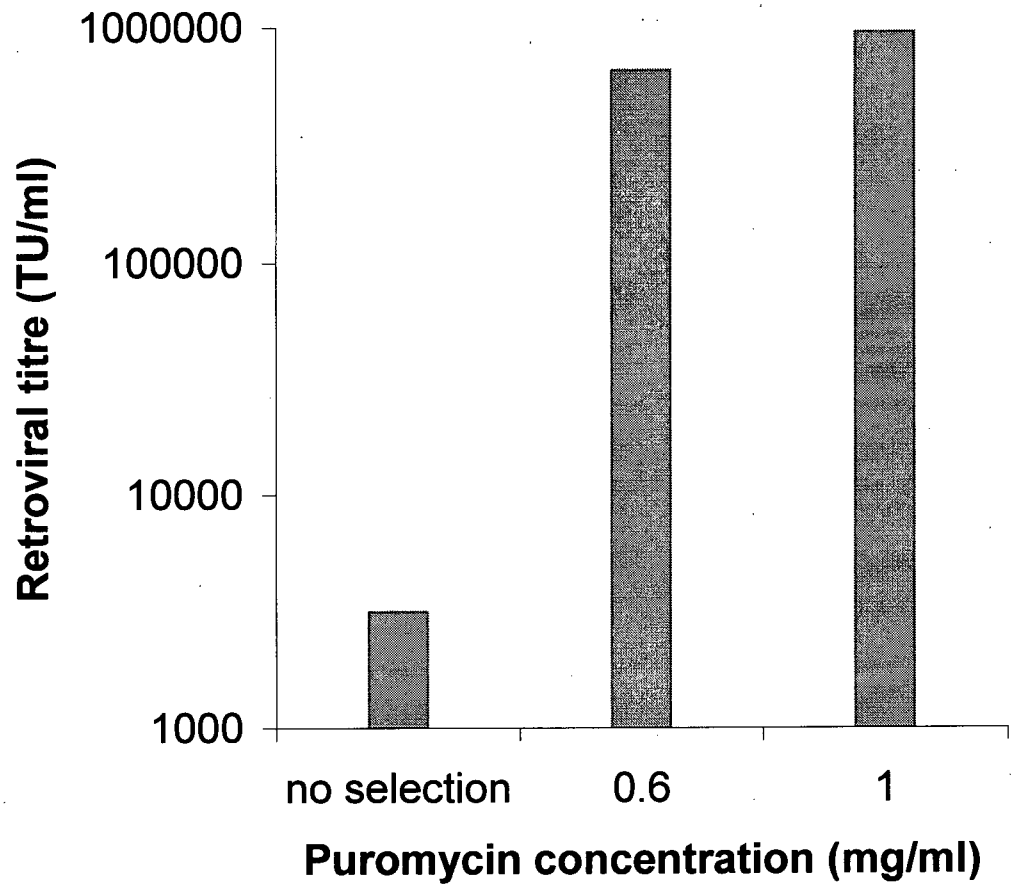

Figure 3-3 High-titre production of TBX2-immortalizing retroviral vectors

LZRS-delta-BamH1-*TBX2*-ires-eGFP was tested for vector production using the ecotropic Phoenix™ retroviral packaging cell line. Vector was collected 48 hr post-transfection (no selection) and after stably selecting the cells carrying the transfected episomal vector with 600 μg /ml puromycin and 1000 μg/ml puromycin. Collected retroviral supernatant was serially diluted and used to infect NIH-3T3 cells. Infected cells were assayed by FACS analysis for GFP expression 48 hr after transduction. Results from one replicate.

Table 3-1 Passage number of non-transformed and *TBX2*-transformed fibroblasts

Mouse Embryonic Fibroblast	Non transformed	After infection with the TBX2-immortalizing vector
Asxl1 -/-	5	25+[a]
Asxl1 +/+	5	25+
rae28 -/-	Not mesured[b]	65+
rae28 +/+	Not mesured[b]	63+
M33 -/-	Not mesured	57+
M33 +/+	Not mesured	55+

[a] The (+) symbol indicate that the cells are still showing a constant growth rate at the number of passages indicated
[b] MEF from unknown passage were used and all cells were infected with the virus produced by transfection of Phoenix™ with LZRS-delta-BamH1-*TBX2*-ires-eGFP

Because the retrovirus expresses GFP, all immortalized cells should be GFP-positive. Moreover, the proportion of GFP positive cells should change over time from a number that reflects the infection rate to 100%. Four days post-infection, fluorescence activated cell sorting (FACS) was used to determine the proportion of infected cells. As shown in Figure 3-4 the initial infection level varied from about 20-60%. For three of the four lines tested (*rae28+/+, M33+/+, M33-/-;* (the *Asxl1* wild-type and mutant cells were not tested in this assay)), the proportion of cells expressing GFP rose as expected to 100% by 122 days post-infection.

However, one line, *rae28-/-* showed the opposite behaviour: no cells expressed GFP after 122 days. Yet these cells were clearly immortalized, and as shown below, are of the

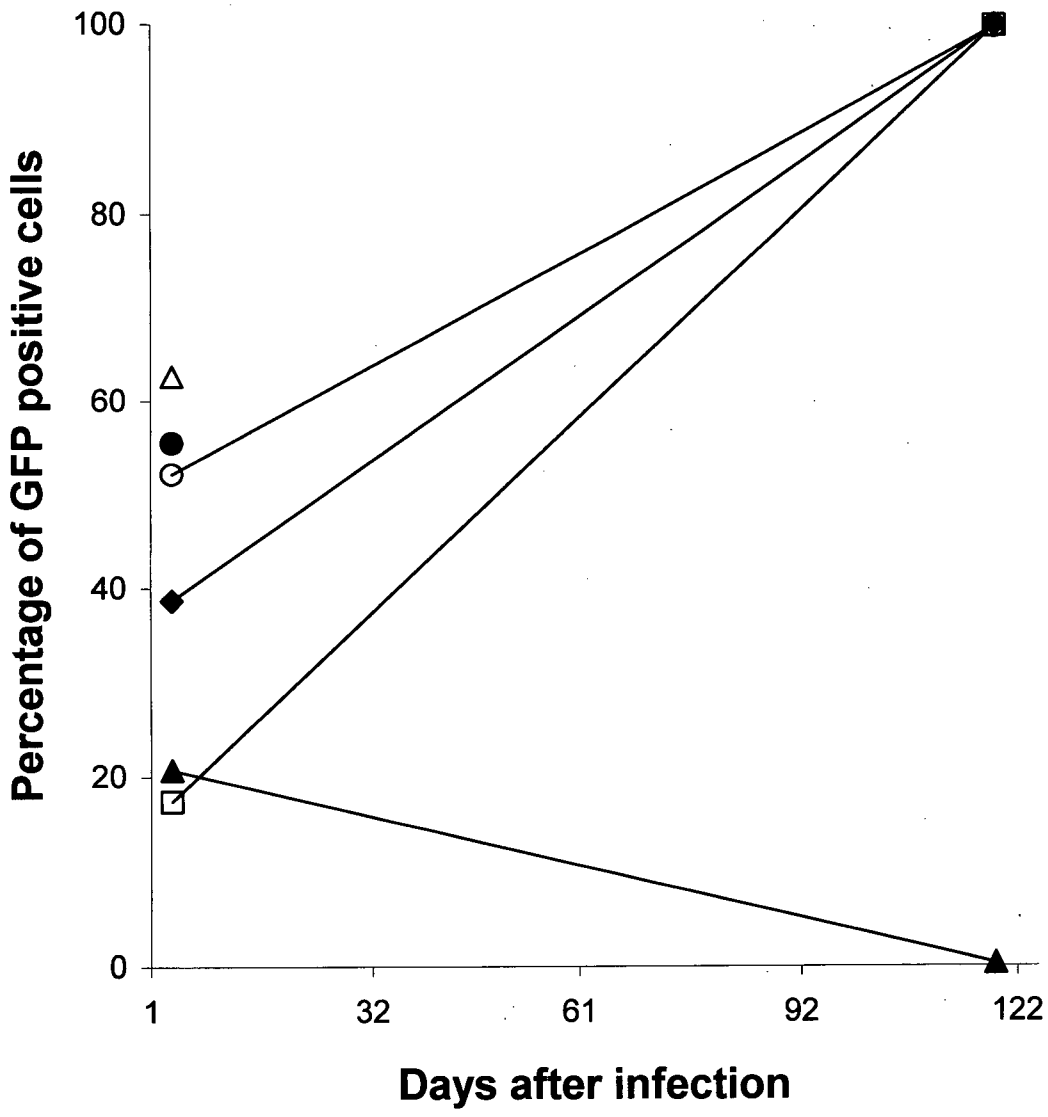

Figure 3-4 Percentage of *TBX2*-transformed cells in immortalized MEF cell lines

Graphical representation of FACS results for the percentage of GFP positive cells in populations of MEF infected with TBX2-immortalizing vector at day 4 and day 119 after infection (*Asxl1* -/- and *Asxl1* +/+ were only measured 4 days after infection). Symbols are (●) for *Asxl1* -/-; (○) for *M33* -/-; (▲) for *rae28* -/- (Δ) for *Asxl1* +/+; (♦) for *M33* +/+ and (□) for *rae28* +/+.

correct genotype. Two explanations are possible. One is that the immortalized cells suffered a mutation or rearrangement of the TBX2-immortalizing vector that caused loss of expression of GFP, but not loss of TBX2, so that the cells are immortalized, but do not express GFP. This possibility was not tested. The other is that the *rae28-/-* cells were immortalized as a result of a random mutation or mutations in the genome that occurred independently of *TBX2,* so the immortalized cells don't have the TBX2-immortalizing vector. Consistent with this possibility, these cells grow at different rates than MEF immortalized by *TBX2*. Immortalized MEF were passaged every 3-4 days at dilutions of 1/10, yet the *rae28-/-* MEF could be passaged at dilutions of 1/40 to 1/60. In addition, wild-type and mutant *Asxl1* and *M33* cells were visually indistinguishable (see Figure 3-5 for the *Asxl1* example), but the *rae28-/-* cells appeared smaller and more rod shaped that the *rae28+/+ cells* (Figure 3-14). Together, the observations support the conclusion that the *rae28-/-* cells were immortalized independently of *TBX2* overexpression. These cells are still useful, but as will be discussed below, care must be taken when interpreting results from these cells.

The variability in cell size, morphology, and overall appearance of MEF shown in Figure 3-5 indirectly suggests that the MEF are a population of cells, rather than being clonal derivatives. This possibility could be tested directly by examining integration sites of retroviruses in our transformed cells using Southern blotting.

Wild-type and mutant MEF were genotyped using PCR to confirm the embryonic genotyping, and to ensure that no labeling errors had occurred during handling (see Materials and Methods for details of DNA preparation, PCR conditions, and Table 2-1 for details on primers). As shown in Figure 3-6, it was possible to unambiguously identify wild-type and

mutant MEF for each of the three PcG mutations using this assay. The results confirm that we have established immortalized cell lines of the appropriate genotypes.

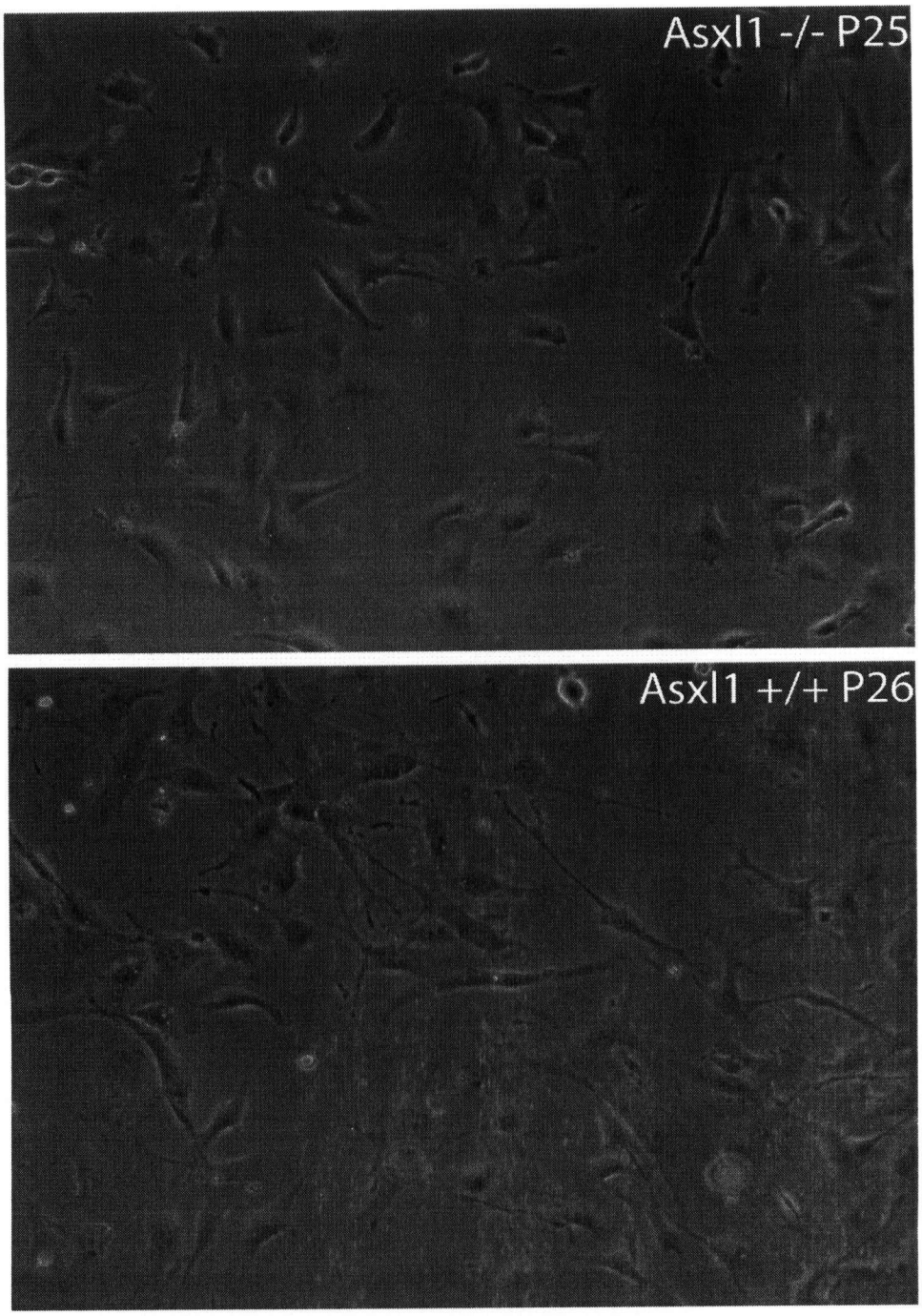

Figure 3-5 Photographs of Immortalized *Asxl1* MEF

Handheld photographs of the *Asxl1* MEF is shown to demonstrate the general phenotype of the immortalized cell lines. The magnification is the same for all cell lines presented in this thesis.

