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Regulation of cellular memory by non-coding transcription through Polycomb group response elements

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Table of contents

Zusammenfassung

Die Bildung funktionell unterschiedlicher Zelltypen ist ein entscheidender Prozess in der Entwicklung eines multizellulären Organismus. Dabei findet die Determination von Zellen gewöhnlich während der frühen Entwicklung statt, wohingegen ihre Differenzierung erst in späteren Stadien beginnt. Auf molekularer Ebene wird die Determination von Zellen durch die Expression einer charakteristischen Kombination von Genen erreicht. Eine zentrale Voraussetzung für die geordnete Differenzierung von Zellen gemäß ihrer Identität ist daher die Aufrechterhaltung der spezifischen Genexpression über viele Zellteilungen hinweg. Diese Aufgabe übernimmt das so genannte Zellgedächtnis.
Ein geeignetes Modellsystem zur Erforschung des molekularen Mechanismus des Zellgedächtnisses ist die Regulation der homöotischen Genexpression in *Drosophila melanogaster*. Zahlreiche Studien belegen, dass die Grundlage des Zellgedächtnisses ein epigenetischer Mechanismus ist. Die Aktivitäten der Polycomb-Gruppen (PcG) Proteine verändern die Chromatinstruktur in einer Weise, die Transkription verhindert, und vermitteln so die Vererbung des reprimierten Zustands. Im Gegensatz dazu führen die Aktivitäten der Trithorax-Gruppen (TrxG) Proteine zur Bildung einer transkriptionell kompetenten Chromatinstruktur, und sind so für die Vererbung aktiver Genexpression verantwortlich. Die Rekrutierung sowohl von PcG als auch von TrxG Proteinen an das Chromatin hängt von identischen *cis*-regulatorischen Elementen ab, den sogenannten Polycomb group response elements (PREs). Dies wirft die Frage auf, wie an einem gegebenen Lokus die Entscheidung zwischen epigenetischer Repression und Aktivierung getroffen wird. Der Grundzustand eines PREs ist vermutlich die PcG-abhängige Repression, während das Umschalten in den epigenetisch aktivierten Zustand externe Signale benötigt. Interessanterweise korreliert die epigenetische Aktivierung von PREs mit nicht-kodierender Transkription durch diese Elemente selbst. Eine zentrale Frage ist, ob diese Transkription eine regulatorische Funktion hat, oder ob sie eine Konsequenz der epigenetischen Aktivierung ist.

Das Hauptziel dieser Arbeit war es, die Funktion der nicht-kodierenden Transkription durch PREs zu klären. Durch die Analyse eines transgenen Reportersystems konnte gezeigt werden, dass die Transkription durch das *Fab-7* PRE als Anti-Silencing Mechanismus fungiert, der der PcG-abhängigen Repression direkt entgegenwirkt. Die Transkription spielt hierbei vermutlich nicht nur eine wichtige Rolle in der Etablierung epigenetischer Aktivierung, sondern wird auch für deren Aufrechterhaltung während der gesamten Entwicklung benötigt. Interessanterweise werden auch PREs ausserhalb der bisher untersuchten homöotischen Genkomplexe transkribiert. Die Transkription durch PREs hat daher vermutlich eine generelle Funktion in der Regulation des Zellgedächtnisses.
Im zweiten Teil der Arbeit wurden die epigenetischen Konsequenzen der Transkription durch das *Fab-7* PRE untersucht. In der transgenen Situation sind sowohl PcG als auch TrxG Proteine an *Fab-7* gebunden, unabhängig vom epigenetischen Status des PRE. Die Hauptfunktion der Transkription durch PREs könnte daher die Modulation der Aktivitäten von PcG und TrxG Proteinen sein.
Um Einsicht in die molekulare Funktion der Transkription durch PREs zu erhalten, wurden die Eigenschaften der nicht-kodierenden RNAs untersucht. Da die Aktivierungsfunktion nicht-kodierender Transkription unabhängig von ihrer Orientierung ist, ist eine sequenz-spezifische Funktion der PRE Transkripte unwahrscheinlich. Die Ergebnisse schließen jedoch eine strukturelle Rolle der RNAs nicht aus. Interessanterweise konnte *Fab-7* RNA an mitotischen Chromosomen detektiert werden. Somit könnte die Vererbung epigenetisch aktiver Chromatinstrukturen durch die Zellzyklus-abhängige Transkription und/oder direkt durch die Assoziation nicht-kodierender RNAs mit dem Chromatin vermittelt werden.

Summary

The formation of functionally distinct cell types from a single zygote is a fundamental aspect of metazoan development. The specification of cell identities usually occurs during early development, long before this information is used during differentiation. The molecular basis of cell fate specification is the activation of characteristic gene expression programs. A prerequisite for the appropriate differentiation of cells according to their fate is therefore the faithful transmission of once established gene expression states throughout phases of cell proliferation and growth. This task is accomplished by the so-called cellular memory.

A paradigm to study the molecular basis of the cellular memory is the regulation of homeotic gene expression in *Drosophila*. A number of studies have shown that the cellular memory is an epigenetic mechanism based on the modification of the chromatin structure. This is accomplished through the antagonizing activities of repressive PcG (Polycomb group) and activating TrxG (Trithorax group) proteins. The recruitment of both PcG as well as TrxG proteins to their target loci depends on the presence of identical *cis*-regulatory elements termed Polycomb group response elements, or PREs. How is the decision between epigenetic silencing and activation at a PRE taken? Presumably, the default state of a PRE is to function as a PcG-dependent silencer, whereas the conversion into the active mode requires incoming signals. It has previously been shown that the epigenetically active state at a PRE correlates with non-coding transcription through these elements themselves. This raised the question whether the non-coding transcription is of functional significance for the epigenetic activation of a PRE, or if it reflects only a consequence of this process.

The major aim of this thesis was to determine whether the transcription through the well-characterized *Fab-7* PRE has any function in the regulation of the epigenetic state of this element. In the first part, a transgenic reporter system was used to answer this question. The results obtained show that the transcription through *Fab-7* functions as a novel anti-silencing mechanism that counteracts the PcG-mediated repression by default. Depending on the tissue and/or on the locus, transcription through PREs may be required throughout development to prevent the re-establishment of PcG silencing. Importantly, PREs located outside the homeotic gene complexes are also transcribed, suggesting that the anti-silencing function of transcription through PREs may be a fundamental aspect of the cellular memory.

In the second part, the transgenic reporters mentioned above were used to analyze the epigenetic consequences of transcription through the *Fab-7* PRE. The results suggest that at least on the transgene, both repressive PcG as well as activating TrxG proteins associate with the *Fab-7* PRE, irrespective of the epigenetic state of this element. The primary function of transcription through the *Fab-7* PRE may therefore be to modulate the activities of PcG/TrxG complexes, rather than regulating their differential recruitment to the chromatin.

To get insight into the precise function of non-coding transcription through PREs, the properties *Fab-7* RNA were investigated in SF4 tissue culture cells *in vitro*, as well as in wildtype embryos *in vivo*. Although a sequence-specific function of *Fab-7* RNA is unlikely, the results obtained do not exclude structural function of non-coding PRE transcripts. Futhermore, *Fab-7* RNA can be detected on mitotic chromatin, suggesting that the transmission of epigenetically activated states through cell division might depend on the timing of non-coding transcription with respect to the cell cycle and/or the association of non-coding RNAs with the chromatin.

Since the functions of PcG/TrxG proteins have been conserved during evolution, the results presented here might have implications on our understanding of epigenetic gene regulation in mammals.

Introduction

1. Introduction

One of the fundamental features of development in multicellular organisms is the specification of distinct cell types in appropriate patterns. The determination of distinct developmental fates is achieved through the establishment of characteristic selector gene expression programs, which in turn are responsible for the activation of effector genes that define the pathway of differentiation (Gellon and McGinnis 1998). Usually, the commitment of cells occurs long before functionally specialized cell types can be observed. This means that the cell-specific transcriptional programs established in the course of determination have to be inherited throughout phases of growth and proliferation. The inheritance of transcriptional states through many rounds of cell division relies on an epigenetic mechanism termed „cellular memory“ (Paro and Harte 1996). As such, the inheritance of gene expression regulated by the cellular memory is independent of changes in the DNA sequence, but acts at the level of DNA packaging within the chromatin (Wolffe and Matzke 1999). What is the molecular basis of this cellular memory? An ideal model organism to study such a fundamental question in developmental biology is *Drosophila melanogaster*, since many of the genes involved in this process have been characterized in the fly.

1.1 The specification of cell fates during Drosophila embryogenesis

The earliest patterning events in the *Drosophila* embryo are regulated by gene products provided by maternal cells. The specification of anterior-posterior (A-P) and dorsal-ventral (D-V) polarity, for instance, occurs in the oocyte, even before fertilization (Ingham 1988; St Johnston and Nüsslein-Volhard 1992). A-P axis formation depends on the localization of maternal mRNAs such as *bicoid (bcd)* and *nanos (nos)* at the prospective anterior and posterior poles, respectively (Driever and Nüsslein-Volhard 1988; Struhl 1989a). After fertilization, these mRNAs are translated, leading to the formation of morphogen gradients along the A-P axis, thus providing a pre-pattern which guides the subsequent development. In addition to their role as translational regulators, these morphogens function as transcription factors at the top of a zygotic transcriptional cascade, whose function is the sequential subdivision of the bipolar embryo into fourteen distinct parasegments (PS).

The first step in the hierarchy is the activation of the gap genes, which define broad domains of contiguous prospective body segments (Driever and Nüsslein-Volhard 1989; Struhl 1989b; Gaul and Jäckle 1990; Rivera-Pomar et al. 1995). The gap gene transcription factors, in turn,

convey positional information on the pair-rule genes, which define the number and borders of PS (St Johnston and Nüsslein-Volhard 1992; Rivera-Pomar and Jäckle 1996). Within individual PS, the subsequent activation of segment-polarity genes then establishes the boundaries between anterior and posterior compartments (Martinez-Arias and Lawrence 1985).

Each embryonic PS defines a single unit of the metameric body plan of the adult fly, and already at this stage, founder cells are singled out that will later form the tissues of the adult (Morata and Lawrence 1975). The identities of individual PS are specified by the expression of a characteristic pattern of homeotic selector genes, or *Hox* genes, along the A-P body axis (Lewis 1978; Kaufman et al. 1990; Kennison and Tamkun 1992). Consequently, the mutation of a homeotic gene does not lead to the loss of segment identity, but instead results in a homeotic transformation, where the affected PS adopts the identity of another segment (McGinnis and Kuziora 1994; Castelli-Gair and Akam 1995).

The establishment of homeotic gene expression is controlled by transcription factors encoded by the gap and pair-rule genes (Qian et al. 1991; Zhang et al. 1991; Müller and Bienz 1992; Shimell et al. 1994; Casares and Sanchez-Herrero 1995). These transcription factors disappear after the first 6 hours of development (Frasch et al. 1987; Gaul et al. 1987; Tautz 1988). Therefore, in order to allow differentiation to proceed correctly, a mechanism has to exist which ensures that the initial homeotic gene expression patterns are transmitted through many rounds of cell division, until differentiation starts.

1.2 The basic components of the cellular memory

Once the transcriptional states of homeotic genes have been established, the cellular memory system takes over the control to ensure their mitotic inheritance. The core components of the cellular memory have been uncovered by virtue of their mutant phenotypes. Proteins encoded by the Polycomb group (PcG) are responsible for maintaining target genes in the repressed state. Therefore, mutations in these genes result in phenotypes reminiscent of ectopic *Hox* gene expression (Lewis 1978; Struhl and White 1985; Glicksman and Brower 1990; Jones and Gelbart 1990; Simon et al. 1992). The counteracting Trithorax group (TrxG) proteins, on the other hand, propagate the transmission of active gene expression states (Kennison and Tamkun 1988). In addition to these *trans*-acting factors, extensive genetic studies have identified chromosomal elements which are required in *cis* to mediate the PcG/TrxG dependent inheritance of transcriptional states (Simon et al. 1990; Zhang and Bienz 1992;

Busturia and Bienz 1993; Simon et al. 1993; Chan et al. 1994; Christen and Bienz 1994; Poux et al. 1996). These *cis*-elements have been termed „PcG response elements“, or PREs.

1.2.1 Keeping the silence – the Polycomb group

To date, 21 different PcG genes have been described in *Drosophila*, and for more than half of these, mammalian homologues have been identified. Although the molecular functions have only been characterized for a subset, it is now well established that PcG proteins silence their target genes by changing the chromatin structure. Biochemical studies have shown that PcG proteins function in the context of large multiprotein complexes. There is accumulating evidence that the composition of these complexes is dynamic and that their activities may be subject to tissue-specific and/or developmental regulation (Strutt and Paro 1997; Otte and Kwaks 2003).

Generally, PcG complexes fall into two classes: Polycomb Repressive Complex 2 (PRC2)- and PRC1-type complexes (Fig. 1.1). The 600kDa PRC2 complex originally purified from *Drosophila* embryos, consists of the core components Enhancer of Zeste (E(Z)), Extra Sex Combs (ESC), and Suppressor (12) of Zeste (SU(Z)12) (Fig. 1.1). The E(Z) subunit of this complex catalyzes the methylation of histone H3 at lysine 27 (H3K27) (and to a lesser extent at lysine 9), a modification which is associated with PREs and other repressed, heterochromatic regions (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002; Müller et al. 2002). In some preparations, the histone deacetylase RPD3 (dHDAC1) co-fractionates with the PRC2 complex (Tie et al. 2001), but it remains unclear if this activity is required for silencing.

The core subunits of the 1-2MDa PRC1-type PcG complexes (Fig. 1.1) are Polycomb (PC), Polyhomeotic (PH), Posterior sex combs (PSC), and *Drosophila* Ring1 (dRING1) (Saurin et al. 2001; Levine et al. 2002). This PRC1 complex, as well as a reconstituted form comprising only the core components (PCC for PRC1 core complex) has been shown to block transcription and the sliding of nucleosomes on chromatin templates mediated by the SWI/SNF ATPase complex *in vitro* (Francis et al. 2001; King et al. 2002). Interestingly, TBP-associated factors (dTAFIIs) can be co-purified with PRC1 in stoichiometric amounts (Saurin et al. 2001). In addition, PC has been found to co-localize with TBP and other general transcription factors at the promoters of PcG-repressed genes in SL2 tissue culture cells (Breiling et al. 2001). This suggests that also *in vivo*, one aspect of PcG silencing is the direct inhibition of the transcriptional apparatus. Direct evidence for this comes from the observation that placing a PRE upstream of a heat-shock promoter on a transgene inhibits transcription from this promoter at the stage of initiation (Dellino et al. 2004).

A human PRC1-like complex (hPRC1) has recently been shown to possess E3-ligase activity, which catalyzes the mono-ubiquitylation of histone H2A on lysine 119 (H2AK119). Knocking down either the Bmi-1 subunit of hPRC1, or the homologous dRING1 subunit of the *Drosophila* PRC1 complex, results in a decrease of H2A ubiquitylation and derepression of target gene promoters (Wang et al. 2004a; Cao et al. 2005). This suggests that the E3-ligase activity of PCR1-type complexes is required for efficient silencing.

In addition to the „classical" PRC1 complexes, a closely related complex termed CHRASCH (Chromatin associated silencing complex for homeotics) has been purified from *Drosophila* SL2 tissue culture cells (Fig. 1.1). This complex consists of PC, PH, RPD3 and the Pipsqueak (PSQ) protein, and is thus the only complex described so far that contains a sequence-specific DNA binding protein (Huang et al. 2002; Huang and Chang 2004).

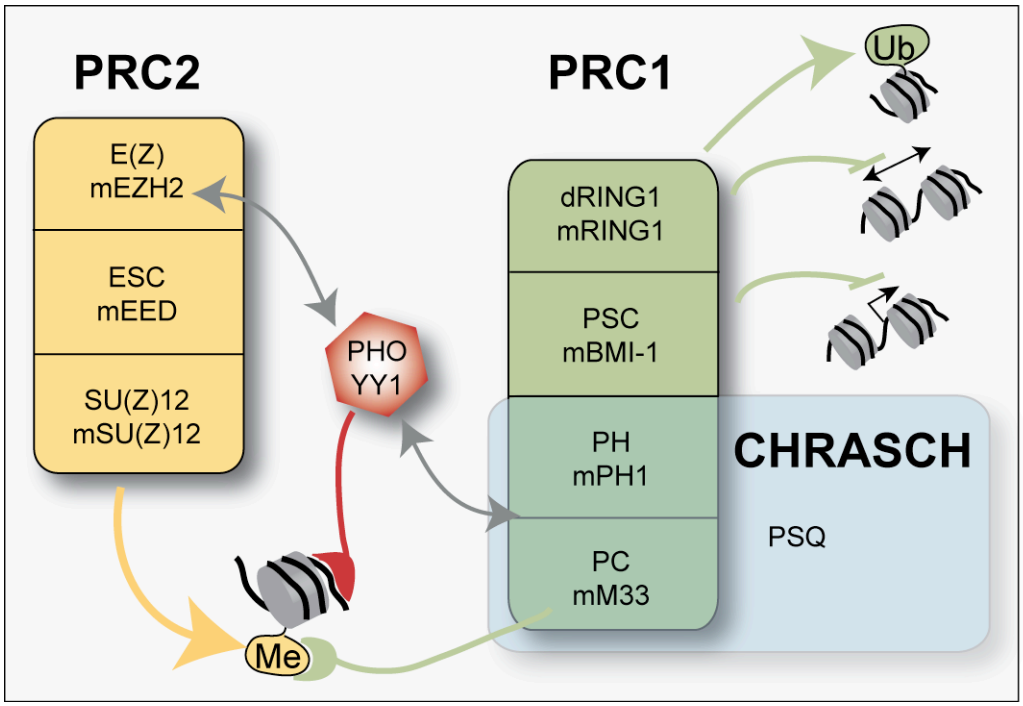

Figure 1.1: PcG complexes form repressive chromatin structures. PHO (red) can directly bind to DNA motifs present in PREs. Via the interaction with E(Z), PHO might recruit PRC2 complexes (orange) to the chromatin, which in turn methylate (Me) H3K27 (left, bottom). This modification is recognized by PC, a member of the PRC1 complex (green). Tethering to the chromatin is further supported by the interaction of PHO with PH and PC. dRING1 catalyzes the mono-ubiquitylation (Ub) of H2AK119 (right, top) which is required for repression. PRC1 might inhibit nucleosome remodeling by BRM complexes (right, middle) and directly block transcription at the stage of initiation (right, bottom). The CHRASCH complex is depicted in blue. Below each *Drosophila* protein name, the mouse homologues are listed.

This raises the question how the „classical" PRC1 and PRC2 complexes find their targets. The best candidate for this task is the Pleiohomeotic (PHO) protein, which specifically binds to DNA motifs commonly found in PREs (Fig. 1.1). Although it does not co-purify with either complex, PHO can interact with the PRC1 subunits PC and PH (Mohd-Sarip et al.

2002), and with PRC2 through binding to E(Z) (Wang et al. 2004b). An attractive model emerging from multiple studies is that the sequence-specific binding of PHO and its homologue PHO-like is the initial event during the establishment of PcG repression. Subsequently, PRC2 complexes become recruited via the interaction with E(Z), which in turn methylates H3K27. It has been shown that this methylation mark is specifically bound by the chromodomain of PC. Thus, the recognition of methylated H3K27 by PC, together with the direct interaction of PC and PH with DNA-bound PHO is thought to be responsible for the tethering of PRC1 to the chromatin specifically at PREs (Cao et al. 2002; Mohd-Sarip et al. 2002; Fischle et al. 2003; Wang et al. 2004b). However, on polytene chromosomes, PC does not completely overlap with sites of H3K27 methylation, indicating that the recruitment of PC-containing complexes to the chromatin likely depends on additional mechanisms (Ringrose et al. 2004).

In summary, PcG proteins act in the context of different multiprotein complexes, which have distinct enzymatic activities linked with the covalent modifications of histones or connected with the general transcriptional machinery. These different enzymatic activities act in concert to render the chromatin structure refractory to transcription.

1.2.2 Propagating the active state – the Trithorax group

The trxG of genes have been defined operationally: Mutations in these genes suppress PcG mutant phenotypes. To date, 14 genes have been described that fall into this class, many of which are conserved in vertebrates (Ringrose and Paro 2004).

Functionally, the TrxG proteins can be divided into four groups (Fig. 1.2). Two TrxG proteins, namely GAGA factor (GAF) and Zeste (Z), are sequence-specific DNA-binding proteins (Fig. 1.2A; Chen and Pirrotta 1993; Katsani et al. 1999). Both GAF and Zeste are not exclusively devoted to the regulation of PcG/TrxG target genes, as their DNA recognition sequences can be found in the promoters of many genes known not to be controlled by the cellular memory system (Ringrose et al. 2003). Although classified as a TrxG protein, GAF and its cognate DNA-binding motifs are required for the repression of reporter genes imposed by a nearby PRE on a transgene (Hodgson et al. 2001). In addition, an *in vitro* reconstituted PRC1 core complex represses a nucleosomal template more efficiently when it is pre-bound to GAF (Mulholland et al. 2003). Similar dual functions have been described for Zeste. For example, Zeste can recruit the TrxG protein Brahma (BRM) to a chromatin template *in vitro* (Kal et al. 2000), and Zeste binding sites are required for the association of BRM to a transgenic PRE *in vivo* (Déjardin and Cavalli 2004). In contrast, Zeste co-purifies with the PRC1 complex and enhances the binding of an *in vitro* reconstituted PRC1 complex to a

chromatin template containing Zeste binding motifs (Saurin et al. 2001; Mulholland et al. 2003).

The second group of trxG genes encodes subunits of the Mediator complex (Fig. 1.2B). The Mediator complex, originally identified in yeast, can function as a co-activator that links specific transcription factors to the basal transcription factors associated with the RNA polymerase II (RNAPII) machinery. Examples are the *kohtalo (kto)* and *skuld (skd)* genes (Kennison and Tamkun 1988), which encode proteins homologous to the TRAP230 (thyroid hormone receptor-associated protein) and TRAP240 subunits of the human Mediator complex (Treisman 2001). As such, these proteins presumably play a role in the global regulation of transcription.

The third group comprises TrxG proteins which act in multiprotein complexes that have chromatin remodeling activity (Fig. 1.2C). In general, chromatin remodeling complexes use the energy of ATP hydrolysis to alter the structure and position of nucleosomes, thereby modulating the access of regulatory proteins and general transcription factors to the DNA in the context of chromatin (Sudarsanam and Winston 2000; Narlikar et al. 2002). Thus, TrxG proteins falling into this class are also involved in the general regulation of transcription. BRM, for instance, largely co-localizes with RNAPII on polytene chromosomes, and a reduction in BRM protein reduces the association of RNAPII with the chromatin (Armstrong et al. 2002). BRM has been shown to be the catalytic subunit of a 2MDa ATP-dependent nucleosome remodeling complex, which also comprises the TrxG proteins Moira (MOR) and Osa (OSA). *In vitro*, the BRM remodeling complex facilitates transcription from nucleosomal templates (Kal et al. 2000). Interestingly, the *in vitro* nucleosome remodeling activity of BRM is inhibited by the presence of a core PRC1 complex (Shao et al. 1999; Francis et al. 2001).

The TrxG proteins of the fourth group are the only TrxG proteins whose functions are solely devoted to the cellular memory (Fig. 1.2D). ASH1 and ASH2 (absent, small, and homeotic) are related proteins which are present in different high molecular weight complexes. ASH1 exists in a 2MDa complex in *Drosophila* embryos. Via its SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domain, ASH1 catalyzes the methylation of lysines 4 and 9 in histone H3 (H3K4, H3K9) and of lysine 20 in histone H4 (H4K20), which is required for the maintenance of *Hox* gene expression *in vivo* (Beisel et al. 2002; Byrd and Shearn 2003; Klymenko and Müller 2004).

A third complex belonging to this group is the TAC1 complex (Trithorax Acetylation Complex). TAC1 is 1MDa in size and consists of Trithorax (TRX), a SET-domain histone methyltransferase (HMTase) that methylates H3K4, dCBP (*Drosophila* CREB-binding

protein), a histone acetyltransferase (HAT), the antiphosphatase Sbf1, and at least four additional subunits (Petruk et al. 2001; Smith et al. 2004). Similar to ASH1, the HMTase activity of TRX is required to prevent the PcG-mediated repression of the homeotic gene *Ultrabithorax (Ubx)* within its normal expression domains *in vivo* (Klymenko and Müller 2004). Interestingly, the combination of *ash1* or *trx* mutations with PcG alleles restores the expression of *Ubx* and even results in severe misexpression. This suggests that ASH1 and TRX are not directly involved in the transcriptional activation of *Hox* genes, but function specifically as anti-repressors to counteract inappropriate PcG silencing (Poux et al. 2002; Klymenko and Müller 2004).

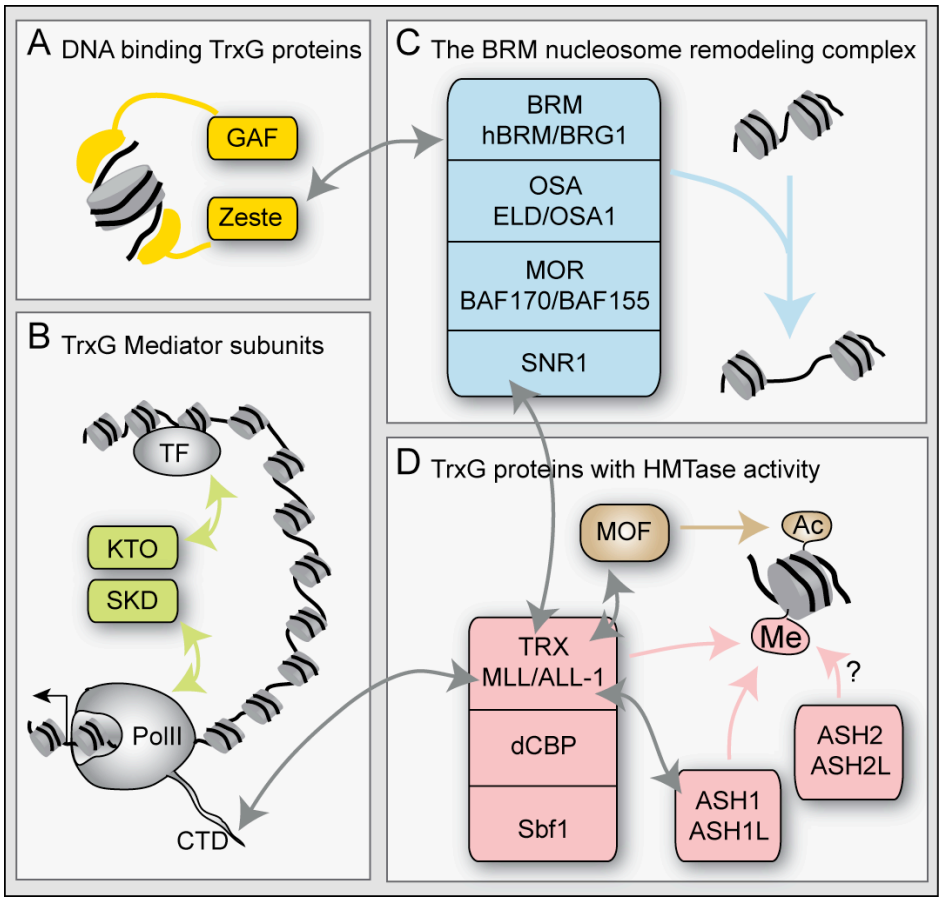

Figure 1.2: TrxG proteins propagate transcriptionally active chromatin states. A) GAF (GAGA factor) and Zeste directly bind to DNA-motifs found in PREs. Zeste enhances the association of BRM (Brahma) remodeling complexes with the chromatin. B) KTO (Kohtalo) and SKD (Skuld) are components of the Mediator complex, which is involved in stimulating transcription initiation. C) The BRM remodeling complex catalyzes the ATP-dependent sliding of nucleosomes and thus regulates the „fluidity" of the chromatin. D) TRX (Trithorax) and ASH1 (absent, small and homeotic) are HMTases which catalyze the methylation (Me) of H3K4, an epigenetic mark which correlates with transcriptionally active chromatin. So far, it is not known whether the paralogue ASH2 possesses HMTase activtiy as well. TRX can interact with ASH1, the SNR1 component of the BRM complex, and the histone acetyltransferase MOF (males absent on the first), which acetylates (Ac) H4K16, a modification commonly found in transcribed regions. Below the *Drosophila* protein names, the mammalian homologues are listed. Human MLL (Mixed Lineage Leukemia) has also been described to interact with the elongating form of RNAPII (RNA polymerase II) via the Ser2 phosphorylated CTD (C-terminal domain).

The human homologue of TRX, MLL (Mixed Lineage Leukemia), also functions as an H3K4 HMTase and is part of a complex which becomes recruited to active *Hox* gene promoters (Milne et al. 2002; Nakamura et al. 2002). In this scenario, the recruitment of MLL depends on active transcription, which leads to a spreading of MLL across wide regions while transcription takes place (Milne et al. 2005). Recently, hMOF (males absent on the first) has been co-purified in a MLL-containing complex (Dou et al. 2005). hMOF is the human counterpart of *Drosophila* MOF, which is a H4K16-specific HAT required for transcriptional hyperactivation of the male X chromosome during dosage compensation (Akhtar and Becker 2000). Both MLL and MOF activities are required for chromatin transcription *in vitro* and the expression of endogenous *Hox* genes *in vivo* (Dou et al. 2005).

Despite distinct TrxG complexes have been purified, there is evidence for cross-talk between them. For instance, TRX is known to interact with ASH1 (Rozovskaia et al. 1999), and it binds to SNR1, a component of the BRM complex (Rozenblatt-Rosen et al. 1998). This indicates that different TrxG complexes might cooperate in maintaining target genes transcriptionally active. The DNA-binding GAF and Zeste, the TrxG Mediator subunits, and TrxG proteins involved in chromatin remodeling such as the BRM complex presumably function as co-activators, which explains their roles in the regulation of global transcription. ASH1 and TRX containing complexes, on the other hand, directly counteract the repression of target genes by PcG proteins. This requires the histone modifying activities of ASH1 and TRX, but the underlying mechanism remains unclear.

1.2.3 Polycomb group response elements set the stage for the cellular memory

Polycomb group response elements (PREs) are the *cis*-regulatory elements through which the PcG and TrxG proteins are recruited to their target genes, and which are essential for the mitotic inheritance of transcriptional states throughout development.

PREs have been originally identified in the homeotic Bithorax Complex (BX-C; Fig. 1.3) through a series of genetic and functional studies (Simon et al. 1990; Busturia and Bienz 1993; Simon et al. 1993; Chan et al. 1994; Christen and Bienz 1994; Busturia et al. 1997). The BX-C comprises the homeotic genes *Ultrabithorax (Ubx), abdominal-A (abdA),* and *Abdominal-B (AbdB)* (Lewis 1978). The characteristic expression pattern of these genes along the A-P body axis is responsible for specifying the identities of PS5-14, which form the posterior half of the thorax and the abdomen of the fly (Fig. 1.3). The PS-specific expression patterns of *Ubx, abdA*, and *AbdB* are controlled by large *cis*-regulatory regions that can be subdivided into nine PS-specific subdomains: *abx/bx* and *bxd/pbx* regulate *Ubx* in PS5 and 6, *iab-2* to *iab-4* direct the expression of *abdA* in PS7-9, and *iab-5* through *iab-8,9* are required

for the proper expression of *AbdB* in PS10-14 (Mihaly et al. 1998). These enhancers are targeted by the gap and pair-rule proteins and are thus responsible for the initiation of *Hox* gene expression, but do not support the PcG/TrxG mediated inheritance of transcriptional states (Qian et al. 1991; Zhang et al. 1991; Müller and Bienz 1992; Shimell et al. 1994; Casares and Sanchez-Herrero 1995). This memory function requires PREs, such as *bxd*, *Mcp*, and *Fab-7*, which delimit the borders of PS-specific enhancers (Gyurkovics et al. 1990; Busturia and Bienz 1993; Simon et al. 1993; Chan et al. 1994; Christen and Bienz 1994; Chiang et al. 1995; Busturia et al. 1997). The current view is that on the one hand, PREs in the BX-C are associated with chromatin domain boundaries, whose function is to prevent the interaction of the early PS-specific enhancers with each other, which would lead to their serendipitous activation or repression in inappropriate PS (Mihaly et al. 1997). On the other hand, the key function of PREs is to recognize the initial expression state of each parasegmental domain and translate this into the establishment of epigenetic silencing mediated by PcG proteins, or the epigenetic maintenance of the active state by TrxG proteins.

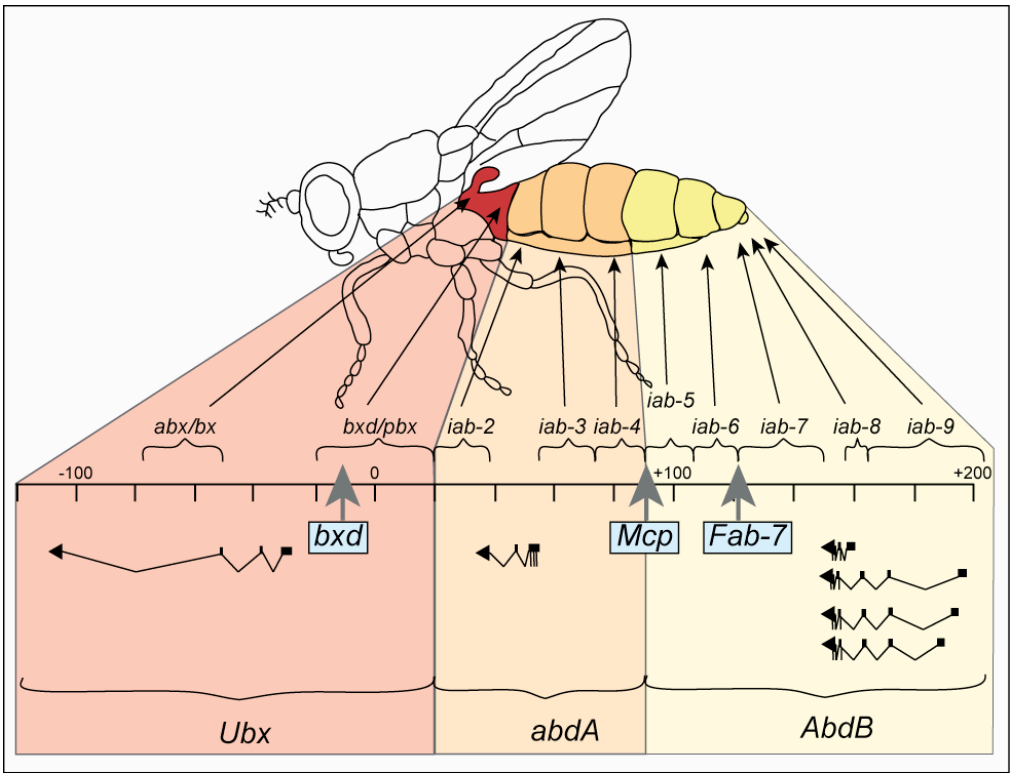

Figure 1.3: Regulatory regions of the BX-C. The 300kb BX-C contains the homeotic genes *Ubx*, *abdA*, and *AbdB* (transcripts are indicated in black at the bottom). The expression patterns of these genes specify the identities of PS5-14. Interspersed in the complex are PS-specific enhancers (*abx/bx*, *bxd/pbx*, *iab-2* to *-8,9*) which are responsible for establishing homeotic gene transcription in response to segmentation gene products during early embryogenesis. The positions of the genetically characterized PREs *bxd*, *Mcp*, and *Fab-7* are indicated by grey arrows. *Bxd* maintains *Ubx* expression active in PS6, *Mcp* and *Fab-7* control the maintenance of *AbdB* expression in PS10 and PS12, respectively (Adapted from Mihaly et al. 1998).

Are the homeotic genes the only genes regulated by the cellular memory? The answer is no: Genetic studies have led to the identification of PREs at the *engrailed (en)* (Kassis 1994), *polyhomeotic (ph)* (Bloyer et al. 2003), and *hedgehog (hh)* (Maurange and Paro 2002) loci. In addition, the binding pattern of PcG and TrxG proteins on polytene chromosomes suggested a much higher number of target genes. Through molecular studies on the characterized PREs, it became evident that DNA-binding motifs for the PcG protein PHO (Fritsch et al. 1999; Mishra et al. 2001), as well as for the TrxG proteins Zeste and GAF (or PSQ) (Rastelli et al. 1993; Hagstrom et al. 1997; Strutt et al. 1997; Huang et al. 2002) are essential for PRE function, but these binding sites occur quite frequently at random in the genome, precluding the identification of PREs solely on the basis of their linear sequence. A major breakthrough was the finding that within PREs, these DNA-binding motifs occur as pairs and are highly clustered, which in turn allowed the *in silico* prediction of potential PREs on a genome-wide basis in *Drosophila*. Thus, 167 potential PREs were predicted, and the target genes assigned to these PREs have diverse functions, ranging from developmental regulators to tumour suppressors (Ringrose et al. 2003).

Although these few DNA-binding motifs allowed the prediction of a large number of new PREs, they are clearly not the only factors defining a PRE. For instance, it has recently been shown that the recognition motif for the Dorsal Switch Protein (DSP1), a homologue of human HMGB2 (high mobility group protein B2) is essential for the silencing function of the *Fab-7* and *en* PREs (Déjardin et al. 2005). In addition, a short element, adjacent to converging PHO binding sites, has been identified in the *bxd* and *Fab-7* PREs. Two of these elements, termed PBE (PCC binding element), in conjunction with two PHO binding sites, are required for the synergistic recruitment of PHO and the PCC complex to chromatin *in vitro*, and for PcG-mediated silencing *in vivo* (Mohd-Sarip et al. 2005).

In summary, PREs are chromosomal elements that are required for the PcG/TrxG-mediated epigenetic inheritance of transcriptional states. In the BX-C, PREs appear in conjunction with chromatin domain boundaries to prevent the interaction between adjacent regulatory regions, which might be specific for such complex gene clusters. The definition of a PRE lies within its DNA sequence, and is characterized by the clustering of motifs for sequence-specific DNA-binding proteins.

1.3 PREs are switchable memory elements

Functional and genetic analyses of the BX-C have shown that each of the homeotic genes is controlled by several PREs, which function as PS-specific units (Mihaly et al. 1998). This subdivision of epigenetic gene regulation into single entities might be a general feature of PRE-controlled loci. In support of this, more than 90% of genes in the genome-wide prediction were associated with at least two PREs, one of which was always found within the promoter region. Alternatively, or in addition to the differential regulation of a gene by PRE „units“, distant PREs might interact with promoter-proximal PREs (Ringrose et al. 2003; Ringrose and Paro 2004). This in turn would bring distant regulatory elements into close proximity and thus facilitate the communication between them.

As explained in the previous section, PREs are platforms for the binding of both repressive PcG- as well as activating TrxG-complexes. The communication between distant PREs with those in the promoter region of a gene might therefore be vitally important for the decision between epigenetic silencing and activation. But how is this decision at a PRE taken?

There is accumulating evidence that the default state of a PRE is to function as a PcG-dependent silencer. On a transgene, for example, the presence of a PRE commonly results in the pairing-dependent repression of linked genes, i.e. silencing is considerably stronger in the homozygous compared with the heterozygous state (Kassis 1994; Zink and Paro 1995). Moreover, this silencing is tissue-independent, as reporter genes controlled by different larval imaginal disc enhancers are potently repressed when linked to the *bxd* PRE (Poux et al. 1996; Sengupta et al. 2004). Interestingly, when an imaginal disc enhancer associated with a reporter gene is combined with an embryonic PS-specific enhancer in addition to the PRE, the activity of the imaginal disc enhancer is restored in those PS in which the embryonic enhancer had been initially activated. This led to the idea that the initial transcriptional state of a target gene promoter during embryogenesis provides the basis for the decision between epigenetic silencing and activation (Poux et al. 1996).

A similar situation was observed when combining a *UAS-lacZ* reporter with the *Fab-7* PRE on a transgene (Fig. 1.4). In the default state, both the *lacZ* reporter and the *miniwhite* transformation marker were repressed in a PcG-dependent manner. Upon the transient activation of *lacZ* transcription by a heat-shock GAL4 pulse during embryogenesis, however, this silencing was abolished. Importantly, this active state was maintained throughout the remainder of development (Cavalli and Paro 1998). Identical results were obtained using the *bxd* and *Mcp* PREs on analogous transgenes (Rank et al. 2002). Thus, PREs are memory elements that can confer the epigenetic inheritance of both repressed and active gene

expression states. The switch of a PRE between functioning as a silencer or activating element is intimately connected with incoming signals and the transcriptional state of its target gene during early development (Cavalli and Paro 1998; Cavalli and Paro 1999).

What are these signals, and how is the transcriptional state of a gene promoter relayed to a PRE? Interestingly, the early transcriptional activation of the *UAS-lacZ* reporter by the transient supply of GAL4 did not only activate the expression of the reporter, but concomitantly resulted in the transcription through the adjacent *Fab-7*, *bxd*, or *Mcp* PREs (Fig. 1.4).

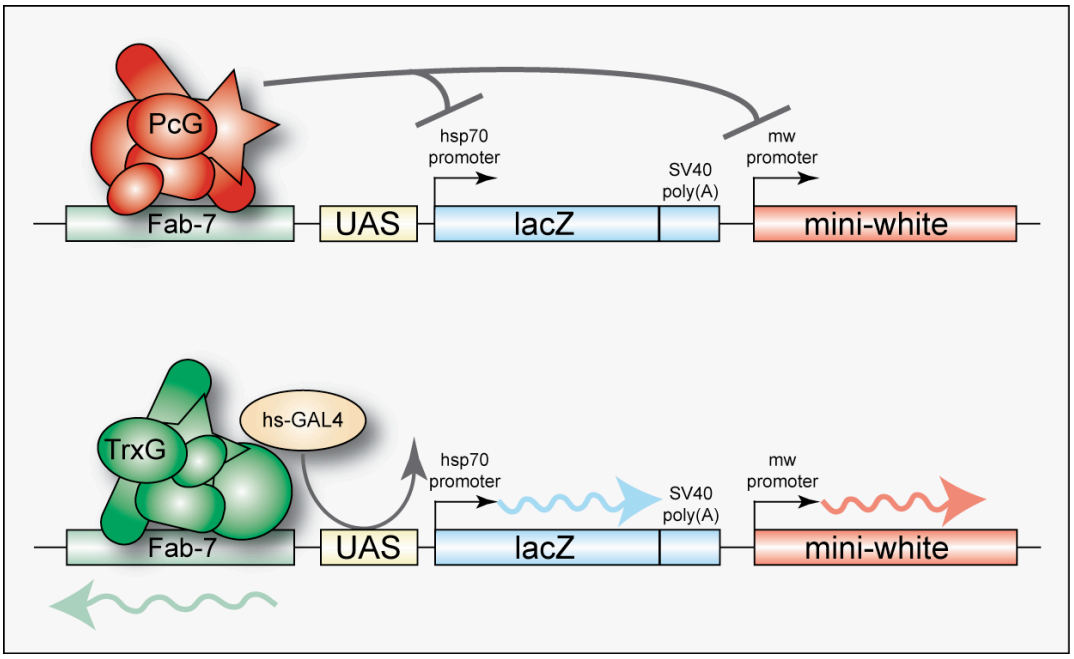

Figure 1.4: PREs are switchable elements. In the default state, the presence of the *Fab-7* PRE on a transgene leads to the PcG-dependent silencing of both the associated *lacZ* reporter and the *miniwhite* transformation marker (top). Upon the transient supply of GAL4 during embryogenesis, this transcription factor binds to its cognate recognition sequence within the *UAS* enhancer and activates expression of the *lacZ* reporter. The transcriptionally active state of *lacZ* is maintained throughout development in a TrxG-dependent manner. In addition, the *miniwhite* transformation marker can now also be expressed. Thus, the *Fab-7* PRE functions as a memory element that can be switched from the silencing into the epigenetically activated mode. Apart from activating *lacZ* expression, the transient GAL4 pulse also induces transcription through the adjacent *Fab-7* PRE.

This was an intriguing observation, since it suggests that the switch of a PRE from the repressed into the activated mode may be intimately connected with the non-coding transcription through these elements themselves (Rank et al. 2002). In support of this, early studies reported that regulatory regions of the endogenous BX-C are transcribed during embryogenesis (Lipshitz et al. 1987; Sanchez-Herrero and Akam 1989; Cumberledge et al. 1990). This non-coding transcription is first detectable at the onset of cellularization, shortly before the homeotic genes become activated. Similar to the expression of *Hox* genes, the transcription through intergenic regions follows the principal of spatial colinearity, with more

proximal sequences being transcribed in more anterior regions within the embryo (Sanchez-Herrero and Akam 1989; Cumberledge et al. 1990). More recently, this analysis has been refined, showing that the intergenic transcripts span the sequences of the characterized *bxd*, *Mcp*, and *Fab-7* PREs. Importantly, the pattern of transcription through these PREs precisely reflected their PS-specific functions, i.e. they were transcribed exactly in those PS, in which they are responsible for maintaining their target genes in the active state (Bae et al. 2002; Rank et al. 2002). Consistent with this, two other studies found that the ectopic transcription through regulatory regions in the BX-C results in homeotic phenotypes reminiscent of PRE-misregulation (Bender and Fitzgerald 2002; Hogga and Karch 2002).