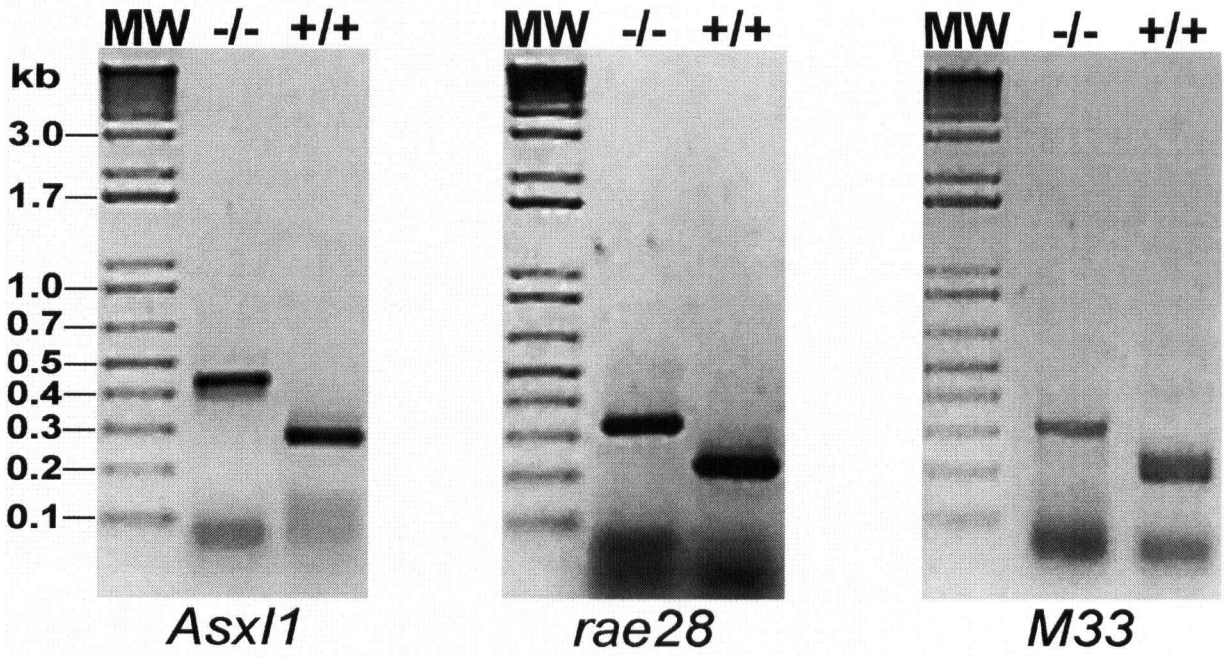

Figure 3-6 Genotype of immortalized MEF

PCR analysis of the genotype of stable *TBX2*-immortalized MEF cell lines. DNA was extracted from confluent cell monolayers on 6cm culture plates. PCR was used to detect the wild-type allele (+/+) and the mutant alleles (-/-) using the appropriate primers described in the Materials and Methods chapter. (A) *Asxl1* MEF, expected fragment size is 253bp for +/+ and 426bp for -/- (B) *rae28* MEF, Expected fragment size is ~225bp for +/+ and ~325bp for -/- (C) *M33* MEF, expected fragment size is ~200bp for +/+ and 325bp for -/-. The position of the molecular weight marker is shown by (MW).

III. Analysis of Gene Expression in PcG MEF

As noted in the Introduction, gene expression in MEF can faithfully reflect gene expression in embryos, suggesting that they are a useful system for examination of gene regulation. Therefore we wished to categorize the expression of PcG and Hox genes in the MEF lines. Details of making RNA, cDNA, PCR reactions, and the primers used are given in the Materials and Methods, and in Table 2-2). The generalized protocol was to compare gene expression in wild-type and PcG mutant MEF, using RT-PCR, using expression of actin to control for equivalent amounts of cDNA in each reaction.

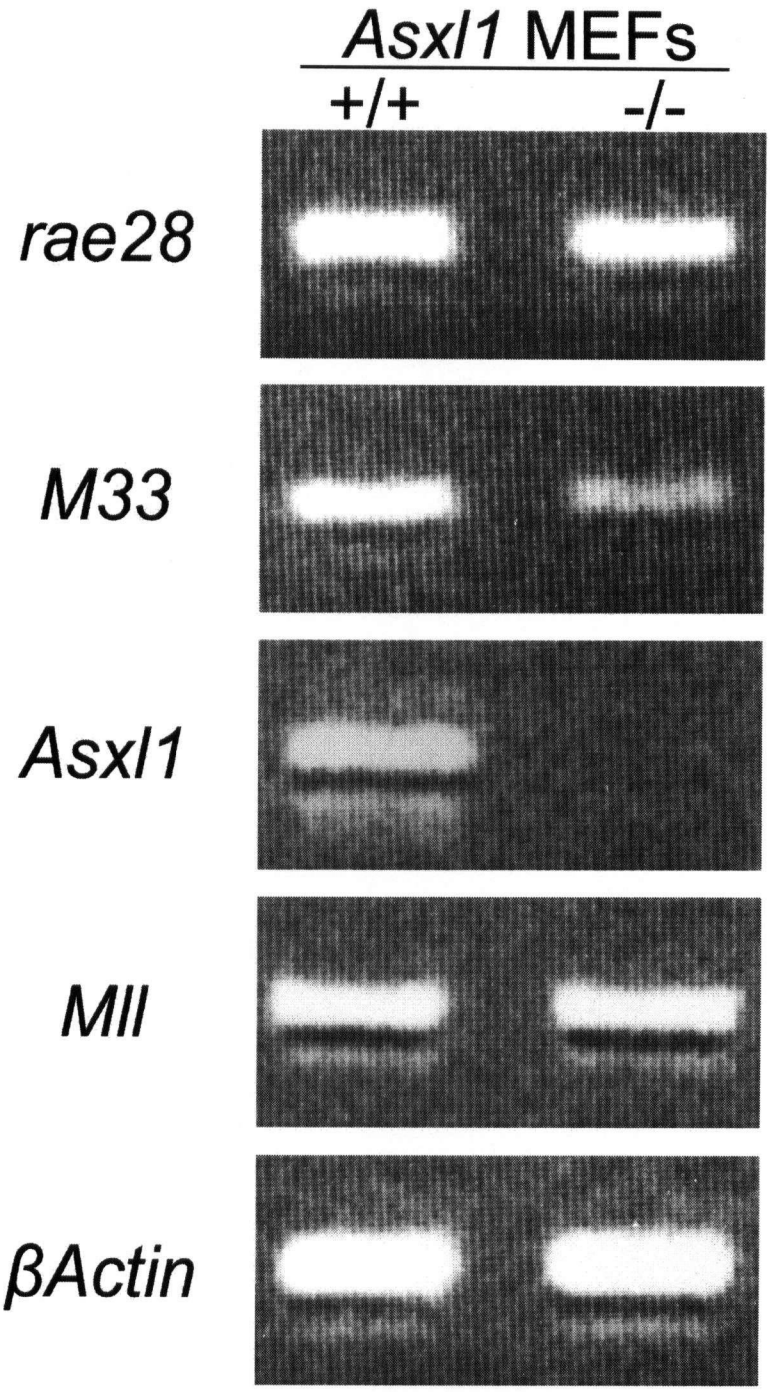

Figure 3-7 Analysis of PcG gene expression in *Asxl1* MEF

RT-PCR analysis of PcG genes expression of in the stably *TBX2*-immortalized *Asxl1* +/+ and *Asxl1* -/- MEF cell lines. PCR-amplified total cDNAs were prepared from total RNAs extracted from the stably immortalized fibroblasts. Primers and expected product sizes are described in the Materials and Methods chapter. Expression of β-Actin is shown to confirm equivalent amount of cDNA in the RT-PCR reactions.

First, the expression of PcG genes in PcG mutant MEF was examined. As shown in Figure 3-7, *rae28* and *M33* were expressed in *Asxl1+/+* and *Asxl1-/-* cell and as expected, the figure shows that *Asxl1* is not expressed in the *Asxl1-/-* MEF.

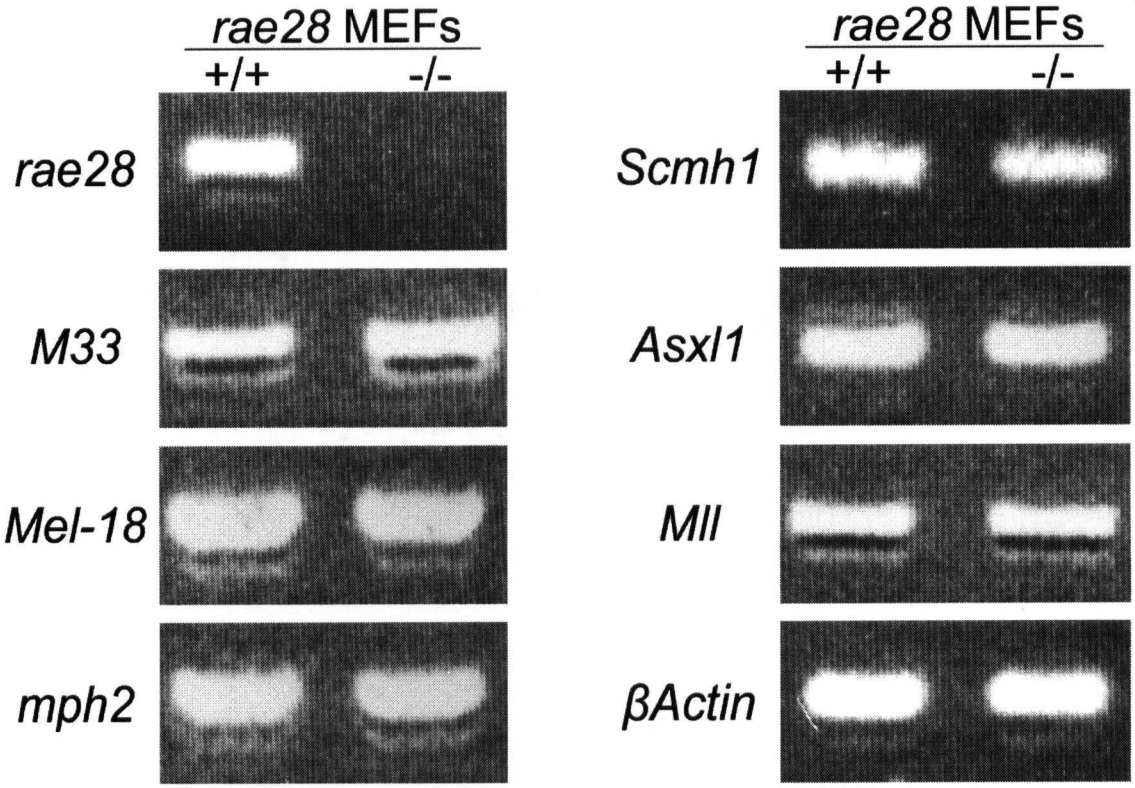

Figure 3-8 Analysis of PcG gene expression in *rae28* MEF

RT-PCR analysis of PcG genes expression of in the stably *TBX2*-immortalized *rae28* +/+ and *rae28* -/- MEF cell lines. PCR-amplified total cDNAs were prepared from total RNAs extracted from the stably immortalized fibroblasts. Primers and expected product sizes are described in the Materials and Methods chapter. Expression of β-Actin is shown to confirm equivalent amount of cDNA in the RT-PCR reactions.

Next, the expression of PcG genes in *rae28+/+* and *rae28-/-* MEF was examined. Unsurprisingly, *rae28* itself is not expressed in *rae28-/-* MEF (Figure 3-8). The expression of *M33, Mel-18* (a murine homolog of *Posterior sex combs*), *Sex comb on midleg homolog 1* (*Scmh1*) and *Asxl1* was unchanged in *rae28* mutant MEF compared to wild-type (Figure 3-8).

The expression of the trxG gene *Mll*, which was expressed in *rae28-/-* MEF was also examined.

Finally, the expression of PcG genes in *M33+/+* and *M33-/-* MEF was examined. The expression of *rae28* was unaffected (Figure 3-9). *M33* expression was detected, because the *M33* KO is a knock-in, and the primers used to detect *M33* in our experiments are downstream of the knock-in.

Next we turned our attention to analysis of Hox genes, because these are well-characterized targets of PcG regulation. We were particularly interested in *Asxl1* because it appears to both positively and negatively regulate Hox gene expression in mouse embryos (C. Fisher and H. Brock, unpublished). As shown in Figure 3-10, there were no differences among the 11 Hox genes tested. As the specific Hox targets of *Asxl1* have not yet been defined, it is not obvious how to interpret these results. One possibility is that *Asxl1* regulates Hox genes that were not assayed. Another possibility is that the MEF do not exhibit *Asxl1*-dependent Hox regulation, but these possibilities cannot yet be distinguished.

Next, Hox gene expression in *rae28+/+* and *rae28-/-* MEF was analysed. No differences were seen in the Hox A and Hox B cluster genes analysed (Figure 3-11).

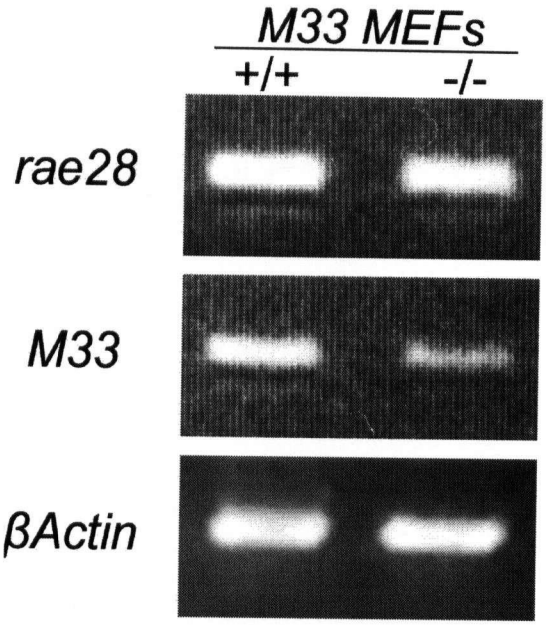

Figure 3-9 Analysis of PcG gene expression in *M33* MEF

RT-PCR analysis of PcG genes expression of in the stably *TBX2*-immortalized *M33* +/+ and *M33* -/- MEF cell lines. PCR-amplified total cDNAs were prepared from total RNAs extracted from the stably immortalized fibroblasts. Primers and expected product sizes are described in the Materials and Methods chapter. Expression of β-Actin is shown to confirm equivalent amount of cDNA in the RT-PCR reactions.

However, strikingly, all Hox C cluster genes analysed were expressed in *rae28*+/+ MEF, but not expressed in *rae28*-/- MEF. This result is surprising, because *rae28* is a repressor of Hox gene expression, so *rae28* mutants would be expected to cause over-expression of Hox genes. One explanation of these results is that they arise from indirect effects of immortalization rather than direct effects of RAE28 on Hox loci. This possibility is explored further, below.

In *M33*+/+ and *M33*-/- MEF, two differences were observed in Hox expression (Figure 3-12). *Hox c5* was not expressed in *M33*-/- MEF, but it was expressed in *M33*+/+ MEF. This result suggests that the effect might be indirect, because one would expect mutations in a silencer to cause increased expression in *M33*-/- cells. In addition, *Hox c9* was expressed in the *M33*-/- mutants, but not in *M33*+/+ MEF. Taken at face value, this is

consistent with M33 being a repressor of *Hox c9*. But if the *M33*+/+ result is wrong, as suggested by the observation that *Hox c9* is expressed in *Asxl1*+/+ and *rae28*+/+ MEF, then this result is likely not significant.

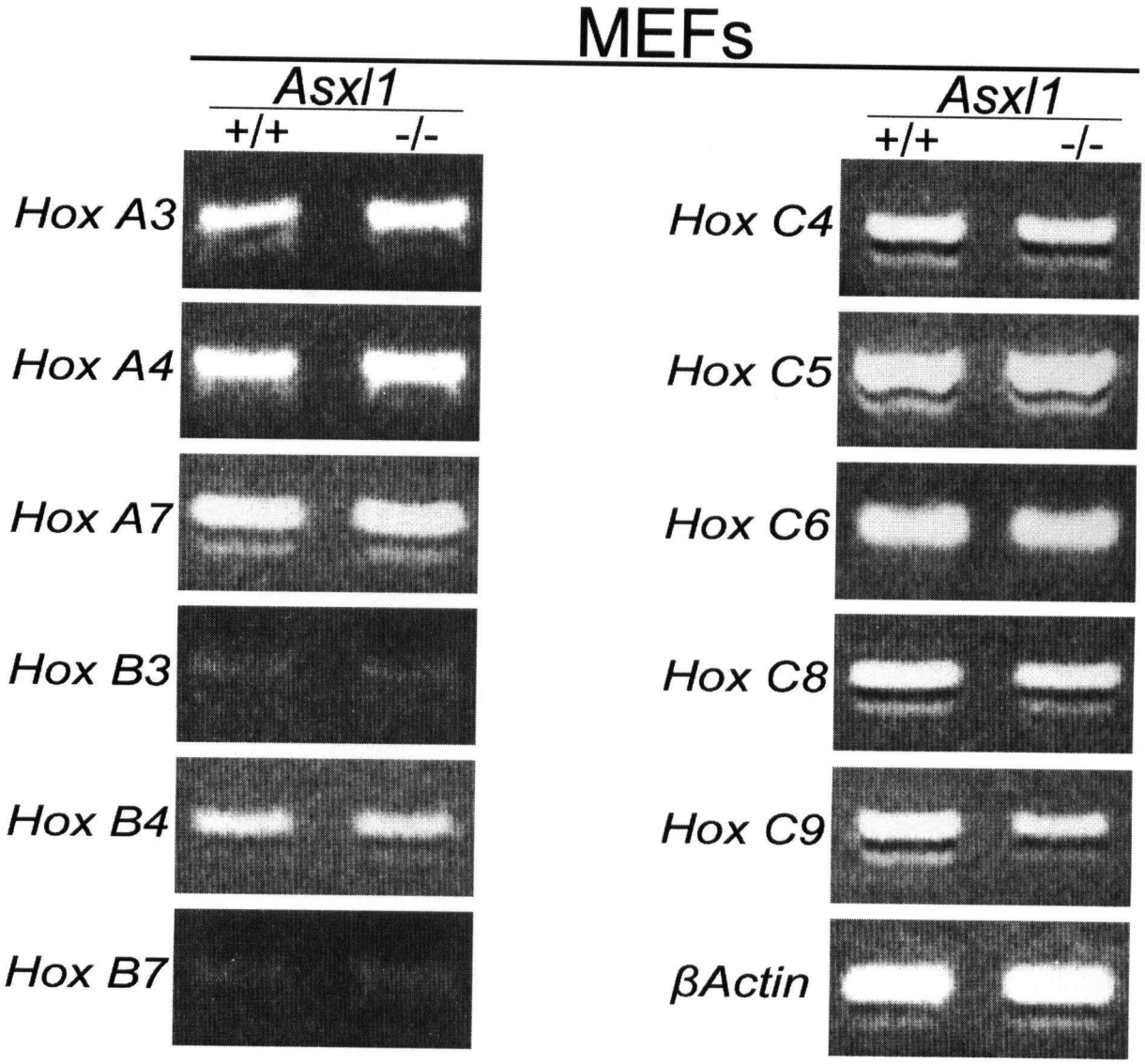

Figure 3-10 Analysis of *Hox* gene expression in *Asxl1* MEF

RT-PCR analysis of *Hox* genes expression of in the stably *TBX2*-immortalized *Asxl1* +/+ and *Asxl1* -/- MEF cell lines. PCR-amplified total cDNAs were prepared from total RNAs extracted from the stably immortalized fibroblasts. Primers and expected product sizes are described in the Materials and Methods chapter. Expression of β-Actin is shown to confirm equivalent amount of cDNA in the RT-PCR reactions.

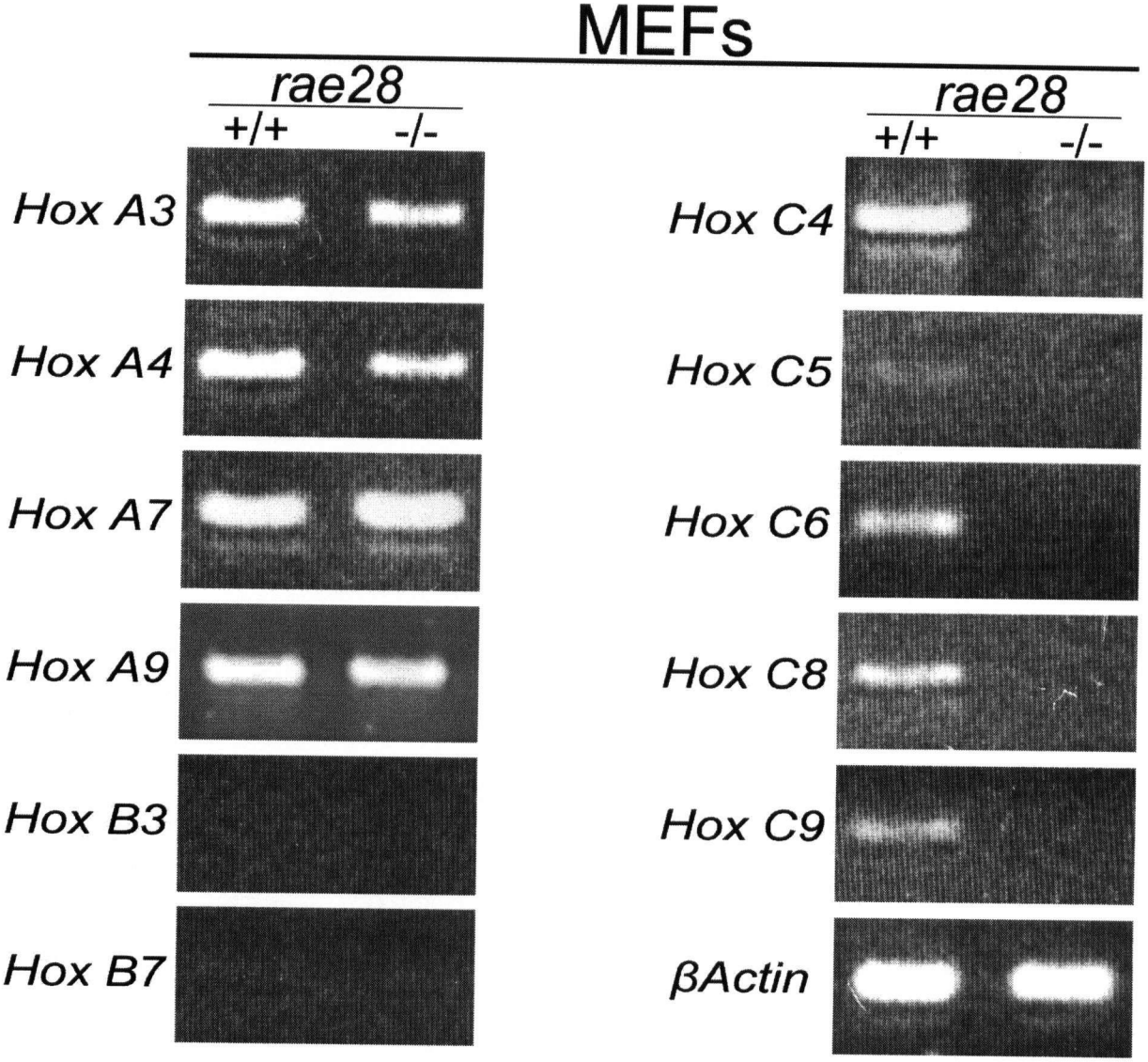

Figure 3-11 Analysis of *Hox* gene expression in *rae28* MEF

RT-PCR analysis of *Hox* genes expression of in the stably *TBX2*-immortalized *rae28* +/+ and *rae28* -/- MEF cell lines. PCR-amplified total cDNAs were prepared from total RNAs extracted from the stably immortalized fibroblasts. Primers and expected product sizes are described in the Materials and Methods chapter. Expression of β-Actin is shown to confirm equivalent amount of cDNA in the RT-PCR reactions.

Because of the striking observation that the Hox C cluster genes were not expressed in *rae28-/-* MEF, we wished to determine if this reflected a direct effect of RAE28, or reflected an indirect effect. We reasoned that if the lack of Hox C expression was a direct effect of RAE28, then supplying *rae28-/-* MEF with an expression vector synthesizing RAE28

should rescue the Hox expression. As shown in Figure 3-13, this was not the case. Expression of RAE28 in *Rae28-/-* cells had no effect on Hox C expression even though RAE28 expression is clearly elevated. These experiments do not rule out the possibility that because the Hox C genes were not expressed, chromatin or DNA methylation changes may have occurred that prevented reactivation when *rae28* was expressed. DNA methylation was shown by Milne et al. (2002) to block activation of *Hox c8* by *Mll* in *Mll-/-* MEF, so a similar explanation is plausible for the failure of *rae28* to activate Hox C genes in *rae28-/-* MEF.