Taken together, these results raise the intriguing hypothesis that the non-coding transcription through PREs may be functionally related to the epigenetic activation of these elements. As such, transcription itself and/or the non-coding RNA generated may provide the signal that decides between the two opposing epigenetic states at a PRE.

1.4 Histone modifications and variants – mnemonics of epigenetic inheritance?

The PcG/TrxG cellular memory system mediates the inheritance of transcriptional states by epigenetic mechanisms. What is the basis of this epigenetic inheritance? The enzymatic activities of PcG and TrxG complexes described in the previous sections clearly point to an important role of covalent histone modifications during this process. The PRC2 complex, for instance, catalyzes the methylation of H3K27 and to a lesser extent, of H3K9 (Czermin et al. 2002; Kuzmichev et al. 2002; Müller et al. 2002). Both these modifications have been correlated with repressive chromatin structures also in other organisms. The maintenance of the active state, on the other hand, depends on the HMTase activities of ASH1 and TRX, which methylate H3K4 (Nakamura et al. 2000; Beisel et al. 2002; Milne et al. 2002; Byrd and Shearn 2003; Klymenko and Müller 2004). Additionally, the switch of the transgenic *Fab-7* PRE from the silent into the activated mode by an embryonic heat-shock GAL4 pulse has been correlated with the hyperacetylation of histone H4 (Cavalli and Paro 1999). Both H3K4 methylation, as well as H4 hyperacetylation have been described as epigenetic marks diagnostic of active euchromatin (Santos-Rosa et al. 2002). What are the roles of these modifications? One function is definitely to serve as binding platforms for chromatin-associated proteins, as has been described for PC (Cao et al. 2002; Fischle et al. 2003).

Another possible function of histone modifications is to serve as „bookmarks“ that signal the cell the epigenetic state of a given locus, so that it is faithfully transmitted into the next cell cycle. Due to their low turnover rates, methylation marks are particularly attractive candidates for such a task (Waterborg 1993; Peters and Schübeler 2005). A histone lysine demethylating enzyme has recently been identified (Shi et al. 2004), but it is not clear if this is involved in the PcG/TrxG memory system. Therefore, methylation marks on histones have been proposed to transmit epigenetic states through consecutive cell divisions, especially during such critical stages as DNA replication, which requires the disassembly of chromatin (Tagami et al. 2004; Wallace and Orr-Weaver 2005), and mitosis when regulatory proteins become displaced from the chromatin (Kellum et al. 1995; Buchenau et al. 1998; Dietzel et al. 1999; Kouskouti and Talianidis 2005; Valls et al. 2005).

Apart from the covalent modification of histones, it has become increasingly clear that the incorporation of specific variants of the canonical isoforms plays an important role in the regulation of the chromatin structure. The variant H3.3 has been intimately related to active euchromatin, as the replacement of canonical H3 by H3.3 is coupled to the transcriptional process itself (Ahmad and Henikoff 2002; Schwartz and Ahmad 2005). This led to the hypothesis that the transcription-coupled exchange of H3 by H3.3 might play an important role in regulating the plasticity of the chromatin structure by removing repressive chromatin marks with otherwise very low turnover rates.

A second histone whose variant isoforms might be involved in the epigenetic inheritance of specific chromatin states, is histone H2A. In many organisms, several isoforms of H2A are known, whereas the *Drosophila* genome encodes only one variant, H2Av (van Daal and Elgin 1992). The role of this variant is not clear, as various studies led to conflicting results. Genetic studies have shown that H2Av is essential for viability, and that its critical functions reside in a C-terminal extended domain, which maps to the region contacting the surface of histone H4 within the nucleosome and is thus critical for the integrity of its structure (Clarkson et al. 1999). This region of H2Av is homologous to H2AZ in yeast, which has been found to be enriched in euchromatin and to function as a boundary preventing the spread of silent heterochromatin into active euchromatin (Meneghini et al. 2003). Similarly, tetrahymena H2AZ is enriched in the transcriptionally active macronucleus, but is absent from the silent micronucleus (Allis et al. 1980), suggesting a function of H2AZ in the formation and/or inheritance of a transcriptionally competent chromatin configuration. In contrast, mammalian H2AZ accumulates in pericentric heterochromatin during early development, and an interaction between this variant and HP1 (heterochromatin protein 1) has been described,

suggesting that mammalian H2AZ might be involved in the formation of a repressive chromatin structure (Rangasamy et al. 2003; Fan et al. 2004). A similar role has been proposed for H2Av in *Drosophila*. For instance, the mutation of the H2Av gene leads to a reduced recruitment of HP1 to centromeric heterochromatin (Swaminathan et al. 2005). Interestingly, H2Av mutations also enhance mutations in PcG genes and suppress trxG mutant phenotypes, suggesting that H2Av might act in concert with PcG complexes in the formation of a repressive chromatin structure (Leach et al. 2000; Swaminathan et al. 2005).
In summary, covalent histone modifications and the incorporation of specific histone isoforms play important roles in setting up particular chromatin structures, and might also be instrumental in transmitting these chromatin structures through critical stages of the cell cycle, such as DNA replication and mitosis.

1.5 Intergenic transcription – a recurrent theme in epigenetic gene regulation

PREs are switchable elements which can mediate the epigenetic inheritance of either repressed or active transcriptional states of target genes. The fact that PREs in the BX-C are transcribed in a regulated manner led to the idea that this non-coding transcription may play a pivotal role in the transition of a PRE from the repressed into the epigenetically activated mode. This is not an isolated example, since non-coding transcription has been described to be involved in the epigenetic regulation of gene expression in numerous systems. Depending on the context, changes in the chromatin structure rely on a molecular function of the non-coding RNAs, or are thought to be mediated by the process of intergenic transcription *per se.*

1.5.1 Intergenic transcription in the β-globin locus – to activate or to silence?

Similar to the *Drosophila* BX-C, the human *β-globin* locus is a cluster of genes (*ε, Gγ, Aγ, δ,* and *β*) whose expression is developmentally regulated, whereby the order of the genes on the chromosome reflects their order of expression. The homeotic genes in the *Drosophila* BX-C are expressed in a characteristic pattern along the A-P body axis, which is colinear with their proximal-distal arrangement within the complex (McGinnis and Krumlauf 1992). In the human *β-globin* locus, this colinearity is of temporal nature: In embryonic red blood cells, the *ε-* and *γ-globin* genes located at the 5' end of the cluster are expressed, whereas during fetal development, *δ-* and *β-globin* at the 3' end are transcribed (Strouboulis et al. 1992). The

expression of all globin isoforms depends on the LCR (locus control region) which defines the 5' end of the cluster (Grosveld et al. 1987).

It has been shown that in addition to the globin genes, regulatory regions within the locus are also transcribed (Gribnau et al. 2000). Intergenic transcription was found to occur in sense direction with respect to the globin genes and was differentially regulated during development. In embryonic cells, intergenic transcripts were detected in the LCR and in the region flanking the *ε*- and *γ-globin* genes, which are also expressed at that stage. During fetal development, globin gene expression switches to the *δ*- and *β-globin* isoforms. Concomitantly with this switch in globin gene transcription, intergenic transcripts were also detected in the regulatory region flanking the *δ*- and *β-globin* genes, but no longer in the *ε*- and *γ-globin* regions. Interestingly, the domains of intergenic transcription coincided with increased sensitivity of the chromatin to DNAse I digestion. These results led to a model whereby the regulated transcription through the LCR and the *ε-/γ-globin* domain at early stages and through the LCR and the *δ-/β-globin* domain at later stages would result in the formation of active chromatin domains. This, in turn would control the switch from early *ε-/γ-globin* to late *δ-/β-globin* expression (Gribnau et al. 2000).

This idea was challenged by a recent study in which the strict correlation between the non-coding transcription through the different domains in the *β-globin* gene cluster and the activation of the chromatin structure could not be confirmed (Plant et al. 2001). Instead, nascent non-coding transcripts were identified in both sense and antisense directions. The knock-down of a key component of the RNA interference (RNAi) machinery resulted in up-regulation of these non-coding transcripts, which was accompanied by an increase in histone modifications diagnostic of active chromatin. In contrast to the previous study, these authors proposed a model in which intergenic transcription leads to the formation of silent heterochromatin (Haussecker and Proudfoot 2005).

These conflicting results make it difficult to conclude what the contribution of non-coding transcription in the *β-globin* locus to changes in the chromatin structure and consequently to the regulation of differential globin gene expression really are.

1.5.2 RNA-mediated hyperactivation of the Drosophila male X chromosome

In *Drosophila*, X chromosome dosage compensation is achieved through the twofold transcriptional hyperactivation of the single male X chromosome compared with the two X chromosomes of a female. The hypertranscription of male X-linked genes is achieved through the coating of the X chromosome with the dosage compensation complex (DCC), consisting

of six proteins and one of two non-coding RNAs, *roX1* (RNA on the X) or *roX2* (reviewed in (Andersen and Panning 2003). The protein components of the DCC are MSL1 (Male-Specific Lethal 1), MSL2, a Ring-finger protein, MSL3, a chromodomain protein, MLE (Maleless), an RNA helicase, MOF (Males Absent on the First), a HAT, and JIL-1, a protein kinase (Andersen and Panning 2003). It has been shown that MSL3 and MOF can directly interact with *roX2* RNA via their chromodomains (Akhtar et al. 2000), which is essential for DCC assembly. The formation of the DCC has been proposed to occur on nascent *roX* transcripts, which would allow its efficient targeting to the X chromosome, on which the *roX1* and *roX2* genes are located (Park et al. 2002). From these two initial entry sites, the DCC spreads along the X chromosome in *cis* via 30 to 40 additional entry sites, until chromosome coating is completed. The recognition of these entry sites could occur, at least in part, through base-pairing of the *roX* RNAs with DNA.

The twofold elevation of X-linked gene transcription is thought to be mainly mediated through the hyperacetylation of histone H4K16 by MOF (Akhtar and Becker 2000). In addition, histone H3 phosphorylation by JIL-1 might play a role, but this enzymatic activity has so far only been shown *in vitro* (Jin et al. 2000).

1.5.3 Mammalian X inactivation is controlled by two opposing non-coding transcripts

In contrast to *Drosophila*, dosage compensation in mammals is achieved through the selective transcriptional inactivation of one of the two female X chromosomes (Lyon 1961). During the first embryonic cleavages, X chromosome inactivation (XCI) is imprinted, and it is always the paternally inherited copy which is inactivated. In the blastocyst, however, the paternal X chromosome becomes reactivated. From this stage onward, XCI is random, so that in roughly 50% of cells, the paternal X becomes inactivated and in the other half, the maternal copy becomes silenced (Mak et al. 2004).

Random XCI can be recapitulated *in vitro* in differentiating female embryonic stem cells (ESCs). This process is controlled by the X inactivation center (XIC) (Brown et al. 1991b), at which the inactive state is initiated and spreads along the whole chromosome, resulting in the formation of the transcriptionally inert and cytologically visible Barr body. The decision between transcriptional inactivation and the maintenance of the active state is regulated by the differential expression of two overlapping convergent non-coding RNAs transcribed from the XIC (Fig. 1.5A; Lee 2003; Stavropoulos et al. 2005). One of these RNAs, termed *Xist (X-inactivation specific transcript)*, is essential for the initiation of heterochromatin formation on

the future inactive X chromosome (X_i) (Brown et al. 1991a; Brockdorff et al. 1992). The maintenance of the active state on the future X_a on the other hand, depends on the transcription of *Tsix* RNA (Lee et al. 1999). *Tsix* transcription is initiated from a promoter located several kb downstream of the *Xist* promoter, leading to the generation of non-coding *Tsix* RNA, which partially overlaps with and is complemetary to *Xist* (Fig. 1.5A; Lee et al. 1999).

Before X inactivation in female ES cells, both *Xist* and *Tsix* RNAs are transcribed (Fig. 1.5B). At the onset of differentiation, one of the two homologous X chromosomes becomes selected for inactivation. This choice depends on the downregulation of *Tsix* and the concomitant upregulation of *Xist* transcription. As a consequence, non-coding *Xist* RNA accumulates on the future X_i (Lee 2003).

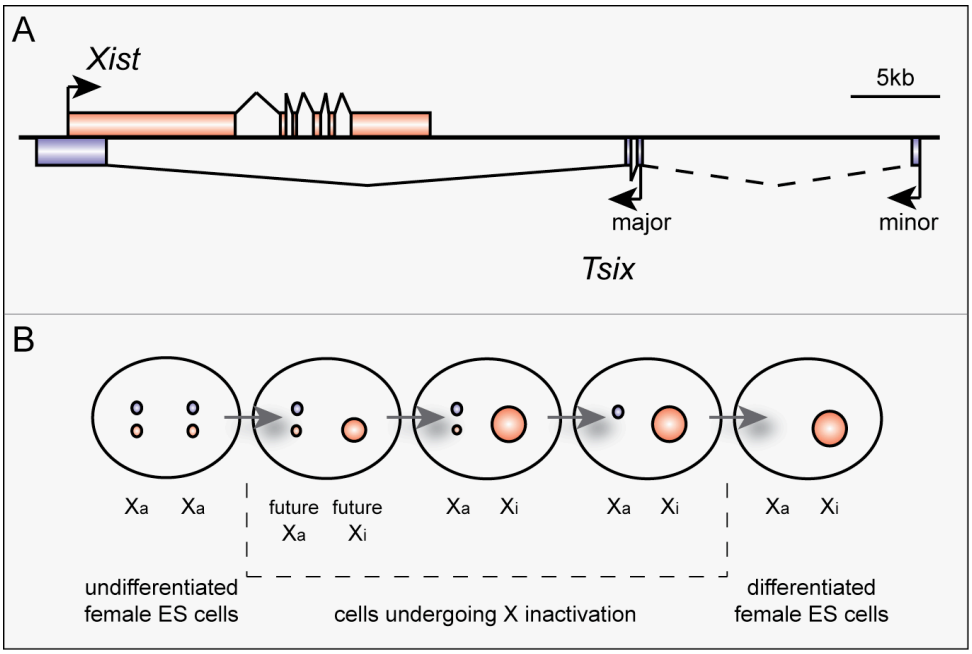

Figure 1.5: X chromosome inactivation in mammals is controlled by the transcription of two non-coding RNAs. A) Schematic of the XIC. *Xist* RNA (red) is transcribed from the top strand. Transcription of *Tsix* RNA (blue) starts from alternative promoters (major and minor) downstream of *Xist* and occurs in the antisense direction. B) X chromosome inactivation can be recapitulated in differentiating female ES cells *in vitro*. In undifferentiated cells, *Xist* (red) and *Tsix* (blue) RNAs are expressed symmetrically and both X chromosomes are transcriptionally active (X_a). At the onset of differentiation, *Tsix* expression becomes asymmetrical and thereby determines the choice of the future inactive X chromosome (X_i). *Xist* RNA accumulates on the future X_i, whereas *Tsix* expression persists on the future X_a. In differentiated female ES cells, only *Xist* RNA is expressed from X_i. (Adapted from Lee 2003 and Stavropoulos et al. 2005).

Interestingly, *Xist* RNA accumulation triggers the transient association of mammalian PRC2 components to the future X_i. In analogy to PcG-mediated gene silencing in *Drosophila*, this transient recruitment of the PRC2 subunits EED (embryonic ectoderm development) and EZH2 (Enhancer of Zeste 2) to the future X_i correlates with an increase of methylated H3K27

(Wang et al. 2001; Mak et al. 2002; Erhardt et al. 2003; Plath et al. 2003; Silva et al. 2003). As differentiation proceeds, EED and EZH2 dissociate from X_i, but the enrichment of methylated H3K27 persists. Concomitant with the appearance of methylated H3K27, PRC1 components become recruited to the X_i in a *Xist*-dependent manner. It has been suggested that, similar to *Drosophila*, PRC1 components might contribute to transcriptional silencing of the Xi by interfering with chromatin remodeling and transcription initiation. Moreover, ubiquitylation of H2AK119 is observed on the X_i, and this modification depends on RING1A and RING1B, the mammalian homologues of *Drosophila* dRING1 (de Napoles et al. 2004; Plath et al. 2004).

As stated above, the initiation of X inactivation depends not only on the accumulation of *Xist* RNA, but also on the downregulation of *Tsix* transcription. On the other hand, it has become clear that persistent transcription of *Tsix* RNA on the future X_a is required to maintain this chromosome in the active state. The function of *Tsix* transcription on the X_a is to negatively regulate *Xist* expression, thereby preventing the formation of heterochromatin. There is increasing evidence that *Tsix* silences *Xist* transcription in *cis* by modulating the epigenetic status at the *Xist* locus (Sado et al. 2005). To date, it is not clear if this process depends on the process of *Tsix* transcription or the non-coding *Tsix* RNA, which overlaps with *Xist* and may thus impair its function through direct base-pairing (Shibata and Lee 2004).

The regulation of *Tsix* transcription in turn depends on yet other intergenic transcripts which are initiated from several promoters upstream of *Tsix*, termed *Xite (X-inactivation intergenic transcription elements)*. Transcription from *Xite* is required for efficient *Tsix* transcription and thus for the maintenance of the active state at the future X_a in *cis*. It has been suggested that the positive effect of *Xite* on *Tsix* expression depends on the process of transcription initiated at *Xite*, rather than the non-coding RNAs generated (Ogawa and Lee 2003).

In summary, the inactivation of one mammalian X chromosome during dosage compensation and the epigenetic silencing of homeotic genes in *Drosophila* show intriguing similarities. In both cases, PcG proteins become targeted to the chromatin, resulting in the modification of the chromatin structure. There is however one fundamental difference between these two systems: PcG recruitment during X inactivation crucially depends on the presence of non-coding *Xist* RNA, whereas the non-coding transcription through PREs has been correlated with the epigenetically active state.

1.5.4 Non-coding RNAs and genomic imprinting

Genomic imprinting describes the mono-allelic expression of genes in a parent-of-origin specific fashion. Imprinted genes commonly occur in clusters which are expressed specifically either from the maternally or the paternally inherited chromosome.

Non-coding RNAs have been identified at six autosomal imprinted loci (O'Neill 2005), among which the non-coding *Air (Antisense Igf2r RNA)* RNA (Lyle et al. 2000) at the mouse *Igf2r* (*Insulin-like growth-factor type-2 receptor)* locus is characterized best. The *Igf2r* locus is a 400kb cluster comprising three imprinted genes (*Igf2r, Slc22a2,* and *Slc22a3*), which are expressed from the maternal chromosome only. The monoallelic expression of these genes is controlled by a *differentially methylated region (DMR)* located within the *Igf2r* gene. This *DMR* functions as a promoter from which transcription of the 108kb long non-coding *Air* RNA initiates and proceeds in antisense direction to *Igf2r* (Wutz et al. 1997; Lyle et al. 2000). Transcription of *Air* on the paternal chromosome is required for silencing of the overlapping *Igf2r* gene, as well as the distally located *Slc22a2* and *Slc22a3* genes. Truncation of *Air* to less than 5kb residual sequence leads to abrogation of silencing (Sleutels et al. 2002), but the transcriptional overlap with *Igf2r* is not required for its function (Sleutels et al. 2003). This led to a model reminiscent of *Xist*-mediated X inactivation, in which *Air* RNA possesses intrinsic silencing properties, thereby generating a *cis* silencing effect that can repress susceptible gene promoters within the imprinted cluster (Sleutels et al. 2003). However, the precise function of *Air* has not been elucidated and it is not known how the silencing function of *Air* becomes restricted to the *Igf2r* domain.

1.6 Aims of the thesis

PREs mediate the mitotic inheritance of both repressed and active transcriptional states, and thus maintain determined cell fates. In the default state, PREs repress their target genes by recruiting PcG silencing complexes to the chromatin. Upon receiving an unknown activating signal, however, PREs are converted into the epigenetically activated mode. This in turn triggers the TrxG-dependent modification of the chromatin structure, which renders it competent for transcription. Although the nature of this activating signal is not known, previous results suggest a role of non-coding transcription through PREs themselves in this process.

The major aim of this thesis was to determine whether the transcription through the *Fab-7* PRE has any function in the regulation of the epigenetic state of this element. Are these two

processes functionally connected, or is the transcription through PREs merely a byproduct of an activated chromatin structure? I will describe the analysis of a transgenic reporter system which allows to distinguish between these two possibilities. The results will show that the transcription through *Fab-7* functions as a novel anti-silencing mechanism to counteract PcG-mediated repression.

To get insight into the molecular basis of this anti-silencing mechanism, it is important to understand the epigenetic changes induced by transcription through a PRE. Does the association of PcG/TrxG proteins with the chromatin change, or does transcription modify the chromatin structure more directly, for example by altering the composition of nucleosomes?

Apart from knowing the consequences, it is essential to understand the precise function of non-coding transcription through PREs. Does the anti-silencing function rely on the process of transcription, the non-coding RNA generated, or a combination of both? The third part of this thesis describes a first characterization of non-coding transcripts spanning PREs, which will help to further our understanding of the mechanism underlying the cellular memory.

Results

2. Results

Polycomb group response elements (PREs) convey epigenetic memory of both repressed and active gene expression states. To understand the molecular mechanism underlying this cellular memory, it is important to know how the decision between epigenetic silencing and activation at at PRE is taken. Previous studies have shown that the conversion of a PRE from the repressed into the epigenetically activated mode is accompanied by the non-coding transcription through these elements themselves (Rank et al. 2002). This raised the question whether this non-coding transcription through PREs is functionally related to the epigenetic activation of these elements, or if it merely reflects fortuitous transcription as a consequence of the activation of the chromatin structure.

2.1 A transgenic reporter system to study the function of non-coding transcription through PREs

To analyze the function of non-coding transcription through PREs, a transgenic reporter system was designed. In this reporter system, transcription through the well-characterized *Fab-7* PRE is controlled by a promoter with defined activity. The expression levels of the *lacZ* and *miniwhite* genes transcribed in the divergent orientation on the transgene served as a direct read-out of the epigenetic state of the PRE (Schmitt 2002).

2.1.1 Constitutive transcription through the Fab-7 PRE results in derepression of the miniwhite gene

To assess if constitutive transcription through the *Fab-7* PRE would have any effect on the epigenetic state of this element, the *actin5C* promoter was used (Fig. 2.1). The *actin5C* promoter is ubiquitously and constitutively active, but nevertheless, the level of transcription from this promoter shows developmental regulation to some extent (Vigoreaux and Tobin 1987; Burn et al. 1989).

During early embryogenesis in wildtype *Drosophila*, transcription through the endogenous *Fab-7* PRE occurs in both sense and antisense directions with respect to its target gene *Abdominal-B* (*AbdB*) (Rank 2002). To determine whether sense or antisense transcription through *Fab-7* on the transgene would have the same effect on the epigenetic state of this

element, the PRE was cloned in both orientations, so that either sense (pFAs; Fig. 2.1) or antisense *Fab-7* RNA (pFAas; Fig. 2.1) would be produced. In the divergent orientation on the transgene, the *lacZ* reporter is controlled by a minimal *hsp70* promoter, while the *miniwhite* transformation marker is regulated by its endogenous promoter. The expression levels of the *lacZ* reporter and the *miniwhite* transformation marker should reflect the activity of the *Fab-7* PRE on the transgene, thus serving as a direct read-out of the epigenetic state of this element (Cavalli and Paro 1998).