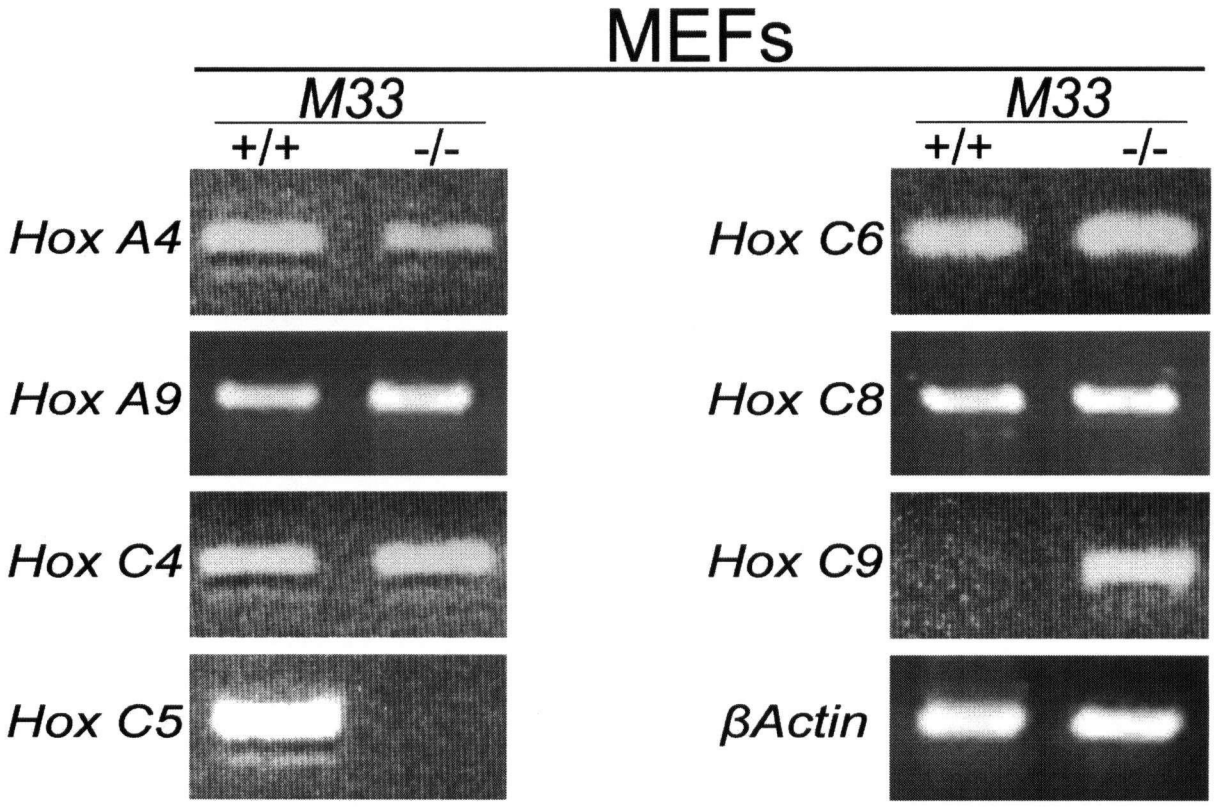

Figure 3-12 Analysis of *Hox* gene expression in *M33* MEF

RT-PCR analysis of *Hox* genes expression of in the stably *TBX2*-immortalized *M33* +/+ and *M33* -/- MEF cell lines. PCR-amplified total cDNAs were prepared from total RNAs extracted from the stably immortalized fibroblasts. Primers and expected product sizes are described in the Materials and Methods chapter. Expression of β-Actin is shown to confirm equivalent amount of cDNA in the RT-PCR reactions.

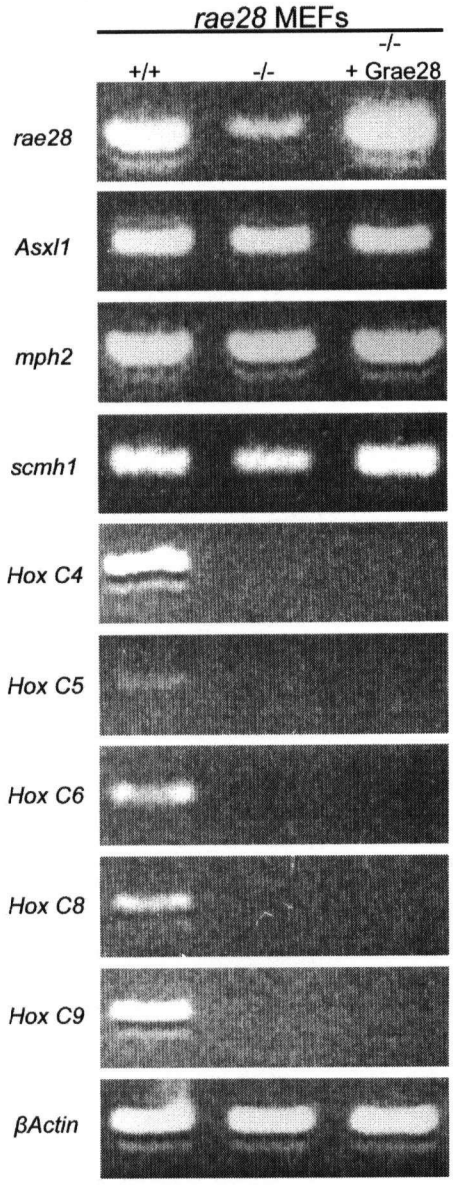

Figure 3-13 Analysis of PcG and *Hox* gene expression in rescued *rae28*-/- MEF

RT-PCR analysis of PcG and *Hox* genes expression of in the stably *TBX2*-immortalized *rae28* +/+, *rae28* -/- MEF cell lines and in the same *rae28* -/- fibroblast that have been complemented with a *rae28* expression vector (MIG-*rae28*). PCR-amplified total cDNAs were prepared from total RNAs extracted from the stably immortalized fibroblasts. Primers and expected product sizes are described in the Materials and Methods chapter. Expression of *β-Actin* is shown to confirm equivalent amount of cDNA in the RT-PCR reactions. 35 cycles of amplification were preformed and the presence of a faint band for the *rae28* RT-PCR in the *rae28*-/- MEF is most likely due to a small contamination of the primer mix or to well overflow when loading the gel.

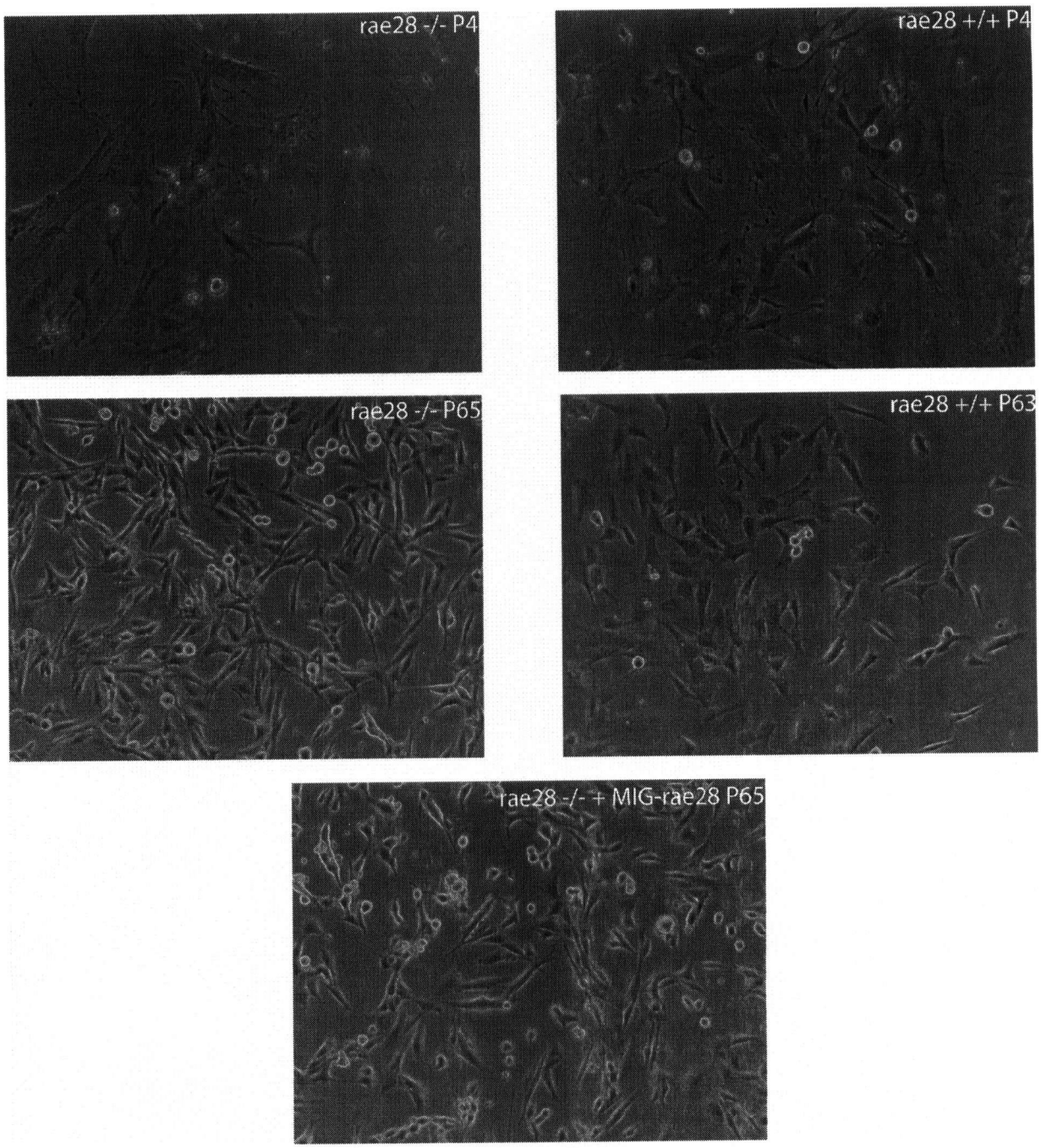

Figure 3-14 Photographs of Immortalized *rae28* MEF

Handheld photographs of the *Asxl1* MEF is shown to demonstrate the general phenotype of the immortalized cell lines. The magnification is the same for all cell lines presented in this thesis.

However, as noted above, the *rae28-/-* cells were probably immortalized by a different route than the *rae28+/+* MEF. This observation raises the possibility that some event

accompanying or following immortalization causes the change in Hox C expression in the *rae28-/-* cells compared to *rae28+/+* cells. Although the evidence is indirect, the *rae28+/+*

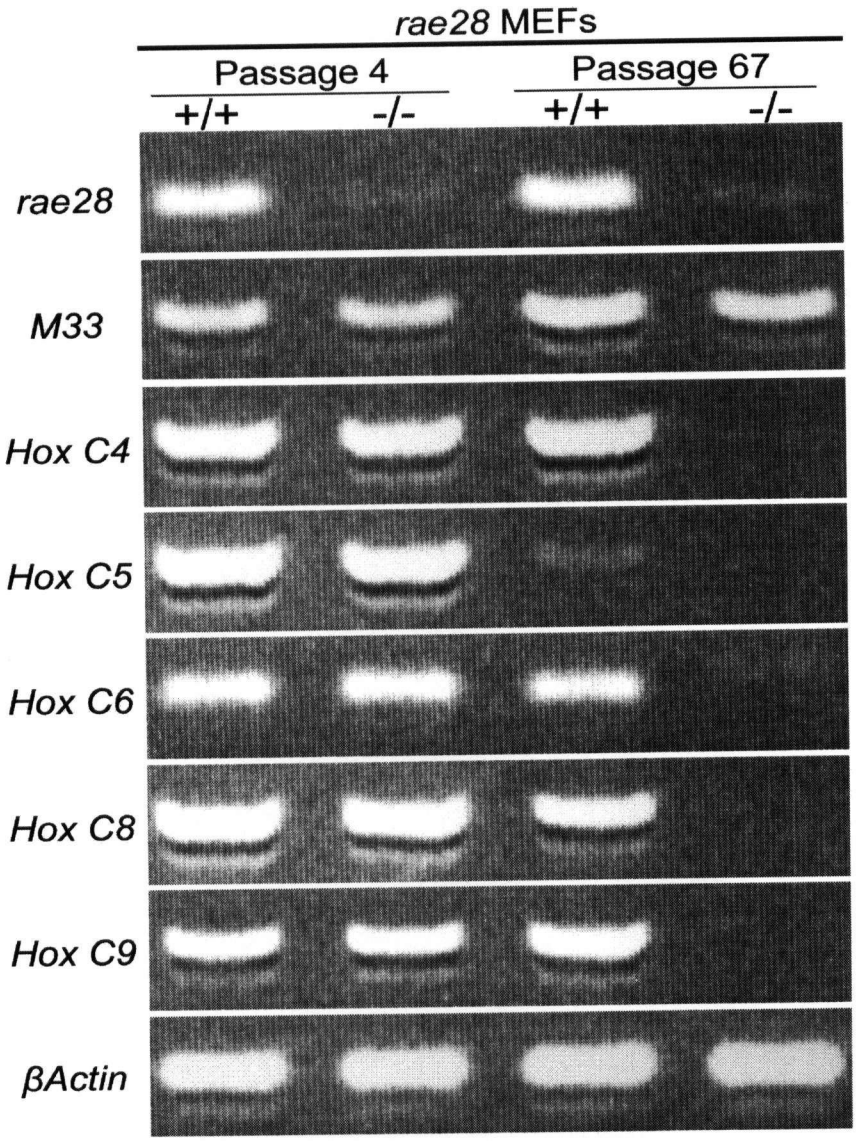

Figure 3-15 Comparison of PcG and *Hox* gene expression in *rae28* MEF of P4 *vs* P67

Comparison of PcG and *Hox* genes expression by RT-PCR analysis in the stably *TBX2*-immortalized *rae28* +/+ and *rae28* -/- MEF cell lines at passages 4 and 67. PCR-amplified total cDNAs were prepared from total RNAs extracted from the stably immortalized fibroblasts. Primers and expected product sizes are described in the Materials and Methods chapter. Expression of β-Actin is shown to confirm equivalent amount of cDNA in the RT-PCR reactions.

and *rae28-/-* cells were indistinguishable at passage 4 after infection (Figure 3-14). However, by passage 63, the *rae28-/-* cells are smaller, more spindle-shaped, and grow much more rapidly than the *rae28+/+* cell. This change in morphology and growth rate is not reversed by expression of wild-type *rae28* in the *rae28-/-* MEF, shown in Figure 3-14 for passage 65 cells, suggesting that the morphological change is independent of *rae28*. The latter observation is also consistent with the demonstration in Figure 3-13 that *rae28* does not rescue expression of Hox C genes.