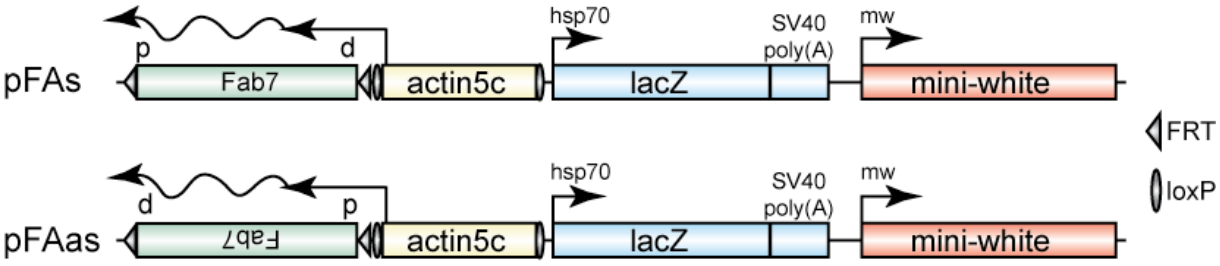

Figure 2.1: Transgenic reporter constructs to determine the effect of constitutive transcription through *Fab-7* on its epigenetic state. The *Fab-7* PRE is located downstream of the *actin5C* promoter. The orientation of *Fab-7* on the transgene is either proximal-distal (p-d) or distal-proximal (d-p) in relation to its orientation within the Bithorax Complex (Martin et al. 1995). As such, either sense (d-p; pFAs) or antisense (p-d; pFAas) *Fab-7* RNA with respect to the *Abdominal-B* mRNA is produced. The *Fab-7* PRE is flanked by *FRT* sites (triangle), whereas the *actin5C* promoter is flanked by *loxP* sites (oval), allowing the separate excision of these sequences from the transgene by Flp/*FRT* or Cre/*loxP* recombination, respectively. In the divergent orientation, the *lacZ* reporter is controlled by a minimal *hsp70* promoter, while the *miniwhite* transformation marker is regulated by its endogenous promoter.

An inherent problem in the analysis of transgenic PRE constructs is that the degree of silencing imposed by a PRE is dependent on its chromosomal environment. To overcome this problem, it was necessary to compare the epigenetic state of the *Fab-7* PRE in the presence or absence of transcription at the identical insertion site of the transgene. To achieve this, the *actin5C* promoter was cloned between *loxP* sites, whereas the *Fab-7* PRE is flanked by *FRT* sites (Fig. 2.1). Thus, the *actin5C* promoter and the *Fab-7* PRE can be separately excised from the transgene by Cre/*loxP* or Flp/*FRT* recombination, respectively (Chou and Perrimon 1996; Siegal and Hartl 1996).

To verify that the transgenic *Fab-7* PRE was transcribed in the expected pattern only in the presence but not in the absence of the *actin5C* promoter, RNA *in situ* hybridizations on embryos and eye imaginal discs from third instar larvae were performed (Fig. 2.2). In stage 10 wildtype embryos, transcription through the endogenous *Fab-7* PRE was only detected in the sense orientation with respect to its target gene *AbdB* (Fig. 2.2A and B). In the pFAs transgenic embryos, *Fab-7* sense RNA was transcribed in a pattern that reflects the expected expression from the *actin5C* promoter: It was ubiquitously active, but showed increased

activity in the anterior midgut primordium of the embryo (Fig. 2.2C; Burn et al. 1989). In addition, higher *Fab-7* sense RNA levels were also found in parasegments (PS) 12-14, in which transcription through the endogenous *Fab-7* PRE is known to occur (Rank et al. 2002).

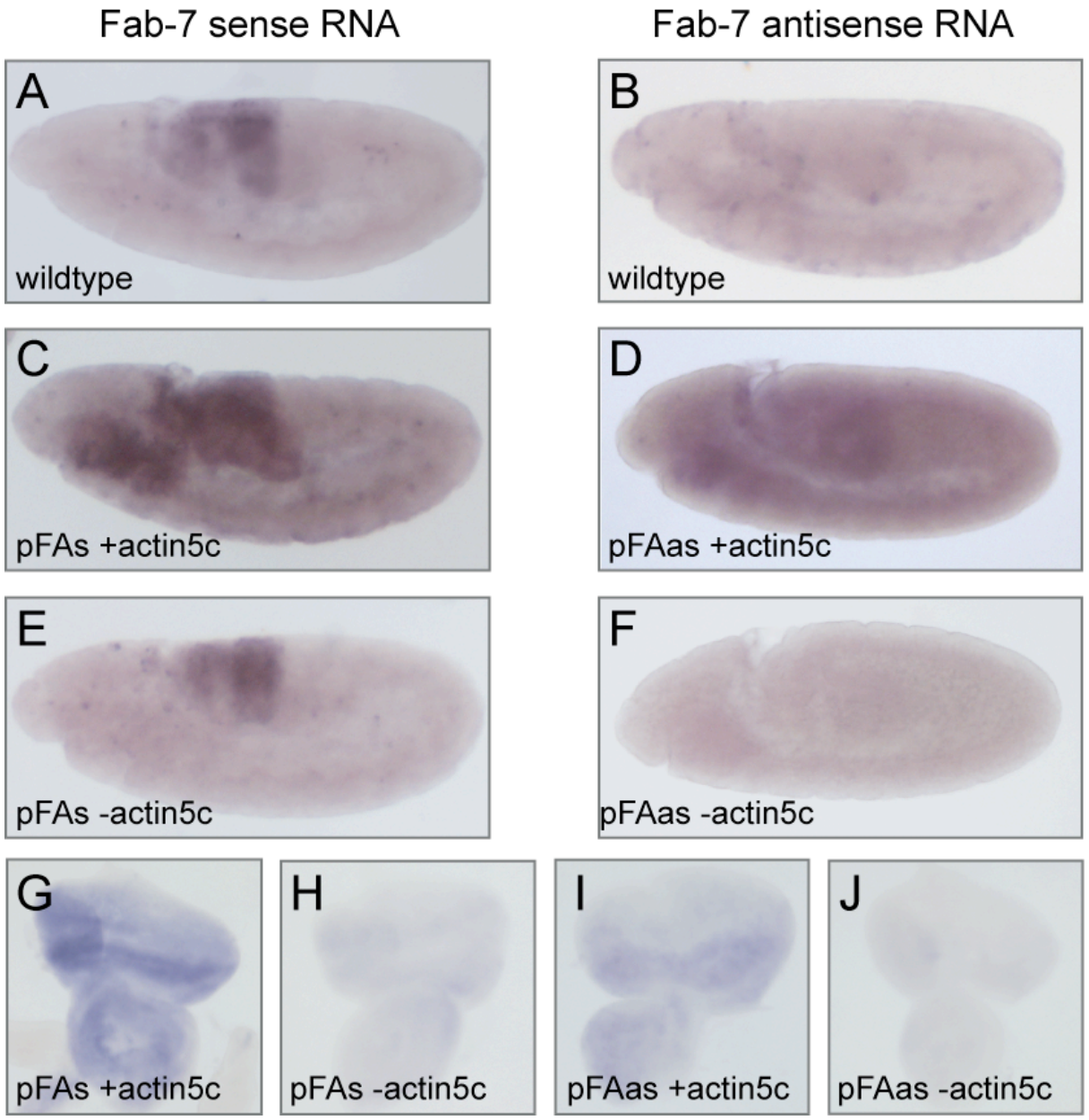

Figure 2.2: RNA *in situ* hybridization to verify *Fab-7* transcription in pFAs and pFAas transgenic lines. A) *Fab-7* sense RNA transcription in wildtype embryos; B) *Fab-7* antisense RNA is absent in the wildtype at this stage; C) and D) *Fab-7* sense and antisense RNA expression in pFAs and pFAas transgenic embyos, respectively; E) and F) Excision of the *actin5C* promoter from the pFas and pFAas transgenes abolished ectopic *Fab-7* transcription; G) and I) *Fab-7* sense and antisense transcription in eye imaginal discs of pFAs and pFAas transgenic lines, respectively; H) and I) *Fab-7* was not transcribed in pFAs and pFAas lines in the absence of the *actin5C* promoter from the transgenes. Embryos are always oriented with anterior pole to the left and dorsal to the top. Eye imaginal discs are shown with posterior to the top.

In the pFAas transgenic line, *Fab-7* antisense RNA was also detected ubiquitously with slight enrichment in the anterior midgut primordium (Fig. 2.2D). As expected, excision of the

actin5C promoter from the pFAs and pFAas transgenes by Cre/*loxP* recombination led to abrogation of the transgenic *Fab-7* RNA signals (Fig. 2.2E and F). In the eye imaginal discs of third instar larvae, *Fab-7* sense and antisense transcription driven by the *actin5C* promoter was detected in all cells in the pFAs and pFAas lines, albeit at varying levels (see Fig. 2.2G and H, I and J).

The *in situ* hybridizations demonstrated that the *Fab-7* PRE was ubiquitously transcribed in the pFAs and pFAas lines only when the *actin5C* promoter was present on the transgenes. To determine the effect of this transcription on the epigenetic state of the PRE, the expression of the *miniwhite* transformation marker in the pFAs and pFAas lines before and after all possible recombination steps was examined.

Remarkably, before recombination, none of the pFAs or pFAas transgenic lines (>20 independent lines for each construct) showed pairing-sensitive silencing of the *miniwhite* gene. Instead, in individuals homozygous for the transgene, *miniwhite* was expressed at very high levels, resulting in a dark eye colour (Fig. 2.3A and E). Upon excision of the *actin5C* promoter, the *miniwhite* gene was almost completely repressed, leading to a pale yellow or white eye colour (Fig. 2.3B and F). Thus, in the absence of transcription, the *Fab-7* PRE functioned as a silencer. After excision of the *Fab-7* PRE from the transgene, the *miniwhite* gene was highly expressed in the presence of the *actin5C* promoter (Fig. 2.3C and G). The subsequent removal of this promoter from the transgene resulted in a moderate decrease in eye pigmentation (Fig. 2.3D and H), indicating that the vicinity of the *actin5C* promoter had a slight activating effect on the expression of the *miniwhite* promoter.

To quantify the effect of continuous transcription through the *Fab-7* PRE on *miniwhite* expression levels, photometric pigment measurements in four independent transgenic fly lines were performed (Fig. 2.3I). This showed that the continuous transcription through *Fab-7* led to a strong elevation of eye pigmentation, which varied considerably depending on the insertion site of the transgene (2.9- to 19.3fold). In the absence of the *Fab-7* PRE, the increase in the *miniwhite* expression levels caused by the presence of the *actin5C* promoter on the transgene remained relatively constant between individual fly lines (1.9- to 3.2-fold). Comparison of eye pigmentation in flies bearing the *Fab-7* PRE transcribed from the *actin5C* promoter with those bearing the *actin5C* promoter but no PRE on the transgene, illustrates that the transcription through the *Fab-7* PRE resulted in a complete relief of silencing. Interestingly, the pFAs and pFAas transgenic lines behaved very similarly, demonstrating that the transcription of either sense or antisense *Fab-7* RNA has the same activating effect.

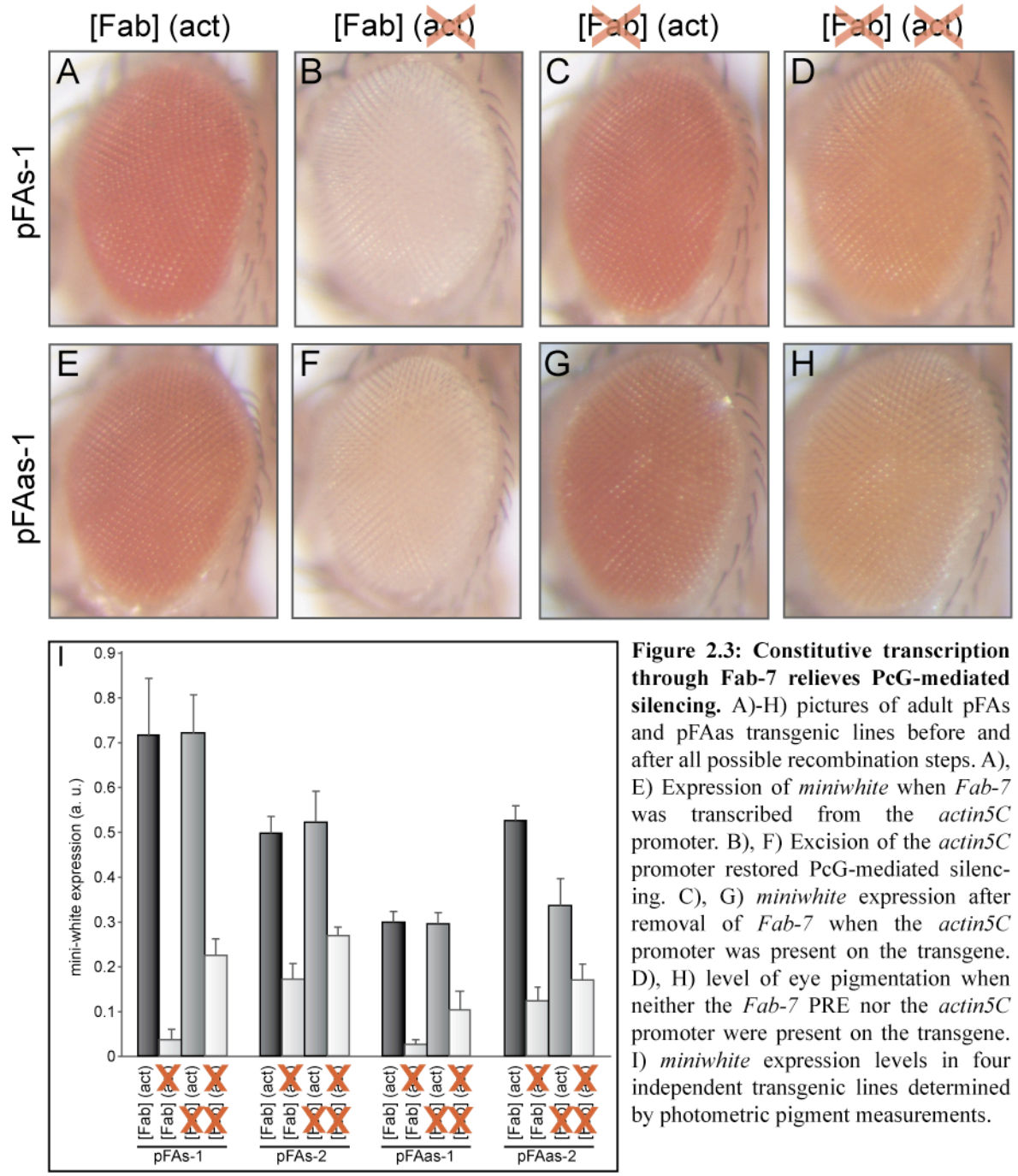

Figure 2.3: Constitutive transcription through Fab-7 relieves PcG-mediated silencing. A)-H) pictures of adult pFAs and pFAas transgenic lines before and after all possible recombination steps. A), E) Expression of *miniwhite* when *Fab-7* was transcribed from the *actin5C* promoter. B), F) Excision of the *actin5C* promoter restored PcG-mediated silencing. C), G) *miniwhite* expression after removal of *Fab-7* when the *actin5C* promoter was present on the transgene. D), H) level of eye pigmentation when neither the *Fab-7* PRE nor the *actin5C* promoter were present on the transgene. I) *miniwhite* expression levels in four independent transgenic lines determined by photometric pigment measurements.

2.1.2 Transcription from the actin5C promoter is uni-directional

As mentioned above, the expression of the *lacZ* reporter was initially thought to also reflect the epigenetic state at the *Fab-7* PRE on the transgene. However, β-galactosidase expression analysis showed that the *lacZ* gene was never expressed in the pFAs and pFAas transgenic lines. After heat-shock, the minimal *hsp70* promoter (lacking heat-shock factor binding sites) regulating the *lacZ* gene was not activated above background levels, even when the *Fab-7* PRE was transcribed from the *actin5C* promoter (Fig. 2.4). Although this renders the *lacZ*

gene useless as a reporter, it proves that the activity of the *actin5C* promoter was uni-directional and did not directly influence the transcriptional acitivity of neighbouring genes.

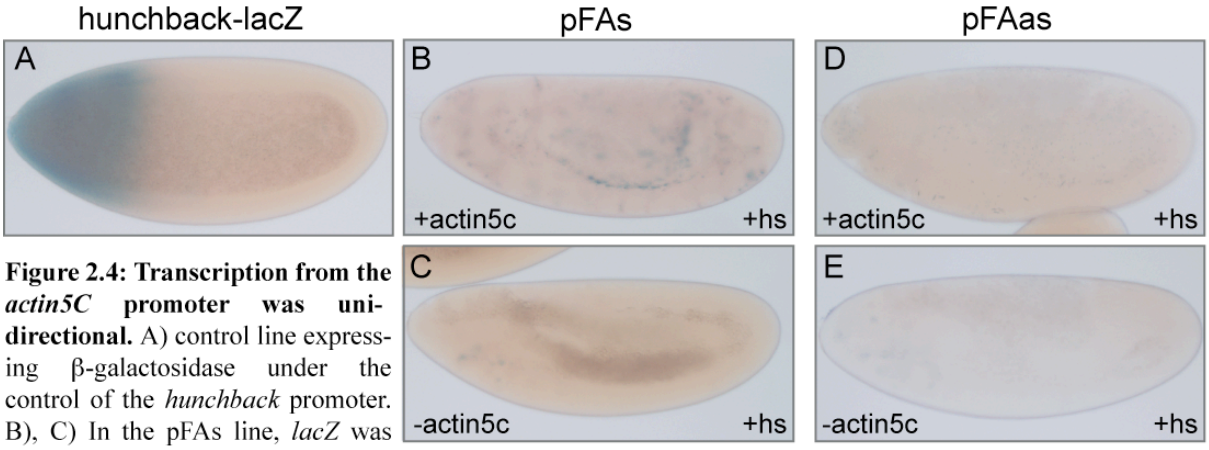

Figure 2.4: Transcription from the *actin5C* promoter was uni-directional. A) control line expressing β-galactosidase under the control of the *hunchback* promoter. B), C) In the pFAs line, *lacZ* was not expressed in the presence or absence of the *actin5C* promoter on the transgene, even after heat-shock induction. D), E) The pFAas line also showed no expression of *lacZ* following heat-shock, irrespective of the absence or presence of the *actin5C* promoter. Embryos are oriented with anterior pole to the left and dorsal to the top.

2.1.3 Relief of silencing requires the transcriptional machinery to pass through the Fab-7 PRE

The *actin5C* promoter employed in the pFAs and pFAas transgenes is a strong promoter which serves as a binding platform for multiple transcription factors (Chung and Keller 1990). It has previously been shown that the recruitment of the yeast GAL4 transcriptional activator to a *UAS-GFP* transgene can counteract the heterochromatin-mediated silencing of this reporter (termed position-effect variegation, or PEV) and even of a neighbouring *miniwhite* transformation marker (Ahmad and Henikoff 2001). To test whether the relief of silencing of the *miniwhite* marker imposed by the *Fab-7* PRE would similarly be caused by the recruitment of transcriptional activators into the vicinity of the PRE, or if this requires processive transcription through this element, the pFTA transgenic construct was designed (Fig. 2.5).

In this construct, a 2kb transcription termination sequence from the 3'UTR of the *Drosophila hsp70* gene (Struhl and Basler 1993) was inserted between the *actin5C* promoter and the *Fab-7* PRE, so that transcription from the *actin5C* promoter was initiated, but should terminate before the PRE is encountered. In two out of six total transgenic lines obtained, the adult flies had a very light eye colour, indicating that in this situation, the silencing function of the *Fab-7* PRE is restored (Fig. 2.5A). In contrast, the excision of the transcription terminator sequence led to a strong derepression of the *miniwhite* gene (Fig. 2.5B).

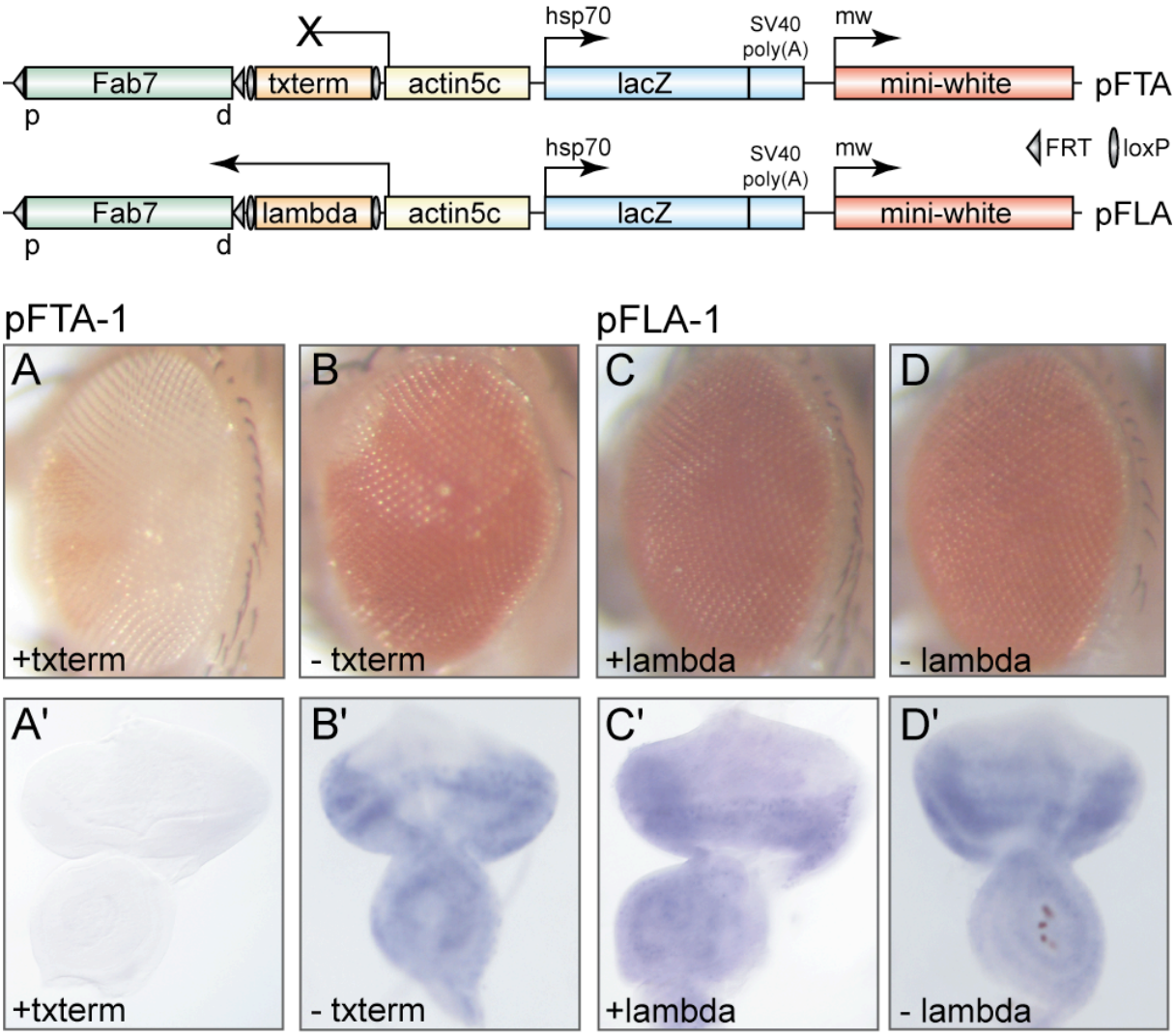

Figure 2.5: The transcriptional machinery has to pass through *Fab-7* to prevent silencing. Top: Schemes of the pFTA and pFLA transgenic constructs. In the pFTA transgene, a transcription terminator (txterm) was inserted between the *actin5C* promoter and the *Fab-7* PRE. In the pFLA construct, the promoter and PRE were separated by *lambda* DNA. In adult flies, *miniwhite* was repressed when the terminator was present on the pFTA transgene (A). Excision of the terminator resulted in a strong increase in eye pigmentation (B). A') RNA *in situ* hybridization in eye imaginal discs showed that *Fab-7* was not transcribed when the terminator was present on the transgene. B') After Cre/*loxP* recombination, *Fab-7* RNA was readily detectable. C), D) The level of *miniwhite* expression in the pFLA-1 line did not change, even when the distance between the *Fab-7* PRE and the *actin5C* promoter was increased by the insertion of *lambda* DNA on the transgene. C'), D') *Fab-7* was transcribed in the pFLA-1 line, both in the presence as well as in the absence of *lambda* DNA. Eye imaginal discs are shown with posterior to the top.