Therefore, we decided to compare expression of early and late passage *rae28-/-* cells. Expression of Hox C genes in cells from passage 4 (likely prior to immortalization) and passage 67 was compared and shown in Figure 3-15. The results for passage 67 cells confirm those shown in Fig. 2-9. Strikingly, there is no difference in Hox C expression in *rae28+/+* and *rae28-/-* cells in passage 4 cells. Because the genotype of the cells is the same in passage 4 and 67 is the same, these results demonstrate that the change in expression of Hox C genes in *rae28+/+*and *rae28-/-* cells is *rae28* independent.

IV. Comparison of Drosophila and murine regulatory regions of *ph/rae28*

To test the possibility that the region upstream of *rae28* is a PRE using the PcG mutant MEF that we isolated in the experiments reported above, vectors with the design shown in Figure 3-16 were obtained. A luciferase reporter in a promoterless vector (pGL3), was modified by cloning 500 bp of the region surrounding the *rae28* transcription start from coordinates -400 to +100 upstream of the luciferase. In addition, the vector incorporates the insulator sequence from the beta globin locus (Chung *et al.* 1997) to try to reduce insertional position effects. Because in stable transfections, the vector is concatemerized, the result will

be that most inserts are flanked by two insulator sequences. This formed the control vector. This vector was constructed by Dr. H.W. Brock. Then Dr. Leonie Ringrose added the 3.8kb *rae28* upstream fragment, or the ph418 fragment to generate two experimental vectors, and generously made these vectors available to me.

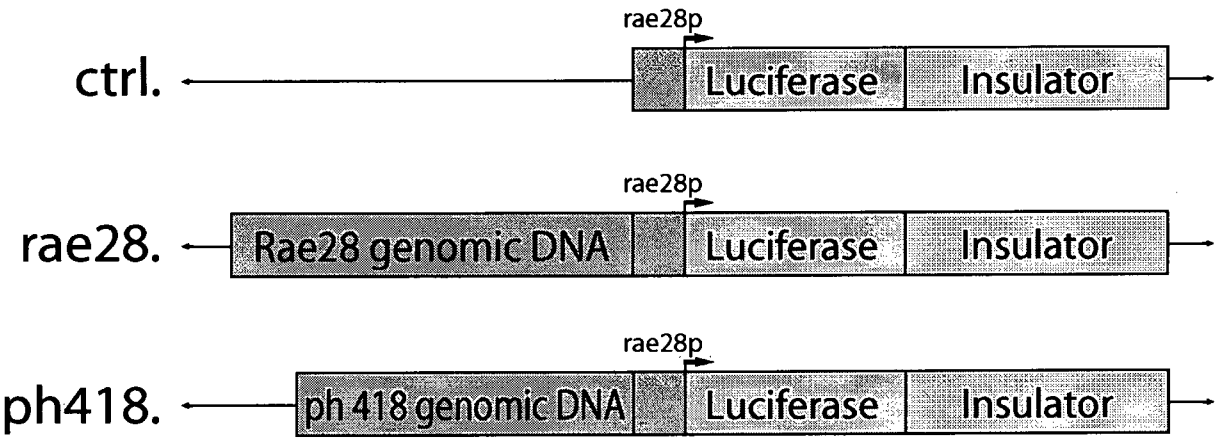

Figure 3-16 PRE assay reporters

Schematic representation of the three vectors used in the reporter assays. Each reporter has the *luciferase* reporter under the control of the *rae28* promoter. (ctrl) is the pGL-R28-3'IN vector and it is used as control. (rae28) is the pGL-Rae28-R28-3'IN vector and it contains the mouse rae28 3.8 kb upstream sequence. (ph418) is the pGL-ph418-R28-3'IN vector and it contains the *ph*418 fly fragment known to have PRE activity in flies.

V. Transfection Optimization

MEF are difficult to transfect by the calcium phosphate method or by electroporation, but they can be transfected using liposome-mediated transfection. Liposome mediated transfection offers several advantages, including relatively high efficiency in a variety of cell types, the ability to transfect cell types resistant to calcium phosphate, and requirement for less DNA. Disadvantages include cytotoxicity, and the need to optimize the DNA-to-liposome

charge ratio, the amount of DNA, cell density, and the transfection period for each type of liposome (Gao and Huang 1995). Because the success of the experiments below depended on high transfection efficiency, we optimized transfection conditions, using Effectene™, a proprietary liposome preparation (Qiagen), which has been previously tested on MEF (H.W. Brock, personal communication).

First, we compared DNA/Effectene™ ratios, with different amounts of DNA. In each case, transfection was carried out on *rae28+/+* MEF at 60% confluence in 6 well culture dishes. We compared 0.2, 0.4, and 0.8 micrograms of MSCV-IRES-RFP (MIR) DNA, in 1:10, 1:25 and 1:50 ratios of DNA to Effectene™. Transfection efficiency was monitored using FACS analysis two days after transfection. As shown in Figure 3-17, for a given amount of DNA, maximal transfection was achieved at a 1:50 ratio of DNA to Effectene™, and transfection efficiency increased as DNA amount increased. However, the number of cells surviving began to decrease if we used more than 0.8 micrograms of DNA. Similar experiments were carried out on the *rae28-/-* cells, because as argued above, they did not appear to be transformed by *TBX2*. Conditions were identical to those just described. As shown in Fig. Figure 3-18, transfection optima were similar to those observed for *rae28+/+*.

A similar set of experiments were carried out for *M33+/+* and *M33-/-* cells, except that more DNA was used, and a ratio of 1:10 DNA to Effectene™ was not tested for the highest concentration of DNA. As shown in Fig. Figure 3-19, and Figure 3-20, the highest amount of DNA, and a ratio of 1:50 DNA to Effectene™ yielded highest transfection efficiency. Notice that the highest efficiencies achieved were somewhat lower in *M33+/+* compared to *rae28+/+*, *rae28-/-* or *M33-/-*.

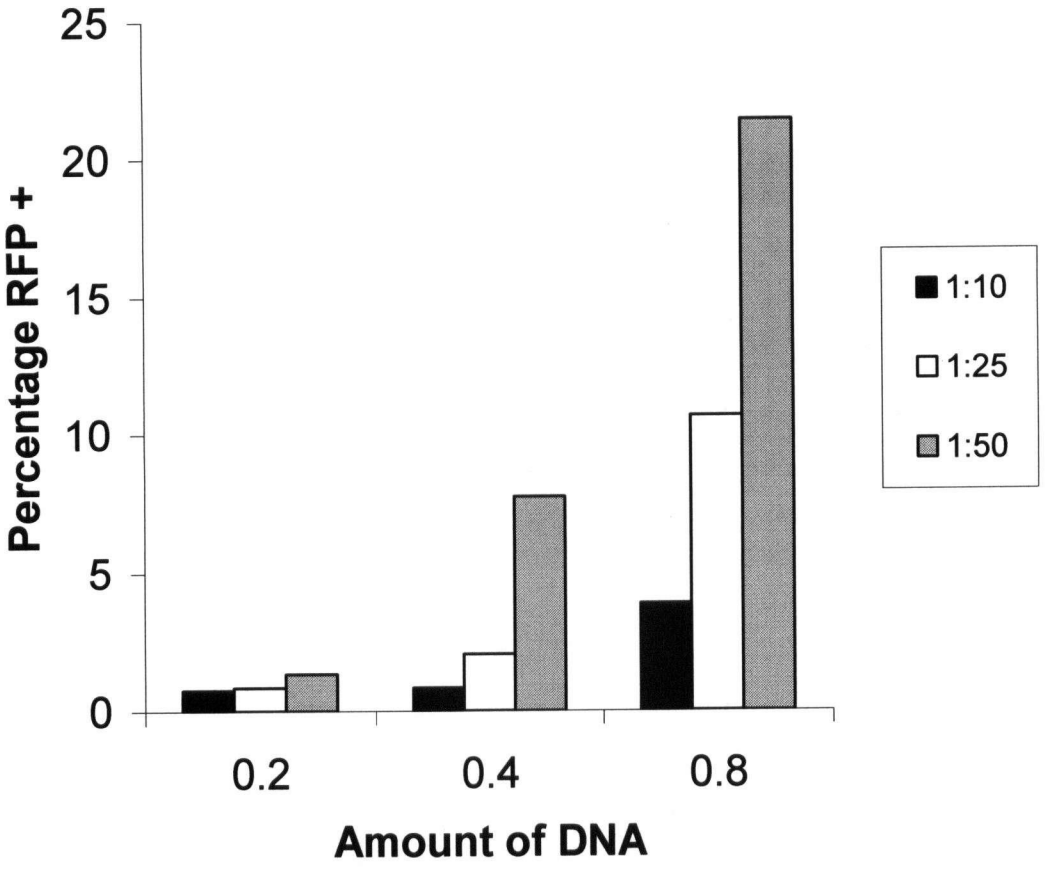

Figure 3-17 *rae28* +/+ MEF transfection optimization

Graphic representation of transfection optimization for the *rae28* +/+ MEF. Cells were 60% confluent in 6-well plates prior to transfection with MSCV-IRES-RFP (MIR). Transfection efficiency was measured by FACS two days after transfection. Different ratios of DNA/Effectene™ (indicated on the right side) where tested using the quantities (in μg) of DNA indicated. Higher quantities of DNA resulted in more than 50% cell mortality. Results form one replicate.

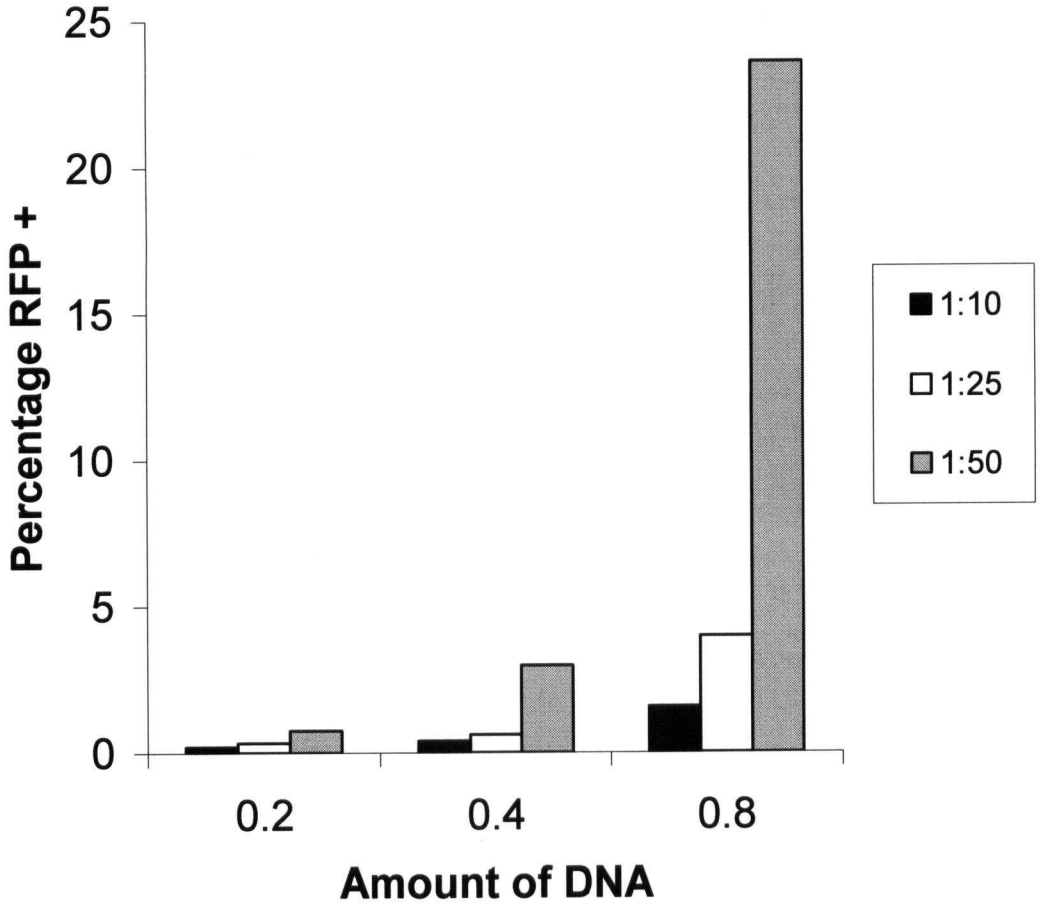

Figure 3-18 *rae28* -/- MEF transfection optimization

Graphic representation of transfection optimization for the *rae28* -/- MEF. Cells were 60% confluent in 6-well plates prior to transfection with MSCV-IRES-RFP (MIR). Transfection efficiency was measured by FACS two days after transfection. Different ratios of DNA/Effectene^{TM} (indicated on the right side) were tested using the quantities (in μg) of DNA indicated. Higher quantities of DNA resulted in more than 50% cell mortality. Results form one replicate

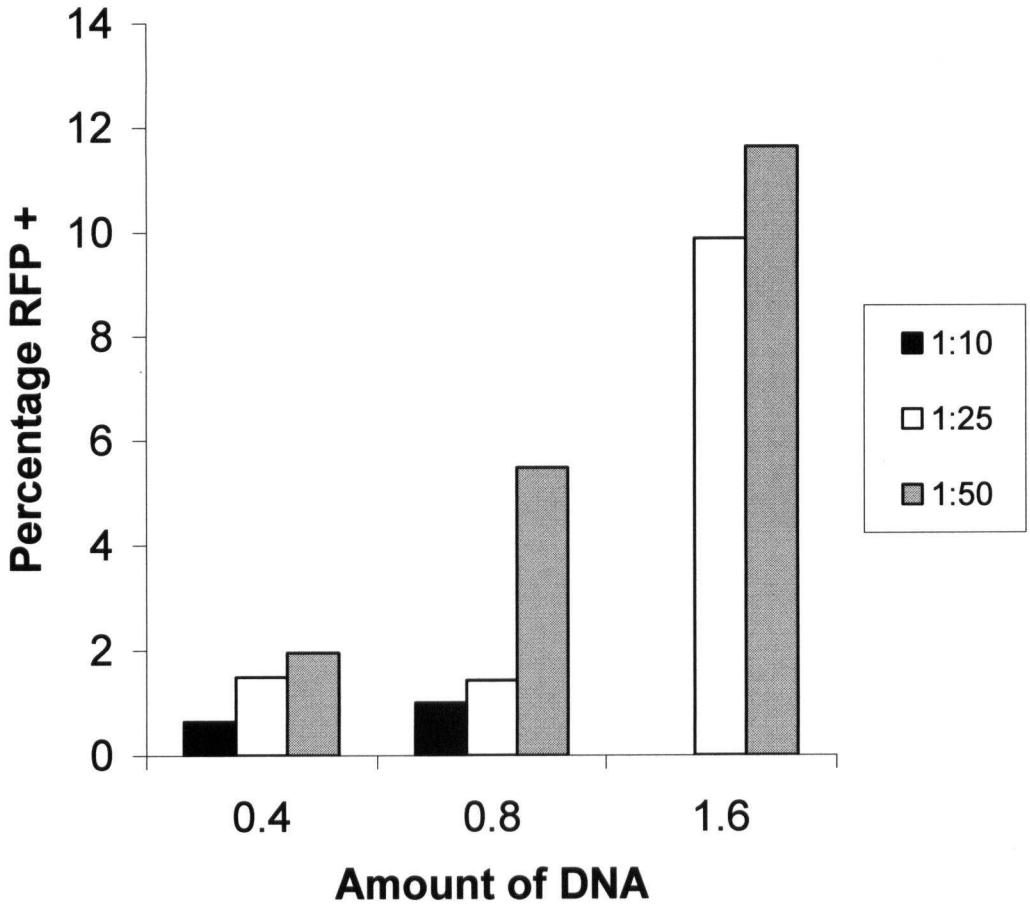

Figure 3-19 *M33* +/+ MEF transfection optimization

Graphic representation of transfection optimization for the *M33* +/+ MEF. Cells were 60% confluent in 6-well plates prior to transfection with MSCV-IRES-RFP (MIR). Transfection efficiency was measured by FACS two days after transfection. Different ratios of DNA/Effectene™ (indicated on the right side) were tested using the quantities (in μg) of DNA indicated. Higher quantities of DNA resulted in more than 50% cell mortality. The DNA/Effectene™ ratio of 1:10 and 1.6μg of DNA was not tested. Results form one replicate

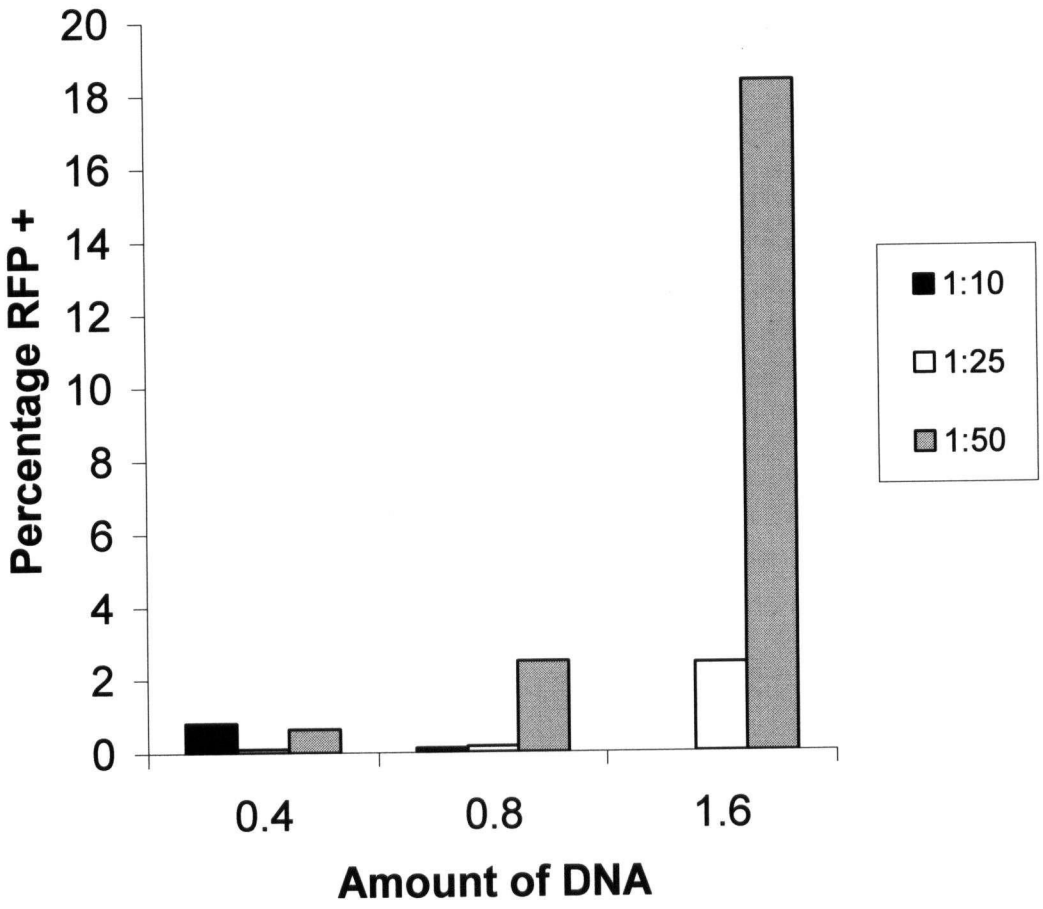

Figure 3-20 *M33* -/- MEF transfection optimization

Graphic representation of transfection optimization for the *M33* -/- MEF. . Cells were 60% confluent in 6-well plates prior to transfection with MSCV-IRES-RFP (MIR). Transfection efficiency was measured by FACS two days after transfection. Different ratios of DNA/Effectene™ (indicated on the right side) were tested using the quantities (in μg) of DNA indicated. Higher quantities of DNA resulted in more than 50% cell mortality. The DNA/Effectene™ ratio of 1:10 and 1.6μg of DNA was not tested.

For the transfections described below, 750 ng of reporter vector, and 50 ng of the vector expressing hygromycin resistance were used. The transfection medium was left on the cells for 6 hours, and then the cells were washed, and fresh medium was added.

One more set of controls was carried out to determine a killing curve for hygromycin. Because not all vectors used in these experiments contained a selectable marker suitable for

selection of stable transfectants, a plasmid containing the hygromycin gene (pMSCVhyg, Clontech) was cotransfected with the other vectors, and cells were selected for hygromycin resistance. MEF of various genotypes were exposed to varying concentrations of hygromycin for 12 days, and survival was measured by trypan blue exclusion. As shown in Figure 3-21, cells of different genotypes showed differential sensitivity to hygromycin. *M33-/-* cells were resistant to hygromycin, exhibiting 50% mortality between 600 and 700 micrograms/ml of hygromycin, *M33+/+* showed 50% mortality at just under 100 micrograms/ml, and both *rae28+/+* and *rae28-/-* were very sensitive to hygromycin, even at concentrations of 50 micrograms/ml.

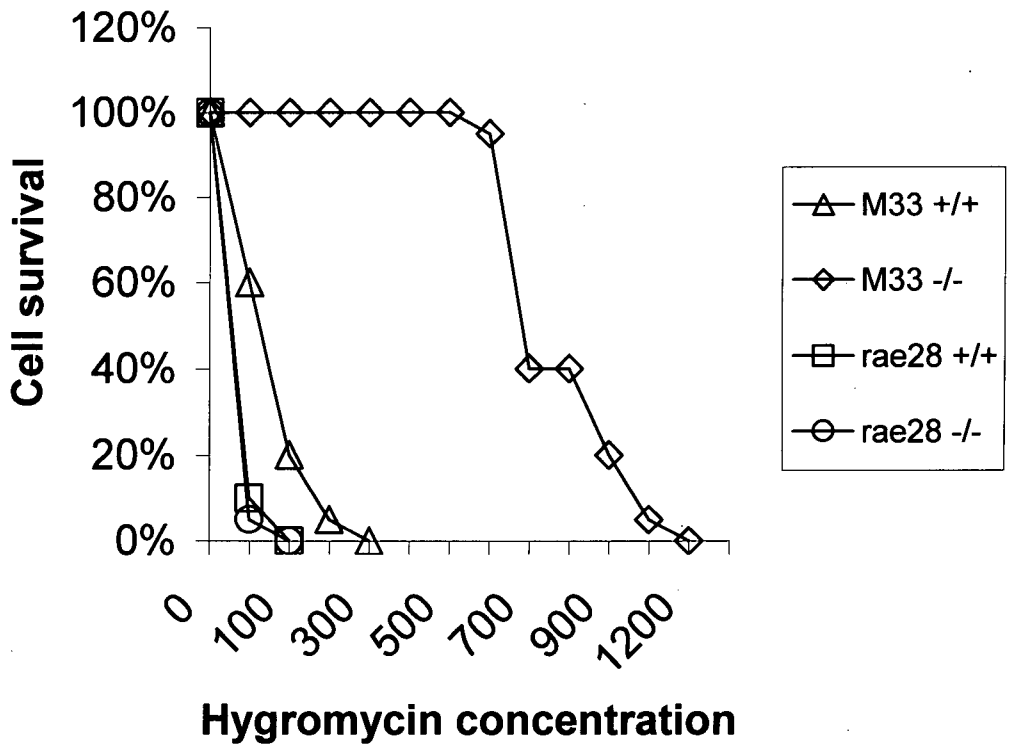

Figure 3-21 MEF dose response curve to Hygromycin B

Immortalised MEF were plated at 80% confluence in 24-well plates. Cellular viability was evaluated after 12 days of culture in medium containing the indicated amounts of Hygromycin B in μg/ml.

For the experiments reported below, 100 micrograms/ml of hygromycin was used, which was added three days after the initial transfections. Cells were kept under constant selection. Mock-transfected cells always showed complete cell death (as determined by staining with crystal violet of the plates after selection) within 10 days after selection started, but transfected cells grew well under selection.

VI. Luciferase assays

Having determined the optimal transfection conditions for MEF, our next goal was to determine if the assay system chosen to identify mammalian PREs worked in practice. All experiments tested the activity of a reporter (CTRL) that contained the *rae28* promoter and the luciferase reporter in *rae28+/+* and *rae28-/-* cells, with and without regulatory sequences from *rae28* (rae28), or *ph* (ph418). The expectation is that comparison of CTRL to either rae28 or ph418 in any cell line would show if the sequence from *rae28* or *ph* has altered expression levels relative to CTRL. If in addition, expression levels are different in *rae28+/+* vs *rae28-/-* cells, then it is possible that the difference in expression levels is dependent on *rae28* expression. If so, introducing MIG-*rae28*, a *rae28* expression vector into *rae28-/-* cells ought to rescue the change in expression in *rae28-/-* compared to *rae28+/+* cells.