Accordingly, RNA *in situ* hybridization verified that transcription from the *actin5C* promoter was efficiently terminated in the pFTA-1 transgenic line. After excision of the terminator sequence by Cre/*loxP* recombination, *Fab-7* RNA was again readily detectable in eye imaginal discs (Fig. 2.5A' and B'). Two other pFTA transgenic lines, in which the *miniwhite* gene had not been repressed were also tested. In these lines, *Fab-7* transcripts were detectable even in the presence of the termination sequence (data not shown), indicating that at these insertion sites, transcription was not efficiently terminated. Moreover, the total number of

pFTA lines recovered was surprisingly low, suggesting that genuine transgenic lines may have been missed due to complete repression of the *miniwhite* transformation marker.

To rule out that the observed effects in the pFTA lines might simply be due to the increased distance between the *actin5C* promoter and the *Fab-7* PRE, control transgenic lines were generated in which the promoter and the PRE were separated by *lambda* phage DNA of the same length as the *hsp70* 3'UTR terminator, but lacking termination signals (Fig. 2.5, pFLA). Importantly, the *miniwhite* gene was not repressed in any of the 10 transgenic lines obtained (Fig. 2.5C). In addition, removal of the spacer DNA from the transgene by Cre/*loxP* recombination had no effect on eye pigmentation (Fig. 2.5D). RNA *in situ* hybridizations verified that the *Fab-7* PRE was transcribed both in the presence as well as in the absence of the *lambda* spacer fragment (Fig. 2.5C' and D'), confirming that the repression of *miniwhite* in the pFTA lines was caused specifically by the presence of the terminator sequence.

These results demonstrate that the relief of epigenetic silencing imposed by the *Fab-7* PRE indeed requires the transcriptional machinery to pass through this element, and that the recruitment of transcriptional activators into its vicinity is not sufficient to overcome silencing. Moreover, these data unambiguously demonstrate that the increase in *miniwhite* expression in the pFAs and pFAas lines (compare Fig. 2.3B and F with Fig. 2.3A and E) is a consequence of the transcription through the *Fab-7* PRE, and is not due to a direct interaction of the *actin5C* promoter with the promoter of the *miniwhite* gene.

2.1.4 An early pulse of transcription is not sufficient to prevent silencing

The previous results showed that silencing imposed by the *Fab-7* PRE on a transgene can be relieved by the forced continuous transcription through this element. In wildtype embryos, endogenous transcription through PREs is first detectable early during nuclear division cycle 14, shortly before transcription of the homeotic target genes begins (Sanchez-Herrero and Akam 1989).

In order to test whether intergenic transcription is only needed to trigger the epigenetic activation of PREs at this early stage, or if it is required throughout development, the pFHs and pFHas transgenic constructs were generated (Fig. 2.6). On these transgenes, transcription through the *Fab-7* PRE is controlled by a 0.7kb fragment of the zygotic *hunchback (hb)* promoter, which is active only during early embryogenesis. Transcription from this promoter starts before cellularization in nuclear division cycle 11-12, and its activity ceases with the beginning of gastrulation (shortly after division cycle 14; Schröder et al. 1988). RNA *in situ* hybridizations showed that the *Fab-7* sense and antisense RNAs were transcribed in the

expected patterns only in the presence of the zygotic *hb* promoter on the pFHs and pFHas transgenes, respectively (Fig. 2.6A to C and D to F).

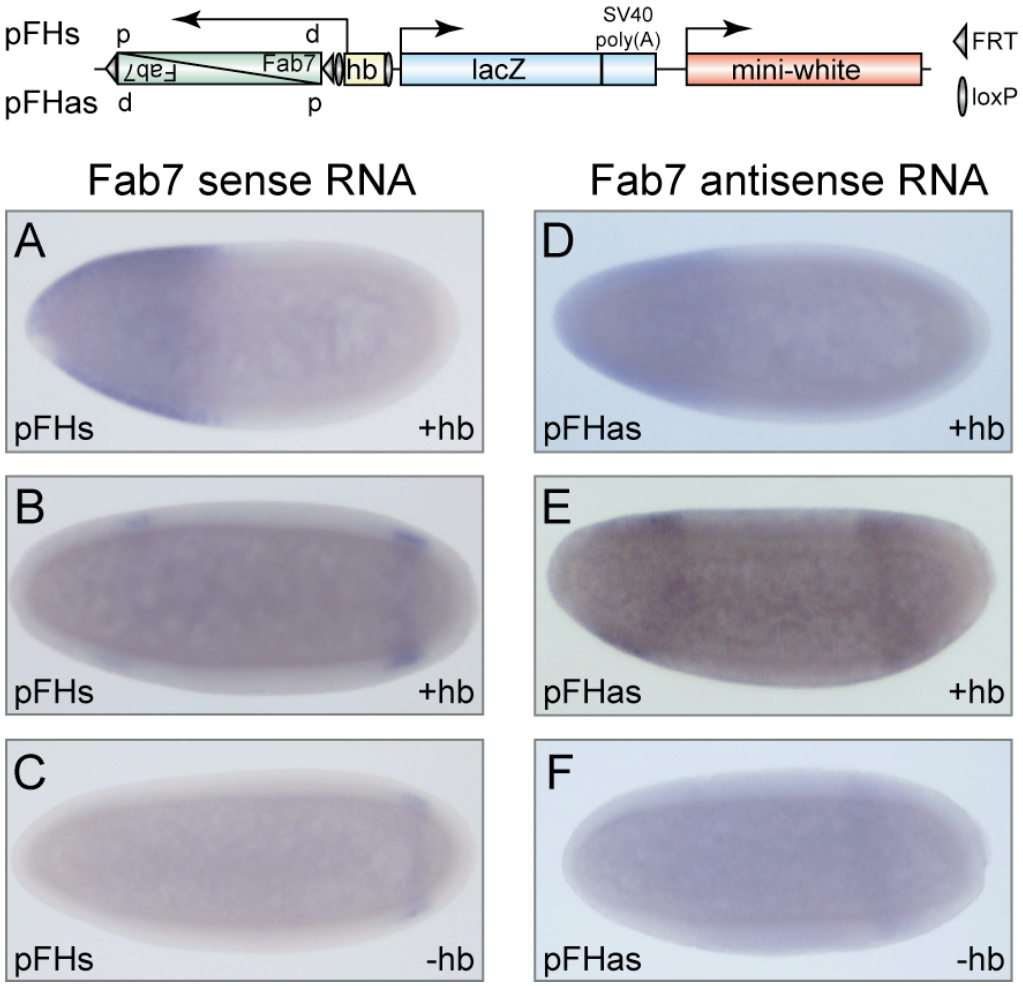

Figure 2.6: Transcription of *Fab-7* in the pFHs and pFHas lines. Top: Scheme of the pFHs and pFHas constructs, in which the *Fab-7* PRE was cloned downstream of the zygotic *hunchback* promoter. A) In the pFHs lines, transcription through the transgenic *Fab-7* PRE occurred in a broad anterior domain until stage 4 which then became restricted to a narrow stripe just before the beginning of gastrulation (B). In addition, endogenous *Fab-7* transcrips were detected in a stripe at the posterior end of the embryo. C) After excision of the promoter from the transgene, only endogenous *Fab-7* RNA was detectable. D) and E) *Fab-7* antisense RNA was transcribed in the same pattern in the pFHas lines. F) Only endogenous *Fab-7* RNA was detected in a posterior domain after excision of the promoter from the transgene. Embryos are shown with anterior to the left, dorsal to the top.

In contrast to the *actin5C* lines pFAs and pFAas, in which the *miniwhite* gene was never repressed prior to recombination, the pFHs and pFHas lines showed pairing-sensitive silencing (PSS) of the transformation marker in 50% (pFHs, n = 12) and 55% (pFHas, n = 11) of the cases. These are the typical rates of PSS that are expected in transgenic reporter lines carrying a PRE (Kassis 2002).

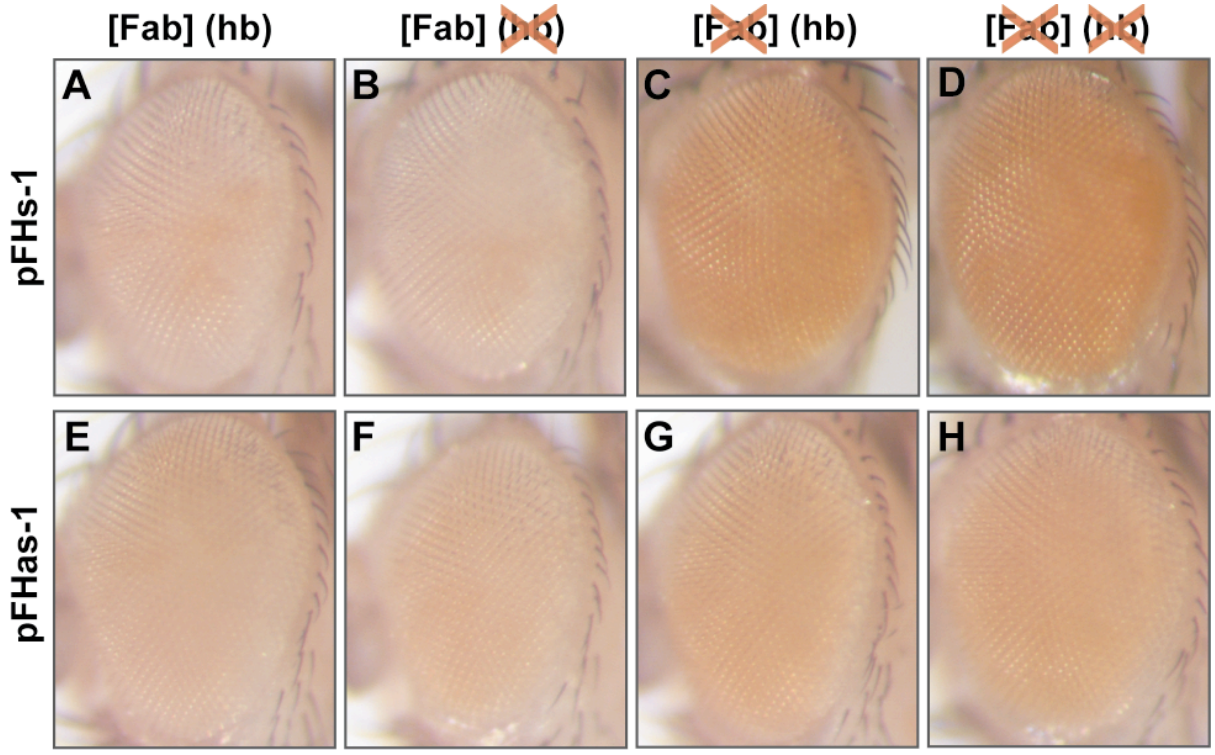

Figure 2.7: Transcription through *Fab-7* from the *hunchback* promoter did not result in a relief of silencing. A), E) The *miniwhite* gene was strongly repressed in the pFHs and pFHas transgenic lines. B), F) Excision of the *hunchback* promoter had no effect on eye pigmentation. C), G) In the absence of *Fab-7* from the transgenes, *miniwhite* expression levels were increased in the pFHs and pFHas transgenic flies. D), H) Further removal of the *hunchback* promoter did not change the expression of *miniwhite*.

After excision of the zygotic *hb* promoter from the transgene by Cre/*loxP* recombination, the eye colours of the transgenic flies did not change, indicating that transcription through the *Fab-7* PRE from this promoter was not sufficient to prevent PcG-mediated silencing (Fig. 2.7A, B, E, F). Removal of the *Fab-7* PRE from the transgene led to an elevation of the *miniwhite* expression level, confirming that the silencing observed in the pFHs and pFHas lines is indeed caused by the PRE (Fig. 2.7C and G). As expected, the further removal of the zygotic *hb* promoter had no effect on the expression of *miniwhite* (Fig. 2.7D and H).
These results indicate that a short pulse of transcription during early embryogenesis is not sufficient to prevent the silencing of *miniwhite* imposed by the *Fab-7* PRE on the transgene.

2.1.5 Transcription through the transgenic Fab-7 PRE until the end of embryogenesis is sufficient to prevent re-silencing

The previous experiments demonstrated that a short pulse of transcription through the *Fab-7* PRE does not efficiently prevent this element from exerting its silencing function. Nevertheless, it was still possible that transcription through this PRE may not be required continuously, but that a longer time frame of transcription, extending over a number of cell divisions, may be sufficient to firmly establish the epigenetic activation of the PRE, which

would then be sustained throughout development. Consistent with this, endogenous non-coding PRE transcripts in the BX-C can also be detected in late stages of embryogenesis (Rank et al. 2002). In addition, in a transgenic assay established previously in our lab, the *Fab-7* PRE can be stably switched into the activated mode through the transient expression of the GAL4 transactivator in embryos, but the highest efficiency of switching was achieved when GAL4 was expressed during late embryogenesis (Cavalli and Paro 1998).

To be able to limit the time frame of transcription through the *Fab-7* PRE up to the end of embryogenesis, the pFAas-1 line was combined with two transgenic lines that allowed the inducible excision of the *actin5C* promoter from the transgene (Fig. 2.8).

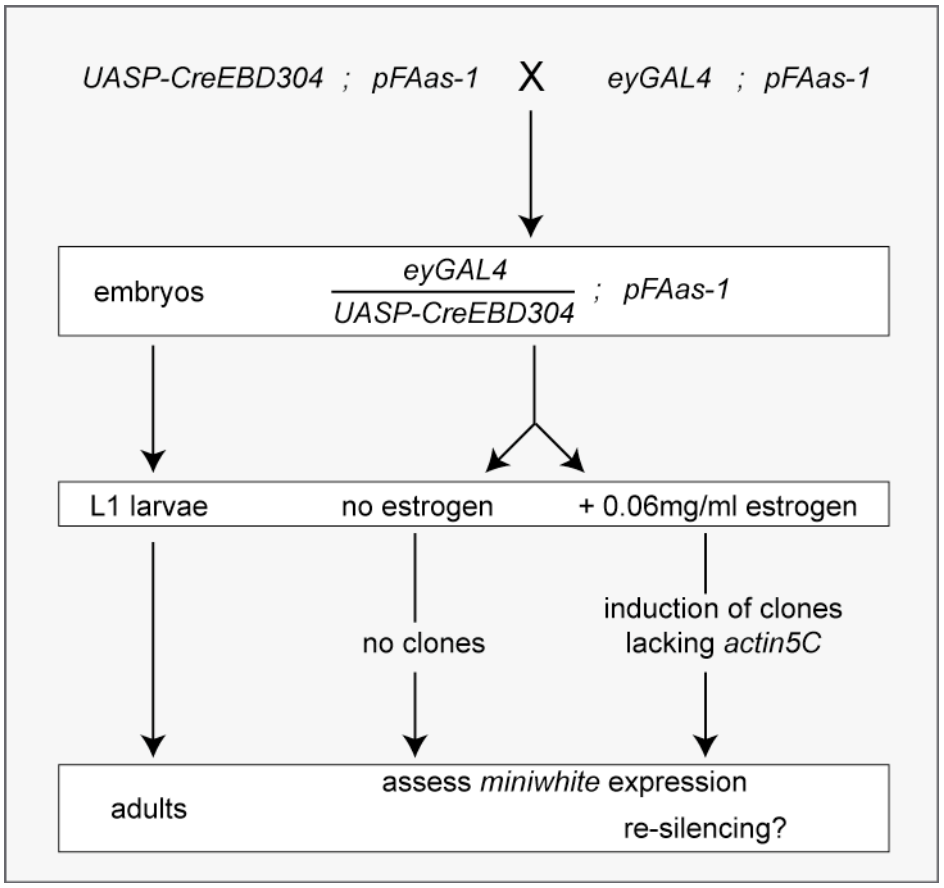

Figure 2.8: Experimental setup to induce Cre/*loxP* recombination in first instar larvae. Flies carrying the *UASP-CreEBD304* transgene on the second chromosome and the pFAas-1 transgene on the third chromosome were crossed to flies in which the *eyGAL4* driver was located on the second chromosome and the pFAas-1 transgene was inserted on the third chromosome. Embryos were collected and freshly hatched first instar (L1) larvae were then transferred onto food containing or lacking 0.6mg/ml estrogen, and *miniwhite* expression was examined in adult flies.

One of these lines *(UASP-Cre-EBD304)* carried a construct encoding a fusion of the Cre recombinase with an estrogen binding domain (EBD) under the control of a yeast *UAS (upstream activating sequence)* enhancer (kindly provided by C. Lehner; Heidmann and Lehner 2001). The second line expressed the yeast GAL4 transactivator under the control of

the *eyeless (ey)* promoter, which is active in all precursor cells of the adult eye of the fly, beginning in early embryogenesis (obtained from Stefan Schönfelder; Quiring et al. 1994; Halder et al. 1998; Schönfelder 2005). Thus, the Cre-EBD fusion protein was expressed in all precursors of the adult eye, but should be active only in the presence of estrogen, and catalyze the specific excision of the *actin5C* promoter from the pFAas-1 transgene (Heidmann and Lehner 2001). In order to induce recombination after the completion of embryogenesis, freshly hatched first instar larvae were transferred onto food containing 0.06mg/ml estrogen.

Genomic PCR on DNA prepared from single heads of adult flies was used to verify that recombination had been induced as expected. In Fig. 2.9, the positions of primer used for this purpose are indicated. To test for successful recombination events, genomic DNA from each sample was amplified in two separate reactions, using the primer pairs 1 and 2 or 1 and 3, respectively. When the *actin5C* promoter is present on the transgene, amplification with primer 1 and 2 should result in a 550bp PCR product. The reaction using primer 1 and 3 should not yield a product, as the PCR conditions chosen did not support the amplification of the expected 3,5kb fragment. In the absence of the promoter from the transgene, PCR using primer 1 and 2 should not lead to the amplification of a specific product, whereas the combination of primer 1 with primer 3 should result in a 900bp fragment.

Fig. 2.9 illustrates the obtained result of 10 individual flies, five of which had been treated with estrogen, and five had not. Lanes A and A' show the expected PCR products obtained from control lines in which the *actin5C* promoter is present in all cells. In contrast, lanes B and B’ represent the PCR fragments amplified from genomic DNA prepared from lines lacking the promoter in all cells. Lanes 1 to 10 show that in all individuals treated or not treated with estrogen, PCR products specific for non-recombined DNA can be faithfully obtained. This was expected, because the expression of the Cre-EBD fusion protein is restricted to eye tissue, whereas the PCR was done on DNA isolated from whole adult heads. Lanes 2’, 4’, 6’, 8’, and 10 illustrate that in 2 out of five individuals tested (lanes 4’ and 10’), excision of the *actin5C* promoter occurred even in the absence of estrogen. This number increased to four out of five when estrogen was present in the food (lanes 1’, 3’, 5’, 7’, and 9’). Although the PCR was performed under saturating conditions, the amount of PCR product in the cases where background recombination occurred was considerably lower than that obtained in the estrogen-treated individuals. This suggests that the number of cells in which recombination had occurred in the absence of estrogen was very low.

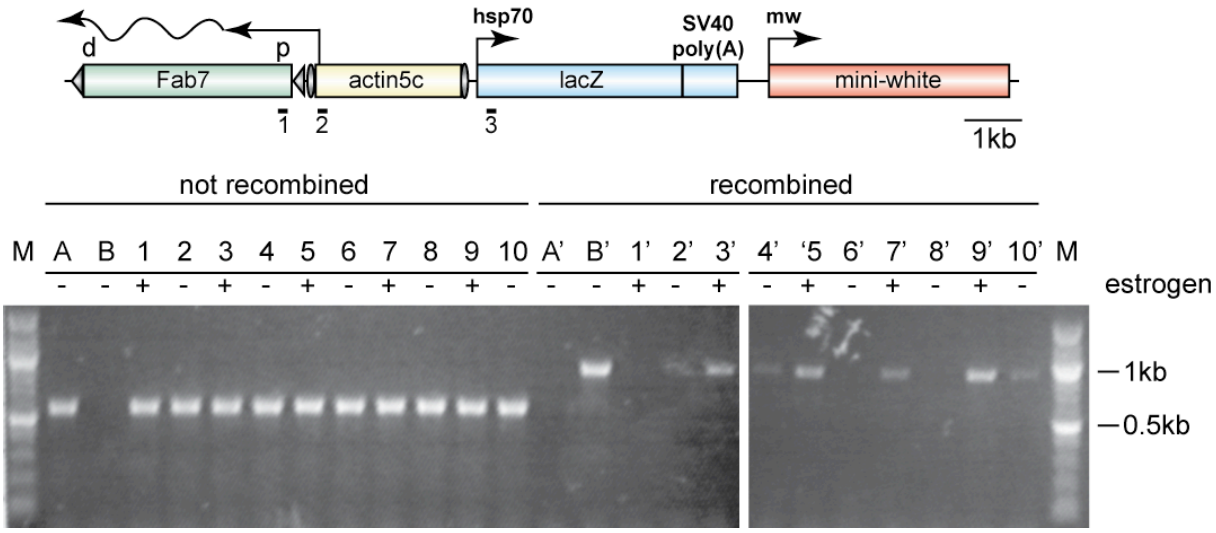

Figure 2.9: Genomic PCR verified the successful induction of Cre-EBD recombinase activity. Top: Scheme of the pFAas construct with the positions of primer used in the PCRs indicated as black lines at the bottom. Lane A shows that in control flies, in which the *actin5C* promoter was present, PCR using primer 1 and 2 yielded the expected 550bp product. Using primer 1 and 3 did not result in any PCR product (lane A'). In flies lacking the *actin5C* promoter, PCR using primer 1 and 2 results in no product (lane B), whereas primer 1 and 3 yielded the expected product of 900bp (lane B'). Lanes 1 and 1', 3 and 3', 5 and 5', 7 and 7', 9 and 9' show the PCR products obtained from individuals treated with estrogen. Lanes 2 and 2', 4 and 4', 6 and 6', 8 and 8', 10 and 10' represent the PCR products resulting from individuals not treated with estrogen.