Previous experiments carried out in *Mll* MEF showed that transient transfection of the reporter did not reveal potential *Mll* response elements, but that stable transfection of the reporter did reveal *Mll* response elements (H.W. Brock, unpublished). This result is consistent with the hypothesis that MLL is a chromatin-modifying protein, and that it exerts its effect only on stably integrated reporters because these assemble normal chromatin structure, whereas unintegrated (transiently transfected) plasmids do not have normal

chromatin structure. Therefore, only stable transfectants of the reporter was assayed. Because the reporter vectors do not have a selectable marker to allow selection of stable transformants, we co-transfected a plasmid with a hygromycin resistance gene (pMSCV-hyg, Clontech) at a ratio of 1/20, and selected for hygromycin resistance. Three days after transfection, the cells were exposed to hygromycin, and grown under selection until all mock-transfected cells died. Usually, very few cells survived selection, and low numbers (1-3) of colonies were detected. This very low transfection efficiency was a continuing problem.

In an initial series of experiments, we limited analysis to the rae28 and Ctrl plasmids in *rae28+/+* and *rae28-/-* MEF. Three independent transfections were carried out in 6 well culture dishes with each plasmid in *rae28-/-* cells and in *rae28+/+* cells. The results are shown in Table 3-2. From this table, it can be seen immediately that there is large variability in luciferase activity. There were always transfections that yielded drug resistant colonies, but no luciferase activity, and there were up to two-log variations in luciferase activity when it was observed. In all experiments, more colonies in *rae28-/-* than in *rae28+/+* transfections were obtained, so this might influence reliability of the results. We expect that more colonies should decrease variation in expression levels, because more integration sites are being sampled for a given transfection.

While clearly, this extent of variability precludes drawing strong conclusions from the data, the data was plotted using the following assumptions, in the hope that underlying trends might be discerned as a basis for planning future experiments. All samples with no activity were removed, reasoning that these likely integrated the drug resistance marker, but not the reporter. Samples with 1 log lower activity than the sample with the highest activity were also removed, arguing that decreased activity could result from plasmid rearrangements, insertion

into regions of the genome that are not active in gene expression ("position effects"), or differences in copy number. None of these possibilities was tested directly. Removing low data has the effect of artificially reducing variability in the data. While both these assumptions are reasonable, it is obvious that removing data from the analysis using arbitrary value is not consistent with normal experimental practice. These data are plotted in Figure 3-22. Both the Ctrl and rae28 plasmids show higher expression in *rae28-/-* cells than in *rae28+/+* cells, suggesting that *rae28* might act as a repressor.

Table 3-2 Results: first series of reporter assays in 6-well plates

MEF Genotype	Vector	Luciferase activity[a]
rae28 -/-	rae28	676531
rae28 -/-	rae28	74126
rae28 -/-	rae28	0
rae28 -/-	ctrl	173862
rae28 -/-	ctrl	256568
rae28 -/-	ctrl	5944
rae28 +/+	rae28	0
rae28 +/+	rae28	180435
rae28 +/+	ctrl	203
rae28 +/+	ctrl	16693

[a] All samples were collected from confluent wells. Three independent transfections were performed for each vector in each cell types. Transfections that did not produce colony were not analyzed for luciferase activity.

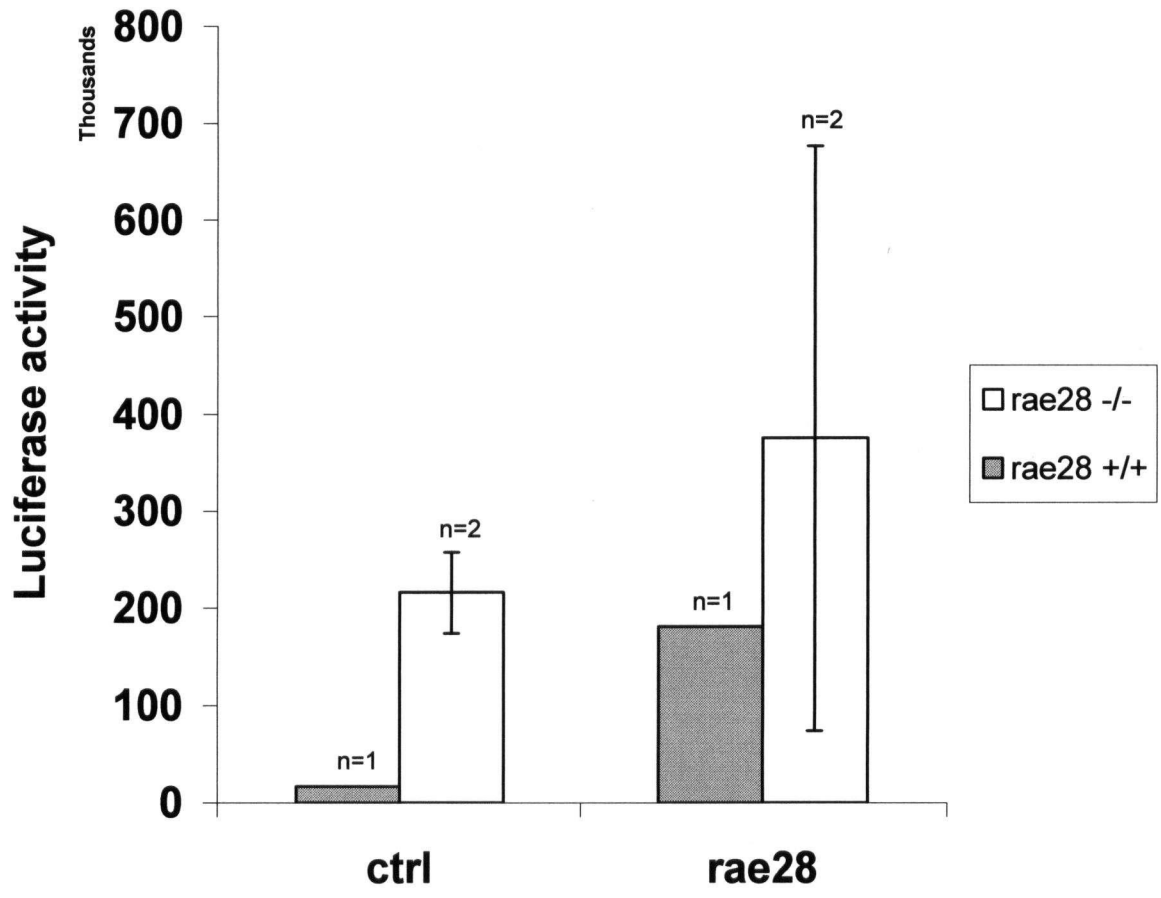

Figure 3-22 First series of reporter assays in 6-well plates

Normalized luciferase activity of stable transfections in immortalized *rae28* +/+ and rae28 -/- MEF of the reporters pGL-R28-3'IN (ctrl) and pGL-Rae28-R28-3'IN (rae28). The error bars indicate the standard error of the mean for the (n) independent experiments indicated. As discussed in the text, samples with low transfection efficiency or activity were not incorporated into this figure.

We repeated these experiments, except that the *ph 418* plasmid was added. As shown in Table 3-3, we had similar problems with differing transfection efficiency comparing *rae28+/+* to *rae28-/-*. Because the colonies grew slowly, and cells in the middle of the colony had different morphology than cells at the edge, viability differences within the colony could cause variability in the results. For this reason, once colonies were visible, cultures were trypsinized, and the cells were replated and grown to confluence, then assayed for luciferase

activity. Once again, the data was plotted, we removed the null data, and data more than one log lower than replicate transfections, and the result is shown in Figure 3-23. In each case, the reporter is more highly expressed in *rae28-/-* than in *rae28+/+* cells, consistent with *rae28* being a repressor of the reporter. Because this is true for the Ctrl plasmid, it implies that Rae28 acts upon the *rae28* promoter. A significant difference is seen comparing rae28 to Ctrl, as about 5 fold higher activity is seen, suggesting that the 3.8kb *rae28* sequence overall is an activator of reporter activity. Finally, it is interesting that the *ph* 418 fragment does not have higher activity than the Ctrl plasmid, suggesting that the Drosophila regulatory sequence does not function similarly to the 3.8 kb *rae28* fragment in MEF. As with the previous experiment, these conclusions must be tentative, given the high variability in the data.

One obvious problem of interpretation comes from our previous conclusion that *rae28+/+* cells are transformed by TBX2, whereas *rae28-/-* cells were probably transformed by an independent mechanism. Therefore the *rae28+/+* and the *rae28-/-* cells may not be directly comparable. One way to sidestep this issue is to compare *rae28-/-* cells with *rae28-/-* cells expressing *rae28*, so that the only difference is in presence or absence of *rae28.* Accordingly, similar experiments were carried out, except that *rae28-/-* cells infected with MIG-rae28, an expression vector that makes wild-type murine *Rae28* were also examined. Ecotropic Producer cells $GP^{+}E86$ (Markowitz *et al.* 1988) containing the MSCV-rae28cDNA-IRES-GFP (E86-MIG-rae28) vector and constitutively producing the MIG-*rae28* virus were obtained from Dr. Yoshihiro Takihara (produced in Dr. Keith Humphries' Laboratory). Using procedures detailed in the Materials and Methods, supernatant containing the virus was obtained from cells, filtered and immediately used for infection of the *rae28-/-*

Table 3-3 Results: second series of reporter assays in 6-well plates

MEF Genotype	Vector	Luciferase activity[a]
rae28 -/-	rae28	4034620
rae28 -/-	rae28	2215026
rae28 -/-	rae28	4739968
rae28 -/-	ph418	542506
rae28 -/-	ph418	602737
rae28 -/-	ph418	1180980
rae28 -/-	ctrl	769878
rae28 -/-	ctrl	559519
rae28 -/-	ctrl	764874
rae28 +/+	rae28	169212
rae28 +/+	rae28	22301
rae28 +/+	ph418	17274
rae28 +/+	ph418	0
rae28 +/+	ph418	0
rae28 +/+	ctrl	1308

[a] All samples were collected from confluent wells. Three independent transfections were performed for each vector in each cell types. Transfections that did not produce colony were not analyzed for luciferase activity.

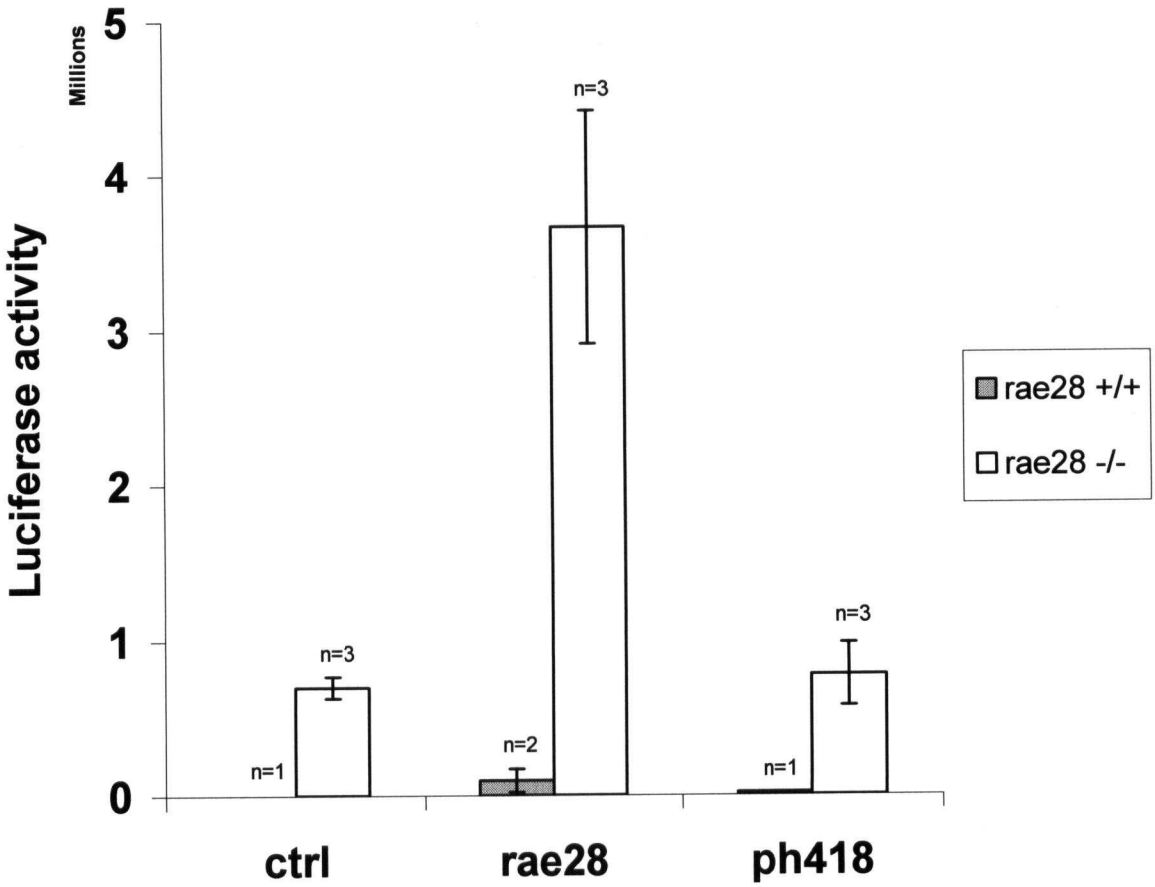

Figure 3-23 Second series of reporter assays in 6-well plates

Normalized luciferase activity of stable transfections in immortalized *rae28* +/+ and *rae28* -/- MEF of the reporters pGL-R28-3'IN (ctrl), pGL-Rae28-R28-3'IN (rae28) and pGL-ph418-R28-3'IN (ph418). The error bars indicate the standard error of the mean for the (n) independent experiments indicated. As discussed in the text, samples with low transfection efficiency or activity were not incorporated into this figure.

fibroblast prior transfection of the reporter vectors. The raw data are shown in Table 3-4. As before, there was high variability in the luciferase activity. To look for trends in the data, the same assumptions as in the two previous experiments were made, with one additional assumption. Because the MIG-rae28 vector expresses GFP, replicates in which more than 25% of cells were GFP negative were eliminated, and the results are plotted in Figure 3-24. No working data for the Ctrl vector in the *rae28-/-* plus MIG-rae28 transfections was

obtained. With these caveats in mind, it is striking that for each reporter (Ctrl, rae28, and ph418), there is more activity in *rae28-/-* than in *rae28+/+* cells, and that the activity is strongly reduced when *rae28* is expressed in *rae28-/-* cells. These results support the conclusion that Rae28 is a repressor, and they also support the conclusion that the 3.8kb rae28 fragment is a Rae28 response element. As before, the ph418 fragment is no more active than the Ctrl fragment, suggesting that this fragment does not function in MEF. If so, Rae28 is presumably acting at the *rae28* promoter.