In the initial characterization of the *UASP-Cre-EBD304* line, estrogen-independent recombination was not observed, whereas upon induction, the recombination rate reached 100% (Heidmann and Lehner 2001). In that study, the clones in the adult eye in which recombination had occurred covered the majority of cells in the eye, even after estrogen treatment for only 3h during the first larval stage. Therefore, after transferring freshly hatched first instar larvae onto estrogen-containing food, recombination was expected to take place in the majority of the eye cells.

To determine the effect of excising the *actin5C* promoter after the end of embryogenesis, the eye colour of adult flies exposed to estrogen from the first larval stage onward was compared with those not treated with estrogen. Control flies kept on food lacking estrogen showed the expected high expression of the *miniwhite* marker in the presence of both the *actin5C* promoter and the *Fab-7* PRE on the pFAas-1 transgene, in combination with the *eyGAL4* driver and the *UASP-Cre-EBD304* construct (Fig. 2.10A). Fig. 2.10C shows the expected eye colour after germ-line excision of the *actin5C* promoter and in the presence of all three transgenes. When first instar larvae were transferred onto food containing 0.06mg/ml estrogen, most of the adult flies had a similar eye colour to those not treated with estrogen (data not shown). However, in 5-10% of the cases, flies with small light-coloured clones in the eyes were obtained, indicating that the *miniwhite* gene had become re-silenced (Fig. 2.10B). The degree of pigmentation in those clones closely matched that observed in control

flies in which the *actin5C* promoter had been excised in the germ-line (Fig. 2.10C). Similar results were observed when the estrogen concentration was varied from 0.03mg/ml to 0.09mg/ml estrogen, which is consistent with the study by Heidmann and Lehner (2001), in which Cre recombinase activity was found to plateau at estrogen concentrations above 0.01mg/ml.

In the control group lacking the *Fab-7* PRE on the transgene, none of the flies hatched with clones in which *miniwhite* had become re-silenced, although large regions in the eyes showed an intermediate pigmentation (Fig. 2.10D and E). The comparison with control flies lacking the *actin5C* promoter suggests that this lighter pigmentation is probably due to the reduction of *miniwhite* expression by the removal of the *actin5C* promoter from the transgene (Fig. 2.10F).

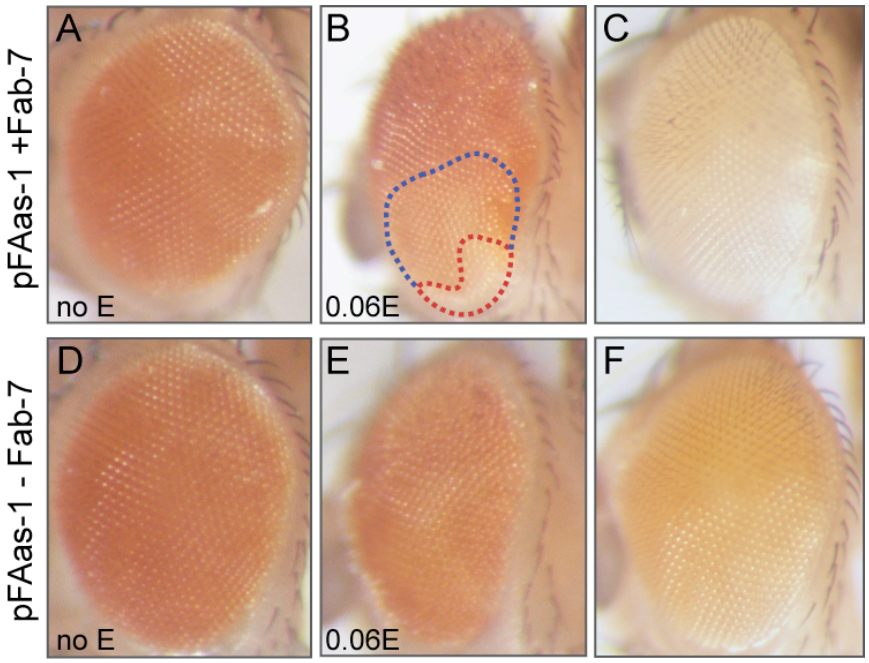

Figure 2.10: Excision of the *actin5C* promoter in L1 larvae resulted in re-silencing of *miniwhite* in rare cases. A) Eye colour of pFAas-1 transgenic flies expressing the Cre-EBD fusion protein not treated with estrogen. B) In rare cases, treatment with 0.06mg/ml estrogen resulted in small clones of *miniwhite* repression in the eyes of adult flies (blue dotted line: Clone of cells in which recombination had occurred; red dotted line: cells within the clone in which *miniwhite* had become re-silenced). C) Without the *actin5C* promoter, the *miniwhite* gene was silenced. D) to F) Eye pigmentation in adult flies lacking the *Fab-7* PRE on the transgene, which had been treated as in A) to C).

As stated above, in the presence of the *Fab-7* PRE on the pFAas-1 transgene, the induction of Cre recombinase activity from the first larval stage onward resulted in adult flies with and without clones in the eyes, in which the *miniwhite* marker had become re-silenced. Genomic PCR indicated that recombination had occurred in flies with light-coloured clones as well as in those without clones (Fig. 2.9). In the latter, the presence of high uniform levels of eye

pigmentation, concomitant with substantial recombination, indicates that despite the excision of the *actin5C* promoter in first instar larvae, the silencing function of the *Fab-7* PRE could not be restored until adulthood in most of the cases. These data suggest that on the transgene, a time frame of transcription through the *Fab-7* PRE at least until embryogenesis is completed is sufficient both for the establishment as well as for the maintenance of epigenetic activation at the PRE. However, in rare cases, the epigenetic features required for the maintenance of the active state may have become diluted through sequential mitotic divisions, which as a consequence, results in the re-silencing of the *miniwhite* marker.

2.1.6 Endogenous PREs in the Bithorax Complex are transcribed in third instar larvae

The analysis of the transgenic reporter system in the present work indicates that in most cases, anti-silencing by transcription through the *Fab-7* PRE until the end of embryogenesis is sufficient to prevent the re-establishment of PcG-mediated silencing until adulthood. However, these data were obtained in a transgenic background on an isolated PRE, raising the question whether endogenous PREs are only transcribed during early development, or if transcription persists until later stages.

In a previous study in our lab, Gerhard Rank and Matthias Prestel showed that the *bxd*, *Mcp*, and *Fab-7* PREs in the BX-C are transcribed in a regulated manner, reflecting the expression pattern of the respective homeotic target genes during embryogenesis (Rank et al. 2002). In analogy to the embryo, the homeotic genes are expressed in a characteristic pattern along the anterior-posterior body axis in the brain of third instar larvae, following the principle of spatial co-linearity (Fig. 2.11A; Duboule and Morata 1994). If the anti-silencing function of transcription is required continuously throughout development, the *bxd*, *Mcp*, and *Fab-7* PREs should also be transcribed in this tissue. Indeed, RNA *in situ* hybridizations demonstrated the presence of non-coding transcripts spanning the *bxd*, *Mcp*, and *Fab-7* PREs in a pattern that reflected the expression domains of the *Ubx* and *AbdB* target genes in the brain, respectively (Fig. 2.11B). As in late embryos, non-coding RNAs were only detected in the sense direction with respect to the orientation of the coding mRNAs.

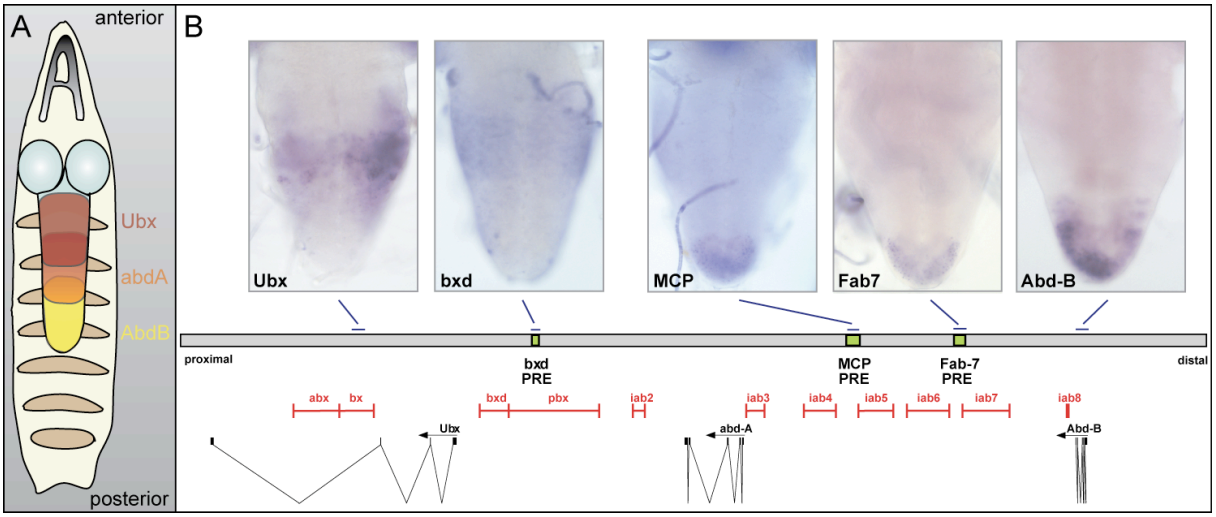

Figure 2.11: Endogenous PREs in the Bithorax Complex are transcribed in third instar larvae. A) Scheme of a third instar larva showing the expression domains of the *Ultrabithorax (Ubx), abdominal-A (abdA),* and *Abdominal-B (AbdB)* genes in the ventral nerve cord of the brain. B) Top: RNA *in situ* hybridizations showing the expression domains of *Ubx* and *AbdB* in the ventral nerve cord. Sense transcripts spanning the *bxd*, *Mcp*, and *Fab-7* PREs were also detected. Bottom: Scheme of the Bithorax complex indicating the positions of the *Ubx*, *abdA*, and *AbdB* genes, as well as segment-specific regulatory elements (*abx/bx*; *bxd/pbx*; *iab2* to *iab8*). Positions of probes used for *in situ* hybridizations are indicated by blue lines, and the locations of the *bxd*, *Mcp*, and *Fab-7* PREs are marked in green.

The finding that transcription through endogenous PREs occurs not only during embryogenesis, but also during third instar larval development suggests that in the context of the endogenous BX-C, the anti-silencing function of non-coding transcription through PREs may be required continuously throughout development to ensure the faithful inheritance of the epigenetically activated state.

2.1.7 Anti-silencing by non-coding transcription through PREs – a general principle?

Apart from the homeotic genes, the PcG/trxG memory system is known to regulate many more target genes (reviewed in Ringrose and Paro 2004). Consistent with this, Polycomb (PC) is associated with over 100 distinct loci in polytene chromosomes, and other PcG proteins such as Polyhomeotic (PH) and Posterior Sex Combs (PSC) show a similar, partially overlapping localization (Zink and Paro 1989; DeCamillis et al. 1992; Martin and Adler 1993). Only in a few cases outside the homeotic Antennapedia and Bithorax Complexes (ANT-C and BX-C), namely at the *engrailed* (*en*; Kassis 1994), the *polyhomeotic* (*ph*; Bloyer et al. 2003), and the *hedgehog* (*hh*; Maurange and Paro 2002) loci, the respective PREs have been identified. However, in a recent *in silico* approach, Leonie Ringrose and Marc Rehmsmeier were able to predict 167 putative PREs in the *Drosophila* genome (Ringrose et al. 2003).

With the sequences of these potential PREs at hand, a few candidates were selected and RNA probes designed in order to test if these elements are transcribed in wildtype *Drosophila*. One selection criterion was that the predicted PREs should have a high score in the prediction and simultaneously lie in a low-complexity region of the genome, as the assignment of target genes to the predicted PREs relied solely on their distances to each other. Secondly, the predicted PREs chosen should all be associated with binding sites of PcG proteins in polytene chromosomes (Ringrose et al. 2003). Moreover, in order to be able to distinguish weak specific RNA signals from homogeneous background in the *in situ* hybridizations, it was important to select PREs potentially regulating genes with defined spatial expression patterns. All selected potentially PRE-regulated genes encode transcription factors. Fig. 2.12B shows that the genetically characterized (Kassis 1994) and predicted PRE in the promoter region of the *en* gene was transcribed bi-directionally, reflecting the expression pattern of the associated *en* mRNA. Similarly, the predicted PRE at the *spalt major* (*salm*; Frei et al. 1988) locus was also transcribed bi-directionally in the same domains in which its potential target gene was expressed (Fig. 2.12C).

At the *slouch (slou)* locus (Dohrmann et al. 1990), the predicted PRE is located around 3kb upstream of the gene, and non-coding transcripts in both sense and antisense directions were detected in those cells in which the Slou transcription factor was expressed (Fig. 2.12D). The predicted PRE at the *tailless* (*tll*; Strecker et al. 1986; Daniel et al. 1999) locus is located in the vicinity of the promoter region and was also transcribed in the same pattern as its cognate *tll* target mRNA (Fig. 2.12E). In contrast to the other PREs analyzed, the predicted *tll* PRE was transcribed uni-directionally, and only antisense RNA with respect to the *tll* mRNA was generated. Interestingly, RNA spanning this PRE was also expressed in the optic lobes of third instar larval brains, suggesting that similar to the *bxd*, *Mcp*, and *Fab-7* PREs in the BX-C, non-coding transcription through the predicted *tll* PRE may also be required continuously throughout development to prevent the re-silencing of this locus (Fig. 2.12E).

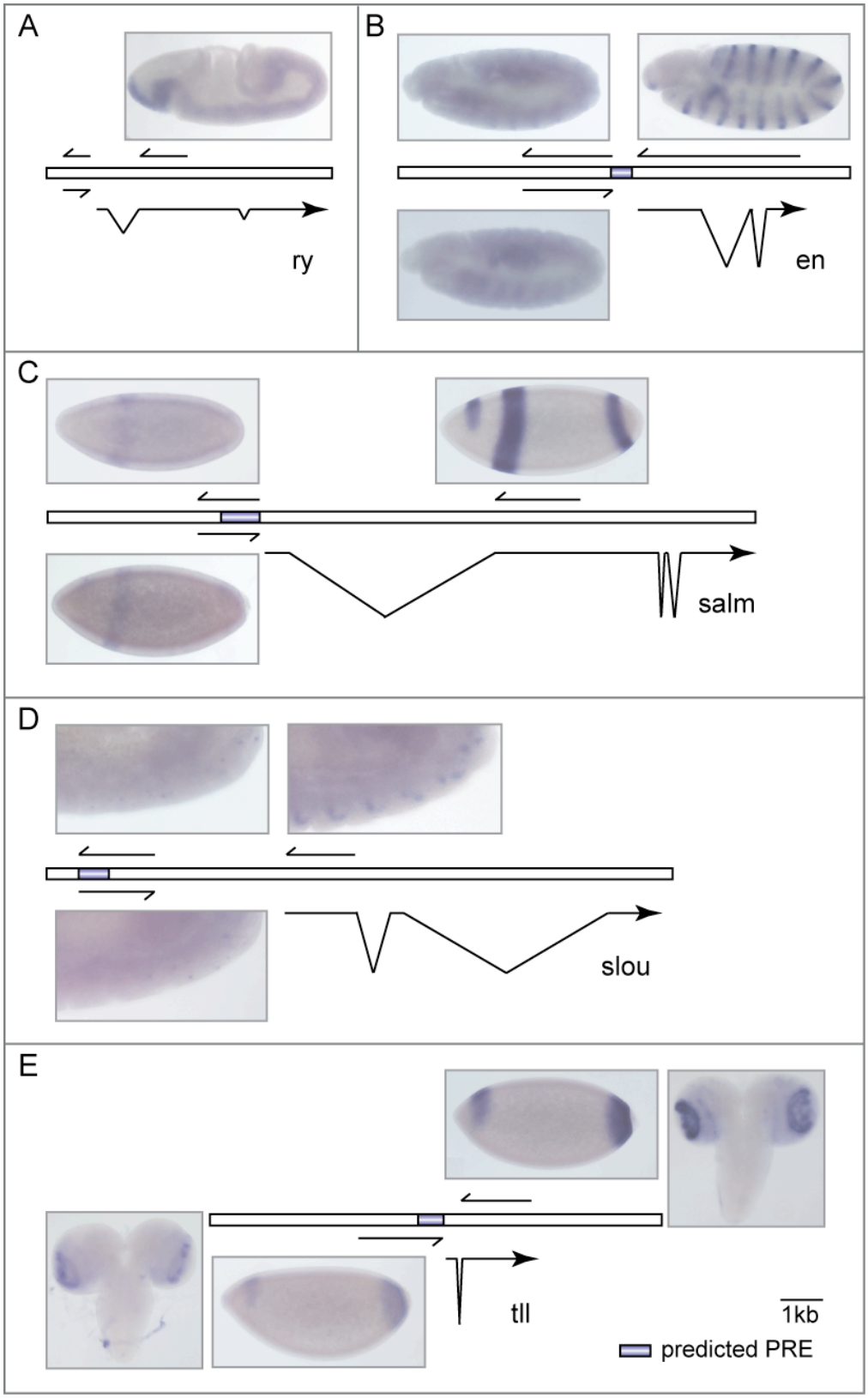

Figure 2.12: RNA *in situ* hybridizations showed that PREs outside the homeotic gene complexes were transcribed. A) There was no spurious transcription through the *rosy (ry)* promoter detectable when the gene was active. B) The *engrailed (en)* PRE was transcribed bi-directionally in a pattern reflecting the expression domain of the *en* mRNA. The predicted PREs at the *spalt major (salm)* and *slouch (slou)* loci were transcribed in both directions in the same patterns as the predicted target genes (C and D). E) The predicted *tailless (tll)* PRE was transcribed in the anti-sense direction only, reflecting the expression pattern of the *tll* mRNA. Transcripts spanning the *tll* PRE were also detected in the optic lobes of third instar larvae, where *tll* mRNA was present as well. Embryos are shown with anterior to the left and dorsal to the top. Brains are shown with anterior to the top.

As the predicted PREs of the *en, salm*, and *tll* genes lie within the promoter regions of the respective target genes, it was important to exclude that upstream non-coding transcription might be a general property of active promoters. As expected, RNA *in situ* hybridizations using a probe directed against the promoter region of the *rosy (ry)* gene, which is known not to be regulated by the PcG/TrxG memory system (Ringrose et al. 2003), failed to detect transcription in this region (Fig. 2.12A). In addition, the RNA *in situ* hybridization analysis of the *en* and *salm* loci showed that these predicted PREs were not transcribed in imaginal discs of third instar larvae, although the respective target mRNAs were expressed in these tissues at high levels (data not shown).

These results suggest that the activation of a gene does not result in the spurious transcription through its promoter region, and that the regulated, non-coding transcription through the predicted PREs may be required to prevent the PcG-mediated silencing of these loci. The fact that non-coding transcription in third instar larvae was only detectable at the *tll*, but not at the *en* and *salm* loci suggests that the maintenance of epigenetic activation may be regulated by different mechanisms in different tissues. Alternatively, the *en* and *salm* genes might be controlled by more than one PRE, which are differentially deployed in different tissues and would thus show a differential pattern of non-coding transcription.

2.2 Does transcription through Fab-7 change the association of PcG/TrxG proteins with the chromatin?

The previous results with the transgenic reporter system demonstrate that the continuous transcription through the *Fab-7* PRE functions as an anti-silencing mechanism that counteracts the establishment of PcG-mediated silencing at the pFAs-1 transgene. To date, it is not clear how this might be achieved mechanistically. One possibility is that anti-silencing by transcription through the transgenic *Fab-7* PRE might be achieved through the specific displacement of PcG proteins and/or the recruitment of TrxG proteins to the chromatin.

2.2.1 Polycomb is associated with the transcribed Fab-7 PRE

To test whether the relief of PcG-mediated silencing at the transcribed *Fab-7* PRE might be caused by the specific displacement of Polycomb (PC) from the chromatin, polytene chromosomes from pFAs-1 transgenic larvae were analyzed.

To be able to compare the binding of PC to the pFAs-1 transgene in the repressed state with the binding to the transcribed *Fab-7* PRE, the site of transgene insertion was mapped by DNA fluorescent *in situ* hybridization (DNA FISH). Fig. 2.13A-C show that the pFAs-1 transgene was located on chromosome arm 2L, and closer inspection reveals that the insertion site mapped to the position 32D (Fig. 2.13D-F).

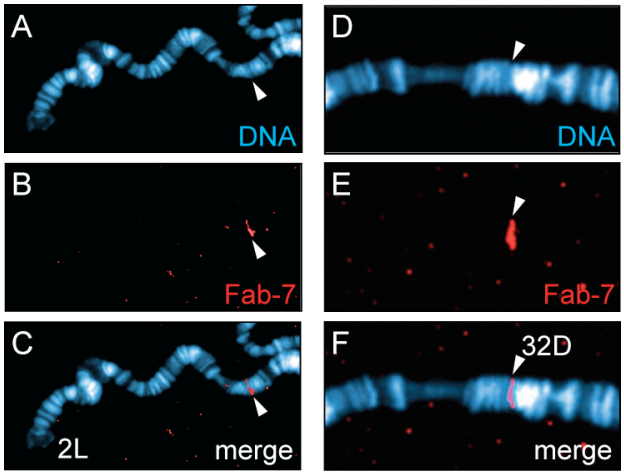

Figure 2.13: Mapping of the pFAs-1 transgene insertion site by DNA FISH on polytene chromosomes. A) to C) DNA FISH using a probe specifically hybridizing with the *Fab-7* fragment resulted in a distinct band on chromosome arm 2L. D) to F) Close inspection revealed that the insertion site of the pFAs-1 transgene mapped to the position 32D. DNA: blue; *Fab-7* DNA FISH: red.

To determine if the insertion of the pFAs-1 transgene generated an ectopic binding site for the PC protein, the DNA FISH technique was combined with PC immunostaining. In the absence of the *actin5C* promoter from the transgene, the *Fab-7* PRE functioned as a silencer and as expected, mediated the recruitment of PC to the chromatin (Fig. 2.14A-D). Consistent with previous findings, binding of PC to the transgene was not impaired when the *Fab-7* PRE was in the epigenetically activated state (Fig. 2.14H-K; Cavalli and Paro 1999). The same analysis with wildtype chromosomes showed that there was no endogenous binding of PC at the site of transgene insertion (Fig. 2.14O-R).

A general problem of the simultaneous detection of DNA and protein is the limited preservation of the immunosignal throughout the DNA FISH procedure. Therefore, a stronger signal for PC binding both to the repressed and transcribed *Fab-7* PRE was obtained when using immunolocalization alone (Fig. 2.14E-G and L-N, respectively). Next to the transgene insertion site, there was a strong endogenous PC binding site, which mapped to the locus encoding the *salm* gene at position 32F (Fig. 2.14) and was used as a „landmark“ for the subsequent immunolocalization analyses.

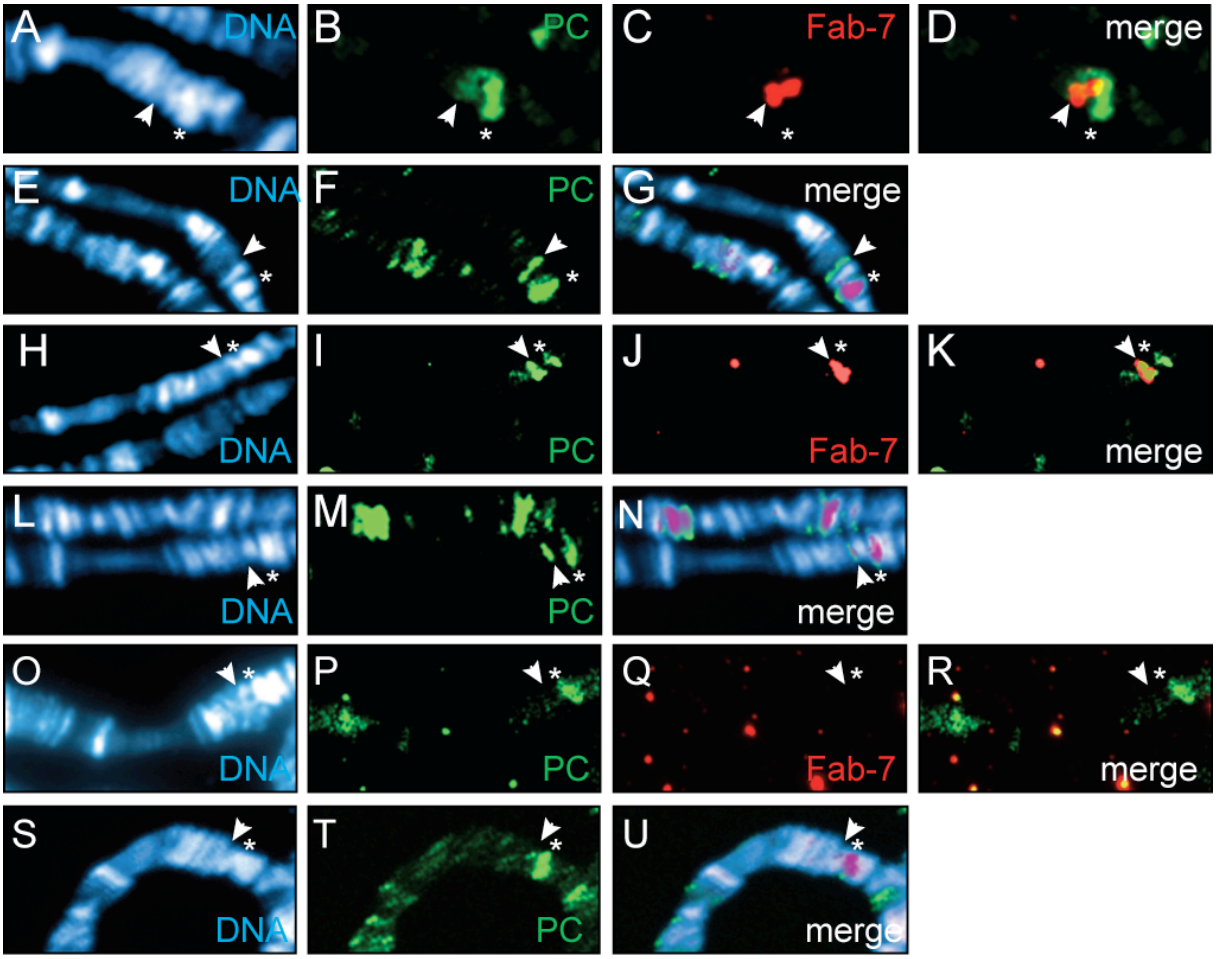

Figure 2.14: Polycomb (PC) was bound to both the repressed and transcribed *Fab-7* PRE. A) to D) DNA FISH combined with PC immunostaining in the pFAs-1 line after excision of the *actin5C* promoter. PC became recruited to the transgene as revealed by overlapping PC and DNA FISH signals (arrows). E) to G) immunostaining alone in the same line showed that PC was strongly bound at the site of transgene insertion (arrow). H) to K) When the *actin5C* promoter was present on the transgene, PC was also associated with the chromatin (arrow). L) to N) immunostaining alone showed strong PC binding to the chromatin at the transgene insertion site. O) to R) In the wildtype, neither PC binding nor a DNA FISH signal were detected at the site of transgene insertion. S) to U) immunostaining alone confirmed absence of PC from the location of the transgene in the wildtype. Asterisks mark a strong endogenous PC band at the *salm* locus at 32F, which was used as a landmark in further experiments. DNA: blue; PC: green; *Fab-7* DNA FISH: red.

2.2.2 Pleiohomeotic is localized to both repressed and transcribed Fab-7 PRE

PC is one of the components of the Polycomb group Repressive Complex 1 (PRC1), which is thought to mediate the „maintenance phase“ of epigenetic silencing (see Introduction 1.2.1). Apart from PC, PRC1 consists of the core components Posterior Sex Combs (PSC), dRING, and Polyhomeotic (PH) (Francis et al. 2001; Lavigne et al. 2004). Increasing evidence suggests that the specific recruitment of the PRC1 complex to a PRE depends on its interaction with the DNA-binding protein Pleiohomeotic (PHO) (Mohd-Sarip et al. 2002; Wang et al. 2004b; Mohd-Sarip et al. 2005).

Immunostaining of polytene chromosomes was used to test whether transcription through the *Fab-7* PRE on the pFAs-1 transgene leads to the specific displacement of this tethering protein from the chromatin. As expected, in the absence of the *actin5C* promoter, the repressed *Fab-7* PRE on the pFAs-1 transgene generated a new binding site for PHO on polytene chromosomes which was absent in the wildtype (compare Fig. 2.15A-C with G-I). When the silencing function of *Fab-7* was abolished by transcription from the *actin5C* promoter, PHO was still strongly bound to the pFAs-1 transgene (Fig. 2.15D-F). Surprisingly, the binding of PHO to the transgenic *Fab-7* PRE appeared to be even stronger in this case. However, due to the high variability inherent to polytene chromosome immunostainings, real quantitative statements cannot be made with this technique.

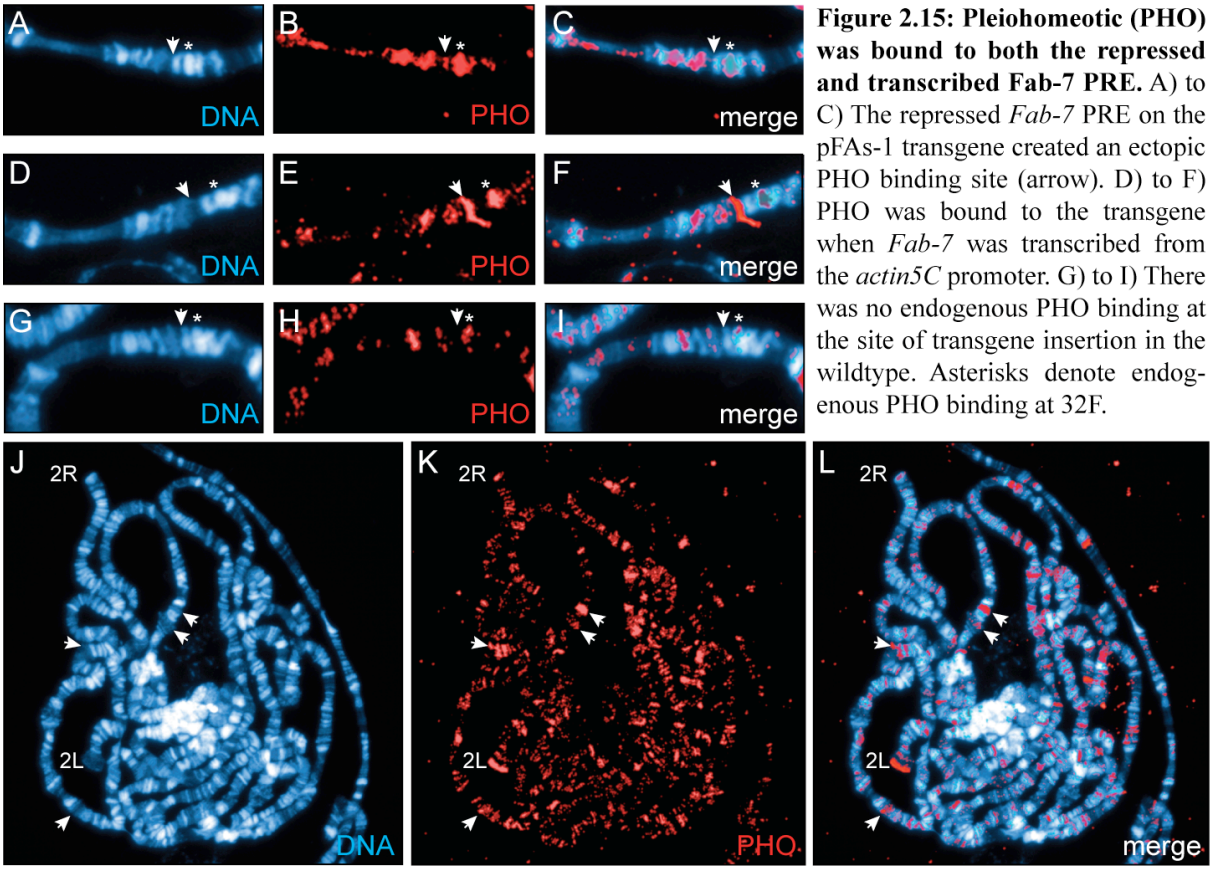

Figure 2.15: Pleiohomeotic (PHO) was bound to both the repressed and transcribed Fab-7 PRE. A) to C) The repressed *Fab-7* PRE on the pFAs-1 transgene created an ectopic PHO binding site (arrow). D) to F) PHO was bound to the transgene when *Fab-7* was transcribed from the *actin5C* promoter. G) to I) There was no endogenous PHO binding at the site of transgene insertion in the wildtype. Asterisks denote endogenous PHO binding at 32F.

J) to L) Complete chromosome spreads revealed enrichment of PHO in puffed regions of the chromosomes (arrows), in addition to strong binding at the telomeres of chromosome arms 2L and 2R. Blue: DNA; red: PHO.

Interestingly, a closer inspection of the association of the PHO protein with chromatin on whole chromosome spreads showed that PHO was strongly enriched in telomeric regions (see telomeres of 2L and 2R in Fig. 2.15J-L). This suggests that apart from its function in the cellular memory system, PHO might be involved in telomeric silencing.

In general, PHO bound to many more loci in polytene chromosomes than other PcG proteins, such as PC or PH (Fig. 2.15J-L; Zink and Paro 1989; DeCamillis et al. 1992). Remarkably, PHO was not only localized to discrete bands on the chromosomes, but showed also a significant enrichment across regions of extreme decondensation, referred to as chromosomal puffs, which are caused by high transcriptional activity (see arrows Fig. 2.15J-L).

Taken together, these results show that at least at the level of polytene chromosomes, forced transcription through the transgenic *Fab-7* PRE does not lead to the displacement of PHO from the chromatin.

2.2.3 Trithorax is bound to the repressed Fab-7 PRE

Trithorax (TRX) is a histone methyltransferase required to maintain the transcriptionally active state of homeotic genes throughout development (Klymenko and Müller 2004; Smith et al. 2004). Polytene immunostaining was used to test whether TRX is absent from the repressed *Fab-7* PRE and becomes specifically recruited to the PRE when it is transcribed.

As expected, TRX was bound to the transcribed *Fab-7* PRE in the pFAs-1 line, thus generating an additional TRX binding site not present in the wildtype (compare Fig. 2.16D-F with G-I). Interestingly, TRX was also associated with the PRE when the *actin5C* promoter was excised from the transgene, i.e. when the *Fab-7* PRE functioned as a silencer (Fig. 2.16A-C).

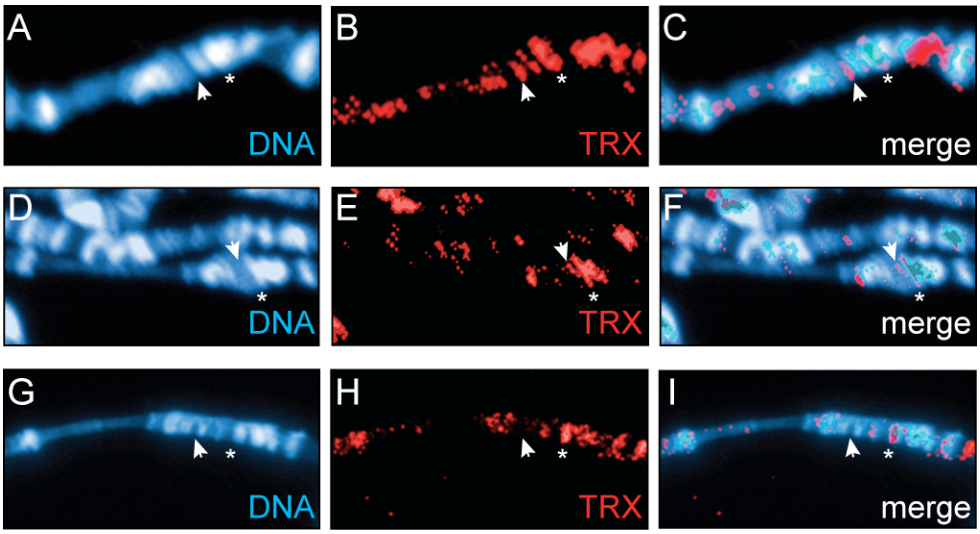

Figure 2.16: TRX was associated with both the repressed and transcribed *Fab-7* PRE. A) to C) In the absence of the *actin5C* promoter, the pFAs-1 transgene generated an ectopic binding site for TRX (arrow). D) to F) TRX was bound to the transcribed *Fab-7* PRE on the pFAs-1 transgene (arrow). G) to H) There was no endogenous binding of TRX at the site of transgene insertion (arrow). Asterisks highlight endogenous TRX binding to the *salm* locus at 32F. Blue: DNA; red: TRX.

Taken together with the results obtained from the analysis of PC and PHO association with the *Fab-7* PRE on the pFAs-1 transgene, these data suggest that the epigenetic state of this PRE is not simply regulated by the specific displacement of PC or PHO from the chromatin, nor by the differential recruitment of TRX. However, due to the variability of signal intensities inherent to the polytene immunostaining technique, quantitative statements regarding the level of binding of a specific protein to the chromatin cannot be made with confidence.

2.2.4 Transcription does not change the levels of Polycomb, Pleiohomeotic, and Trithorax bound to the Fab-7 PRE

In order to analyze the binding of PC, PHO, and TRX to the transgenic *Fab-7* PRE in a more quantitative manner, chromatin immunoprecipitation (ChIP) experiments were performed. The principle of this technique is to precipitate formaldehyde-crosslinked and sheared chromatin with antibodies specifically recognizing a protein of interest. Subsequently, the precipitated DNA is extracted and quantified.

In this work, semi-quantitative PCR was used to evaluate and compare the binding of PC, PHO, and TRX proteins to the repressed and the transcribed *Fab-7* PRE on the pFAs-1 transgene. To exclude adulteration of the results caused by the endogenous *Fab-7* sequence in the BX-C, the pFAs-1 transgenic line containing the *actin5C* promoter and the pFAs-1 transgenic line without the *actin5C* promoter were crossed to the *Fab-7*^{1} line. This line carries a 4kb deletion in the BX-C, and thus lacks the complete *Fab-7* sequence (Gyurkovics et al. 1990). Importantly, the absence of the endogenous *Fab-7* PRE showed no effect in *trans* on the eye pigmentation of the pFAs-1 transgenic flies (data not shown). Thus, in contrast to a previous study (Bantignies et al. 2003), the silencing function of the transgenic *Fab-7* PRE as well as the anti-silencing effect caused by transcription through this element are independent of the endogenous *Fab-7* PRE in the BX-C.

Fig. 2.17 shows a scheme of the pFAs-1 construct with the position of the analyzed PCR fragments indicated by black lines. Within the *Fab-7* element, the first fragment analyzed is located at the proximal end (prox). The second fragment lies within a region that has genetically been shown to function as a boundary element (bound; (Mihaly et al. 1997), and the third fragment maps to the „core“ PRE fragment (PRE) (Mihaly et al. 1997; Mishra et al. 2001; Déjardin and Cavalli 2004). In addition to the *Fab-7* element, primer detecting enrichment of the PC, PHO, and TRX proteins near the minimal *hsp70* promoter regulating the *lacZ* gene (*lacZ*), and in the promoter of the *miniwhite* gene (white) were used. Primer

amplifying the highest-scoring PRE fragment of the endogenous *bxd* element (bxd) (Horard et al. 2000; Ringrose et al. 2003) served as a positive control, whereas a PCR fragment in the *rosy* promoter was used as a negative control (ry). As starting material for the ChIP analysis, 4-20h old embryos were collected to enrich for cells in which the PcG/TrxG memory system has taken over the control of endogenous gene expression (Orlando et al. 1998).

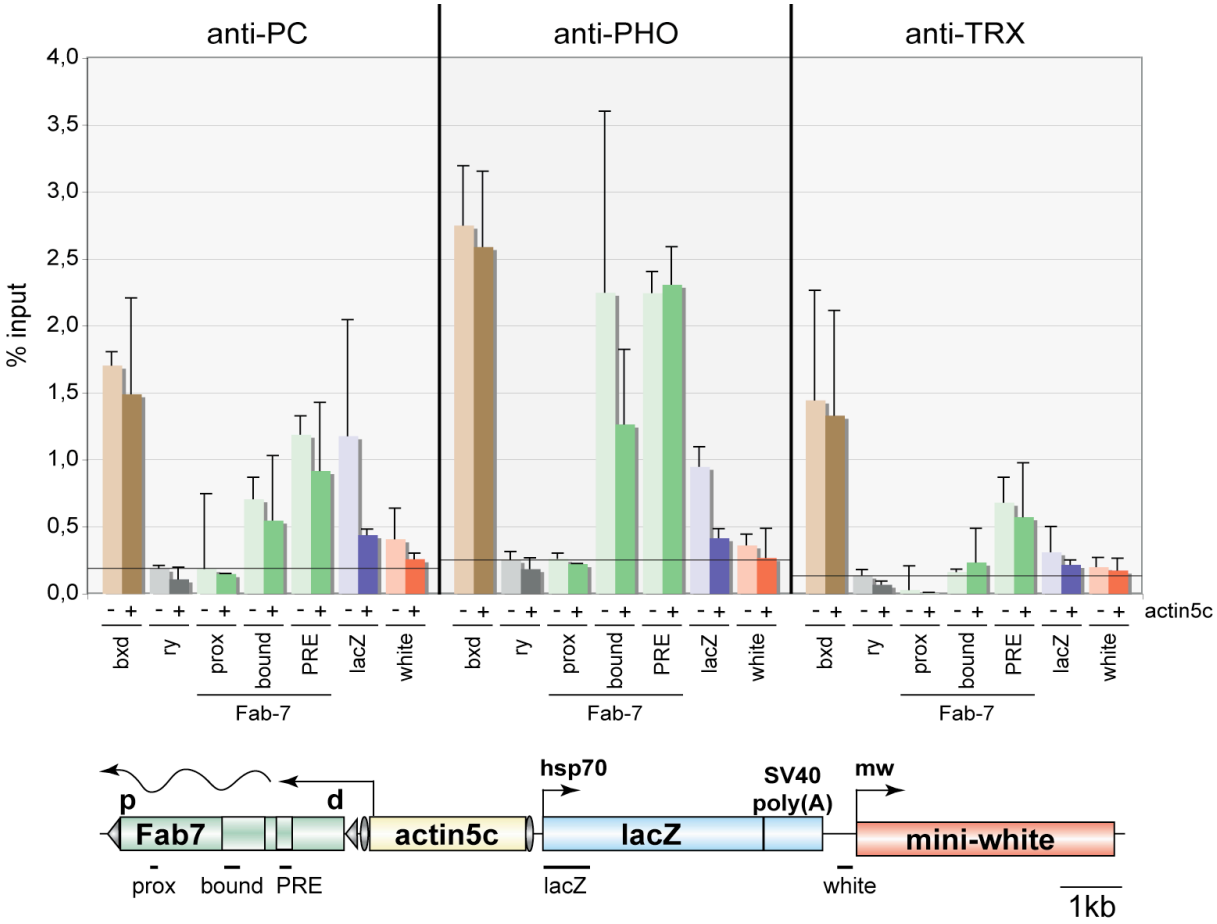

Figure 2.17: ChIP analysis of PC, PHO, and TRX binding to the pFAs-1 transgene. The graph shows the enrichment of immunoprecipitated material as percentage of input material. At the bottom, a scheme of the pFAs-1 transgene shows the position of PCR fragments analyzed denoted as black lines below. A fragment within the *bxd* PRE (brown bars) and one in the *ry* promoter (grey bars) served as positive and negative controls, respectively. Within the *Fab-7* PRE, three fragments were analyzed (green): one located in the proximal region of the element (prox), one localized to the boundary element (bound), and one mapping to the „core" PRE (PRE). Enrichment near the *hsp70* promoter was detected using primer amplifying a fragment distal to this promoter (blue); the last primer pair mapped to the *miniwhite* promoter (red). On the left part of the graphic, enrichment at all fragments following PC immunoprecipitation from pFAs-1 transgenic embryos lacking (-) or carrying the *actin5C* promoter on the transgene (+) are depicted. The middle part represents the enrichments obtained from PHO immunoprecipitation, and the right part illustrates the values obtained from immunoprecipitation with TRX antibodies.