Table 3-4 Results: Third series of reporter assays in 6-well plates

MEF Genotype	Vector	Luciferase activity[a]	Percentage of GFP + cells (MIG-*rae28*)
rae28 -/-	rae28	5596628	
rae28 -/-	rae28	2174484	
rae28 -/-	rae28	3782477	
rae28 -/-	ph418	935567	
rae28 -/-	ph418	2700786	
rae28 -/-	ctrl	1511467	
rae28 -/-	ctrl	1206119	
rae28 +/+	rae28	1457	
rae28 +/+	rae28	49457	
rae28 +/+	rae28	192422	
rae28 +/+	ph418	41107	
rae28 +/+	ph418	305171	
rae28 +/+	ctrl	81621	
rae28 +/+	ctrl	9314	
rae28 +/+	ctrl	808070	
rae28 -/- + MIG-rae28	rae28	553503	90
rae28 -/- + MIG-rae28	rae28	614165	85
rae28 -/- + MIG-rae28	rae28	497687	47
rae28 -/- + MIG-rae28	ph418	26320	78
rae28 -/- + MIG-rae28	ph418	33164	99
rae28 -/- + MIG-rae28	ph418	3671	93
rae28 -/- + MIG-rae28	ctrl	437	98[b]
rae28 -/- + MIG-rae28	ctrl	740	95[b]
rae28 -/- + MIG-rae28	ctrl	6333162	53

[a] All samples were collected from confluent wells. Three independent transfections were performed for each vector in each cell types. Transfections that did not produce colony were not analyzed for luciferase activity.
[b]ells had abnormal phenotype and were not used in further analysis.

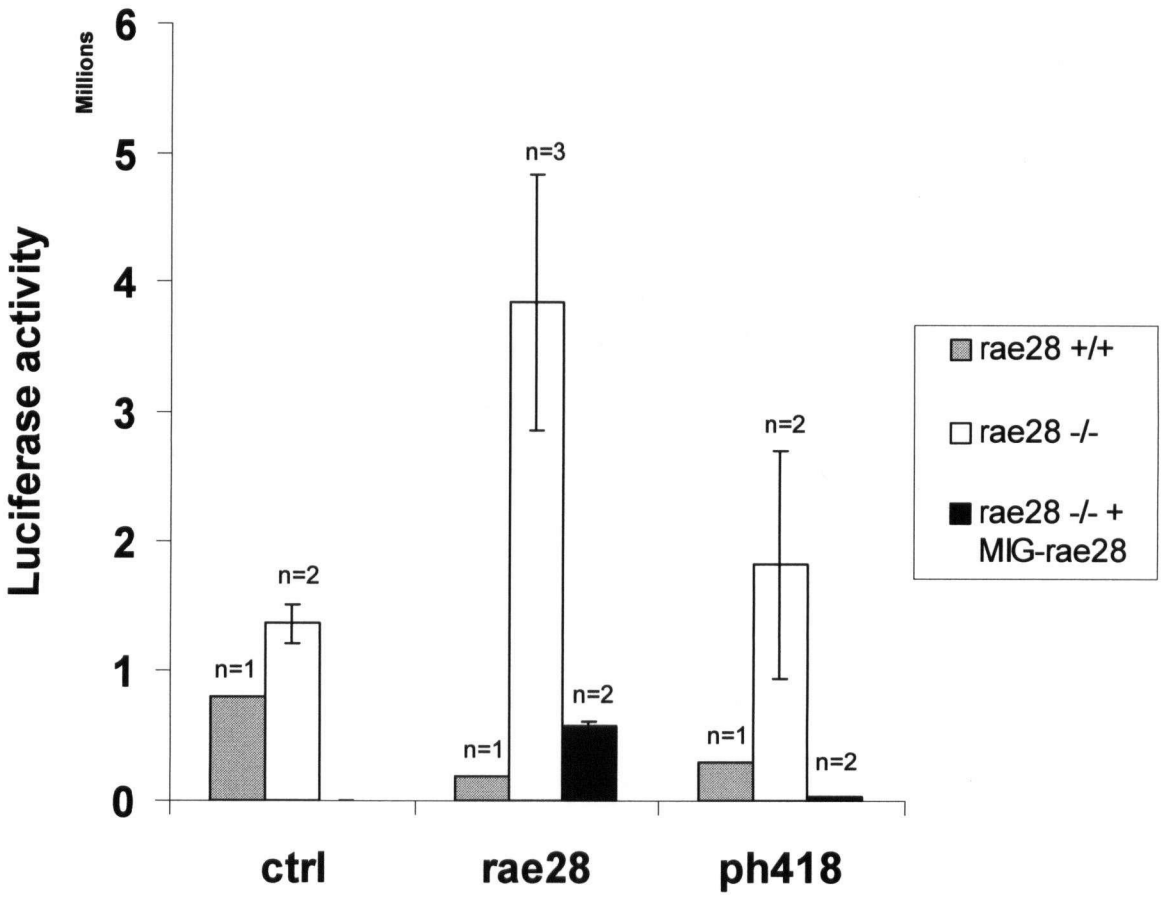

Figure 3-24 Third series of reporter assays in 6-well plates

Normalized luciferase activity of stable transfections in immortalized *rae28* +/+, *rae28* -/- and rae28 -/- + MIG-rae28 MEF of the reporters pGL-R28-3'IN (ctrl), pGL-Rae28-R28-3'IN (rae28) and pGL-ph418-R28-3'IN (ph418). The error bars indicate the standard error of the mean for the (n) independent experiments indicated. As discussed in the text, samples with low transfection efficiency or activity were not incorporated into this figure.

CHAPTER 4 General discussion

The studies outlined in the previous chapter show that it is possible to establish immortalized MEF that are mutant for three different PcG genes: *Asxl1, M33* and *rae28*, using TBX2. Nevertheless, the results with *rae28-/-*, in which it appears very likely that a spontaneous mutation resulted in immortalization, illustrates the need to confirm that TBX2 is expressed in MEF. In our case, having *rae28+/+* and *rae28-/-* cells immortalized by different routes makes it very difficult to compare gene expression data obtained in each cell type, because it is not possible to assign differences to presence or absence of *rae28*, or to changes induced during immortalization.

We now wish to consider some of the potential problems of immortalized cells in general, and to make arguments suggesting that they are not useful for the study of endogenous genes, like those illustrated in Figure 3-7 to Figure 3-13 and Figure 3-15.

In mixed populations, effects owing to insertional mutagenesis of the retroviral vector expressing TBX2 would be minimized, since the population would contain cells with many different insertion sites, supporting the use of mixed populations of MEF in any study. In our studies, mixed populations of fibroblasts were transfected, grown out, and a presumably mixed population of cells with varying growth rates were passaged. It can be expected that over time, the fastest growing cells would be selected, and thus that variation within the population would decrease, perhaps until only one cell established itself. This possibility suggests that in future studies, it would be advisable to determine the clonality of immortalized cells. This argument also suggests that it would be good to establish cloned

lines, and to compare results among clones. If the same results were obtained in multiple clonal cell lines, this would increase the confidence that the results obtained were not a consequence of downstream effects of insertional mutagenesis. Unfortunately, MEF immortalized with TBX2 did not grow well when plated at low density making the cloning of single cells difficult.

A related problem of using immortalized MEF for functional studies of endogenous gene expression is illustrated by the results comparing Hox expression in early passage and late passage *rae28-/-* MEF (Figure 3-15). In cells prior to immortalization, the Hox c cluster genes are expressed, whereas in late passage cells, they are not. Because the genotype of both cells is identical, the differences presumably arise from downstream, indirect consequences of immortalization, rather than from differences in genotype (i.e. *rae28+/+* vs *rae28-/-*). For example, a mutation in a transcription factor may be responsible for both the immortalization of the fibroblast and the reduction in expression of the Hox C cluster. Because of this problem, in our view, MEF of different genotypes are not useful for studying the expression of endogenous genes. However, they may be useful for short-term studies of transgenes.

Even if clonal differences in MEF are observed and controlled for, there is another potential problem for analysis of endogenous genes. Because the MEF are taken from mutant embryos, and PcG genes regulate the activity of many genes, it can be expected that there will be indirect effects of the PcG mutation of downstream genes, that in turn could affect genes further downstream and so on. So it may not be very useful to consider differences between cells with and without a given PcG gene to be direct rather than indirect consequences of the mutation. In turn, this argues against attaching much importance to changes in expression of endogenous genes in mutant versus wild-type MEF. Another problem for analysis of

endogenous genes is that the embryological origin of the MEF is unclear. One can imagine that MEF taken from the anterior of the embryo might express different populations of Hox (or any other) genes than MEF taken from the posterior.

Considering the comments above, we suggest that MEF are best used to study the expression of transgenes. We wish to comment on some of the potential problems in these studies, based on the data we obtained, and on some theoretical considerations. While the results obtained suggest that there might be a PRE upstream of *rae28*, the overall variability of the results precludes any firm conclusions.

Overall, the symptom of the problem was very large variability between independent replicate transfections. There are many potential underlying explanations.

Variation in luciferase levels might be expected because of insertional position-effects, the number of transgenes concatemerized at each insertion site, or whether the hygromycin resistance gene, but not the reporters were successfully integrated. Therefore, independent transfections yielding one or two colonies per well are more likely to have biased samples than wells containing multiple colonies, because with a large enough sample, positional effects and copy number differences should average out. It is notable that in our experiments, the overall variability of the data was less for mutant MEF than for wild-type MEF, and that the mutant MEF had a much higher transfection efficiency. In theory, these experiments would be improved by transfecting many more cells. In practice, this would be expensive, because of the cost of the transfection reagent, the cost of preparing large amounts of DNA, the cost of culturing cells, and the cost of the luciferase assays. For example, to obtain 1000

colonies from transfection of wild-type MEF, it would cost about $5600 for the Effectene™ alone, for one vector, which is clearly impractical.

Because of the difference in transfection efficiency between wild-type and mutant MEF, and because of potential indirect effects, it may be better to avoid comparing expression levels of reporters in wild-type and mutant MEF. Instead, it might be better to compare mutant MEF only to MEF in which the wild-type product is supplied by means of an expression vector, so that the cells are identical, except for the presence or absence of the protein being investigated. Despite this concern, it is notable that the luciferase expression results in *rae28* wild-type and *rae28* mutant MEF supplied with the wild-type expression vector for *rae28* shown in Figure 3-24 are rather similar, suggesting limited indirect effects on our reporter vector.

Overall, we would advise those following in our footsteps that the current development of RNAi offers a much better way to do the experiments undertaken here. The experiment would be to take a cell in which the target gene of interest, plus the PcG genes under test is expressed to serve as the baseline, and compare this to the same cell in which RNAi is used to knock down expression of a PcG gene. While the experiment would be done in immortalized cell lines, all conditions would be the same in cells with and without the RNAi vector. Because the current literature suggests that significant knock down of expression can occur in 48 hours, there is far less time for establishment of potential indirect effects. Moreover, analysis of an endogenous target in its normal chromosomal context should improve the results.

Another improvement on these experiments would be to use a targeting system to ensure that the reporter gene is integrated into one genomic site as a single copy. An example is provided by the FLP-In™ (Invitrogen) system, in which an FLP recombination Target (FRT) site is inserted as a single copy into the genome in the test cell, and then a reporter flanked by an FRT site is transfected into the test cell in the presence of an expression vector expressing the FLP recombinase. The FLP recombinase mediates recombination between the FRT sites in the genome and the reporter, resulting in single copy integration of the reporter. Such a system would eliminate position effects, and combined with RNAi, would yield much more reliable, and repeatable results. This would be a good way to map PREs.

Understanding epigenetic gene regulation will have important implications for human biology and diseases. Chromatin modulators now seem to be frequently involved in tumorigenic pathway. They have a well-established function in modifying the histone and probably a function in regulating cell cycle pathway. Unfortunately, the mammalian epigenetic mechanisms are poorly understood. The discovery of target binding site for mammalian PcG homologs will be an important step for the comprehension of these mechanism.

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