The Chromatin Immunoprecipitation and PCR analysis of PC binding to the pFAs-1 transgene showed that PC was enriched above background in the boundary and PRE regions, but not at the proximal fragment (Fig. 2.17). The levels of PC association with these fragments did not change upon transcription from the *actin5C* promoter (Fig. 2.17). In addition, PC had become

recruited near the minimal *hsp70* promoter and the promoter of the *miniwhite* gene when the associated *Fab-7* PRE functioned as a silencer. Interestingly, the association of PC with the chromatin near the *hsp70* promoter decreased slightly when the *actin5C* promoter was present on the transgene (Fig. 2.17). Since the standard deviation at this fragment was relatively high, it is however not clear if this decrease is really significant. At the *miniwhite* promoter, no change in PC recruitment was detected when the *actin5C* promoter was present, although one has to consider that the enrichment at this fragment was in general very low and only slightly above the background (compare ry with white in Fig. 2.17).

The analysis of the levels of PHO binding to the pFAs-1 transgene gave a similar picture: This protein was also only enriched at the *Fab-7* boundary and PRE fragments, but not in the proximal region (Fig. 2.17). Similar to PC, PHO remained bound to *Fab-7* to a similar degree when the PRE was transcribed from the *actin5C* promoter. In this case, a slight decrease in the association of PHO with the chromatin was only detected near the *hsp70* promoter (Fig. 2.17).

In general, TRX showed only moderate enrichment on the chromatin at the transgenic *Fab-7* PRE and near the *hsp70* and *miniwhite* promoters (Fig. 2.17). As described for PC and PHO, TRX is associated with the *Fab-7* element to the same levels independent of the epigenetic state of this PRE. Similarly, no change in TRX binding near the *hsp70* promoter and at the *miniwhite* promoter was detectable.

Consistent with the results from the analysis of polytene chromosomes, the ChIP data suggest that also in a population of diploid cells, anti-silencing by transcription through *Fab-7* does not impair the binding of PC and PHO silencing proteins to this PRE on a transgene. Furthermore, the binding of TRX to the chromatin does not prevent the *Fab-7* PRE from repressing its target genes. However, the presence of the *actin5C* promoter on the transgene led to a slight decrease in the association of PC and PHO near the *hsp70* promoter, although it is possible that this might be caused by the increased distance of the *hsp70* promoter to the *Fab-7* PRE (from 1.4kb to 4kb), rather than truly reflect the relief of silencing due to transcription through the PRE.

2.2.5 The histone variant H2Av is not directly linked with the PcG/TrxG memory system

Although only a few candidates have been tested, the above results suggest that the association of PcG and TrxG proteins with the transgenic *Fab-7* element does not determine the epigenetically repressed, nor the activated state of this PRE. As the transcription through

the *Fab-7* PRE does not lead to the displacement of PC and PHO proteins from the chromatin, how is this anti-silencing function achieved mechanistically? Apart from the recruitment of chromatin-associated proteins, it has become increasingly clear that the incorporation of histone variants plays an important role in the regulation of the chromatin structure (Henikoff et al. 2004; Henikoff and Ahmad 2005). Due to its proposed role in PcG-mediated silencing (Swaminathan et al. 2005), the histone H2A variant H2Av (H2AZ in mammals) was an attractive candidate for such a function.

To test the involvement of H2Av in the regulation of the epigenetic state of the transgenic *Fab-7* PRE, the pFAs-1 line containing or lacking the *actin5C* promoter was crossed to a transgenic line expressing an H2Av-GFP fusion protein under the control of the endogenous *H2Av* promoter region. This construct has previously been shown to rescue H2Av mutations, indicating that the fusion protein is functional and can substitute for the wildtype H2Av variant (Clarkson and Saint 1999).

To determine if the H2Av-GFP fusion protein is specifically deposited at the transgenic *Fab-7* PRE, larval polytene chromosomes were stained with α-GFP antibodies. Since the combination of GFP immunolocalization with DNA FISH did not lead to satisfying results, co-immunostaining for PC was used to unambiguously identify the site of transgene insertion. As shown above, when *Fab-7* was transcribed from the *actin5C* promoter, PC was bound to the site of transgene insertion (Fig. 2.18E-H). Consistent with a suggested role of H2Av in PcG-mediated silencing (Leach et al. 2000; Swaminathan et al. 2005), there was no enrichment of the H2Av-GFP fusion protein detectable at the activated PRE (Fig. 2.18E-H). However, when the *Fab-7* PRE was not transcribed and thus functioned as a silencer, there was still no specific incorporation of H2Av-GFP detectable at the site of transgene insertion (Fig. 2.18A-D). Immunostaining of control larvae showed that this site was also devoid of H2Av-GFP in the wildtype (Fig. 2.18I-L). These results suggest that at least in the transgenic situation, the H2Av isoform has no direct role in the inheritance of transcriptionally silent chromatin, nor does it act as an „anti-silencer“ at the transcribed *Fab-7* PRE.

It is possible that the enrichment of H2Av-GFP at the pFAs-1 transgene might have been too low to be detectable by immunostaining of polytene chromosomes. However, the investigation of the ANT-C and BX-C showed that even at these large gene complexes containing multiple PREs, H2Av-GFP was completely absent (Fig. 2.18M and N). The same was true for other predicted endogenous PREs, such as the one at the *salm* locus, to which the PcG proteins PC and PHO, as well as the TrxG protein TRX became robustly recruited (see asterisk in Fig. 2.18A-L and Fig. 2.14-2.16).

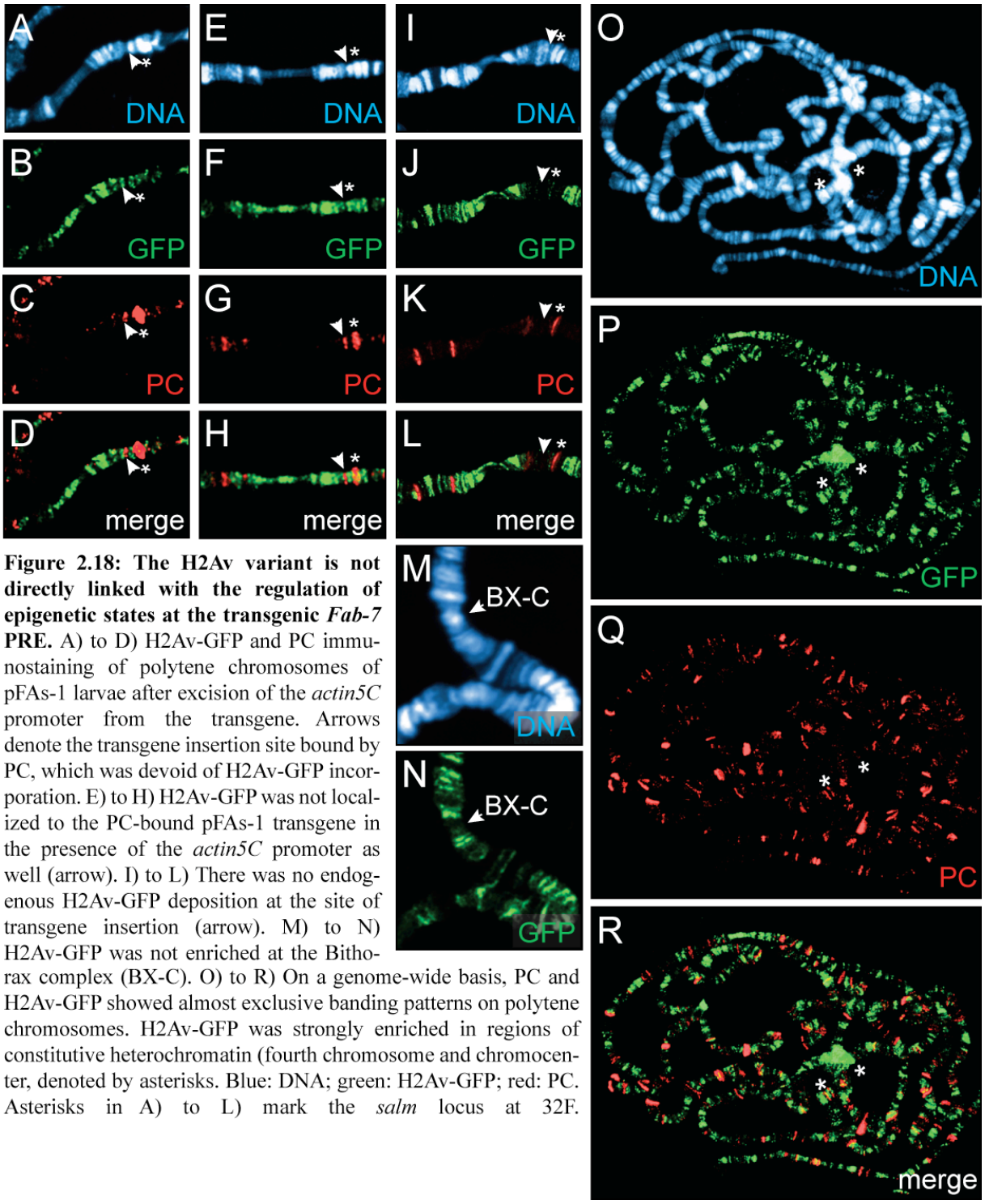

Figure 2.18: The H2Av variant is not directly linked with the regulation of epigenetic states at the transgenic *Fab-7* PRE. A) to D) H2Av-GFP and PC immunostaining of polytene chromosomes of pFAs-1 larvae after excision of the *actin5C* promoter from the transgene. Arrows denote the transgene insertion site bound by PC, which was devoid of H2Av-GFP incorporation. E) to H) H2Av-GFP was not localized to the PC-bound pFAs-1 transgene in the presence of the *actin5C* promoter as well (arrow). I) to L) There was no endogenous H2Av-GFP deposition at the site of transgene insertion (arrow). M) to N) H2Av-GFP was not enriched at the Bithorax complex (BX-C). O) to R) On a genome-wide basis, PC and H2Av-GFP showed almost exclusive banding patterns on polytene chromosomes. H2Av-GFP was strongly enriched in regions of constitutive heterochromatin (fourth chromosome and chromocenter, denoted by asterisks. Blue: DNA; green: H2Av-GFP; red: PC. Asterisks in A) to L) mark the *salm* locus at 32F.

The exclusive binding of PC and H2Av-GFP became even more striking when comparing the genome-wide distribution of these two proteins on complete polytene chromosome spreads (Fig. 2.18O-R). As has been described previously for PH (Leach et al. 2000), there was almost no overlap detectable between H2Av-GFP and PC.

These results indicate that the observed genetic interaction of PcG and TrxG components with H2Av (Leach et al. 2000; Swaminathan et al. 2005) is likely to be caused by indirect effects, and is not due to a direct function of this histone variant in the regulation of the epigenetic states of PREs. Consistent with its described function in the formation of heterochromatin and HP1 recruitment (Swaminathan et al. 2005), the H2Av-GFP fusion protein was strongly enriched at the chromocenter and the heterochromatinized, largely silenced fourth chromosome (see asterisks in Fig. 2.18O-R).

2.3 Characterization of the non-coding PRE transcripts

The analysis of the transgenic pFAs and pFAas reporter lines has demonstrated that the non-coding transcription through *Fab-7* functions as an anti-silencing mechanism to prevent the establishment of PcG-mediated repression. Moreover, the analysis of several predicted PREs suggests a similar function of intergenic transcription outside the homeotic gene complexes. This raises the intriguing question whether this anti-silencing mechanism is solely dependent on the transcriptional process *per se*, or if the non-coding RNAs generated may play a structural role.

In the transgenic reporter system, the transcription of sense and antisense *Fab-7* RNA has a similar activating effect (Fig. 2.3). In addition, in early embryos, endogenous *Fab-7* transcription occurs in both orientations, whereas at later stages, only the sense transcript is generated. The predicted PREs at the *en, slou,* and *salm* loci are transcribed bi-directionally (Fig. 2.12), wheras the *bxd* and *Mcp* PREs, as well as the predicted *tll* PRE are transcribed in one direction only (figs. 2.11 and 2.12; Rank et al. 2002). Thus, transcription of only one strand is sufficient to prevent silencing at some PREs, whereas transcription of both strands may be required at other loci.

How can the fact that the anti-silencing function by transcription through a PRE is not strand-specific comply with the idea that the non-coding RNA generated during this process might have a structural function? One possibility is that the generation of a sufficient amount of non-coding RNA *per se* may influence the efficiency of PcG-mediated repression at a PRE. To tackle such a difficult question, it is important to know more about the properties of the endogenous transcription through PREs and the corresponding non-coding RNAs generated. As to the process of non-coding transcription, previous results suggest that this is catalyzed by the cellular RNA polymerase II machinery, since endogenous intergenic transcripts in the

BX-C are spliced and enriched in the poly(A)$^+$ fraction of RNA originating from wildtype embryos (Lipshitz et al. 1987; Cumberledge et al. 1990; Rank et al. 2002). Similarly, using RT-PCR, non-coding transcripts spanning the predicted PREs at the *en, slou, salm,* and *tll* loci were detected in the polyadenylated fraction of embryonic RNA (data not shown).

2.3.1 Non-coding RNA spanning the Fab-7 PRE is less abundant than the mature AbdB mRNA

Within a developing embryo, the fraction of cells in which individual PREs are transcribed is very low, thus precluding the detailed molecular characterization of intergenic transcription as well as the non-coding RNAs generated. In order to have a more homogenous starting material, SF4 tissue culture cells (kindly provided by P. B. Becker) were chosen to further analyze the non-coding *Fab-7* transcripts. SF4 cells are a derivative of the original Schneider cell line SL-2, which has been isolated from late embryos and presumably originates from the blood cell lineage (Schneider 1972). Previous results from our lab have shown that the *AbdB* gene is active in SL-2 cells and that the endogenous *Fab-7* and *Mcp* PREs are also transcribed (Prestel 2003).

The RNA *in situ* hybridization experiments in wildtype embryos and third instar larval brains reproducibly resulted in much lower signals when detecting intergenic transcripts spanning PREs compared to the corresponding mRNAs (Fig. 2.11 and Fig. 2.12). This suggests that the non-coding RNAs are much less abundant than the respective target mRNAs. Real-time RT-PCR on total RNA isolated from SF4 cells was performed in order to test if this is also true in tissue culture cells and to quantify the differences.

To detect transcripts spanning the *Fab-7* PRE, primer specifically amplifying the „core“ PRE within the *Fab-7* region were designed (Mishra et al. 2001; Déjardin and Cavalli 2004). The mature *AbdB* mRNA was quantified by choosing primer specifically amplifying the last exon of *AbdB* shared by all of the five alternative *AbdB* transcripts, whereas a fragment located in the second last intron shared by all *AbdB* isoforms was used to detect all nascent *AbdB* pre-mRNAs (Zavortink and Sakonju 1989); Flybase: http://www.flybase.org/cgi-bin/gbrowse_fb/dmel?id=FBgn0000015;chr=3R).

Fig. 2.19 shows that the mature *AbdB* mRNA was around 59fold more abundant than the intron fragment of the nascent *AbdB* pre-mRNA. This was expected, because the intron of the *AbdB* pre-mRNA is excised during splicing and subsequently degraded, whereas the exon fragment is part of the mature *AbdB* mRNA which is translocated to the cytoplasm where translation takes place (Aguilera 2005). As the rate of transcription for both the *AbdB* exon

and intron fragments are identical, the 59fold higher steady-state level of mature *AbdB* mRNA can be attributed to a higher stability in comparison with the intron.

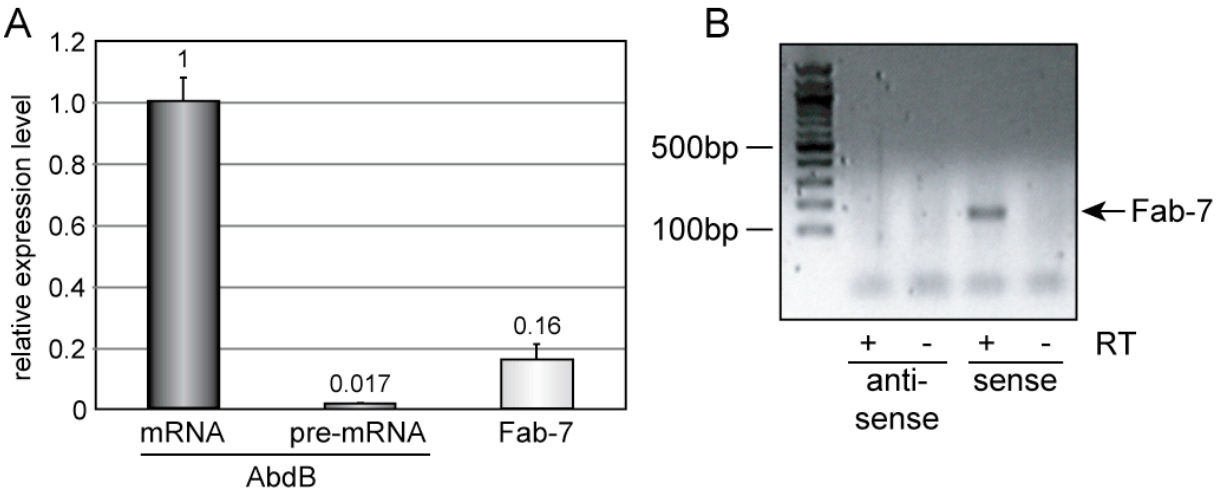

Figure 2.19: Relative levels of *AbdB* mRNA and non-coding *Fab-7* RNA in SF4 tissue culture cells. A) Real-time RT-PCR on total RNA revealed that the mature *AbdB* mRNA (detected by an exonic fragment) was approximately 59fold more abundant than a corresponding intronic fragment of the *AbdB* pre-mRNA. In relation to this, the steady-state level of *Fab-7* RNA was approximately 6fold lower than that of the *AbdB* exon fragment, but around 9fold higher than the level of *AbdB* pre-mRNA (intron). B) RT-PCR using strand-specific primer during the cDNA synthesis demonstrated that only the sense strand of *Fab-7* was transcribed in SF4 cells.

As expected from the RNA *in situ* hybridization data, the non-coding *Fab-7* RNA in SF4 cells was approximately 6fold less abundant than the mature *AbdB* mRNA (Fig. 2.19A). Interestingly, the *Fab-7* transcript levels were around 9fold higher than the level of the *AbdB* intron fragment (Fig. 2.19A). Although previous experiments suggest that the non-coding *Fab-7* RNA is processed (Gerhard Rank, personal communication), the splicing pattern of this RNA is not known. Thus, it is not clear if this higher level of *Fab-7* RNA compared to the *AbdB* intron fragment was caused by a higher stability of the non-coding transcript, a different rate of transcription, or a combination of both.

As mentioned before, the *Fab-7* PRE is transcribed in both orientations during early embryogenesis, whereas toward later stages, sense transcription prevails. As random hexamer primer were used for the cDNA synthesis in the real-time RT-PCR analysis, these experiments did not distinguish between sense and antisense transcription. To exclude that the higher level of *Fab-7* non-coding RNA compared with the *AbdB* intron fragment might be due to the bi-directional transcription through *Fab-7*, RT-PCR on total RNA isolated from SF4 cells was performed, this time using strand-specific primer for the cDNA synthesis reaction. Agarose gel electrophoresis of the PCR reactions indeed showed that as in later stages during wildtype *Drosophila* development, transcription through *Fab-7* in SF4 cells was detectable in the sense direction only (Fig. 2.19B).

2.3.2 Non-coding Fab-7 RNA is more stable than AbdB mRNA

The above experiments illustrated that although the steady-state level of *Fab-7* RNA was 6fold lower than the level of mature *AbdB* mRNA, it was still approximately 9fold higher than the nascent *AbdB* pre-mRNA. In order to determine whether these differences in steady-state RNA levels are due to differences in transcription rates, or in the stabilities of the RNAs themselves, the half-life of each of the individual RNA fragments was determined. To do this, the relative levels of each RNA at different time points following transcriptional arrest by incubation with Actinomycin D (Leclerc et al. 2002) were quantified.

Semi-quantitative RT-PCR of total RNA prepared from thus treated cells demonstrated that after 8hours, the mature *AbdB* mRNA was almost completely degraded, whereas non-coding *Fab-7* RNA could be faithfully detected throughout the time-course (Fig. 2.20).

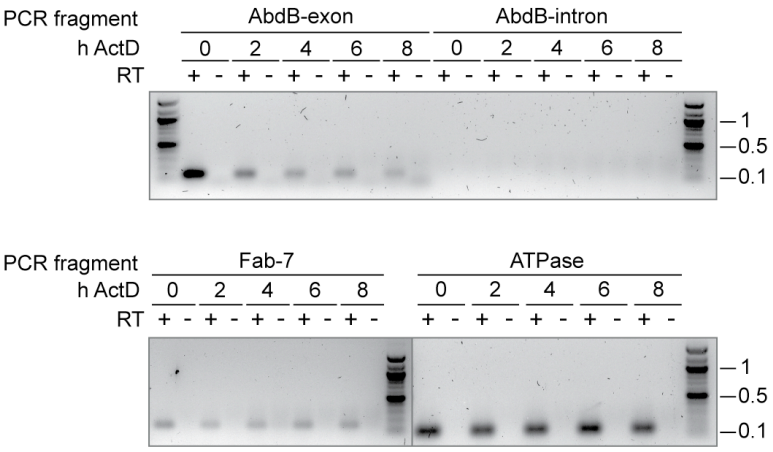

Figure 2.20: RNA stabilities measured by RT-PCR following transcriptional arrest. Total RNA isolated from cells incubated with actinomycin D (ActD) for 0-8h was used for RT-PCR. As negative controls, parallel RT-PCR reactions in which the reverse transcriptase was omitted were performed (-RT). Mature *AbdB* mRNA was degraded during the 8h time course, whereas *Fab-7* RNA and *ATPase* mRNA could be detected throughout the time-course. *AbdB* intronic fragments were below the detection level of this method.

The amount of *AbdB* intron fragment was below the detection level of this assay, and could thus not be determined (Fig. 2.20). As an internal control for the efficiency of cDNA synthesis, PCR primer amplifying the mRNA encoding a mitochondrial ATPase (ATPase cf6), which proved to be a very stable control in previous real-time RT-PCR experiments, were used (Fig. 2.20; Roustan-Espinosa 2005).

To quantify the relative amounts of *AbdB* exon and intron, and the *Fab-7* RNA following transcriptional arrest by Actinomycin D more precisely, real-time RT-PCR analysis was used. By plotting the relative RNA levels as a function of Actinomycin D incubation time, the half-lives of each of the RNAs tested were estimated (Fig. 2.21).

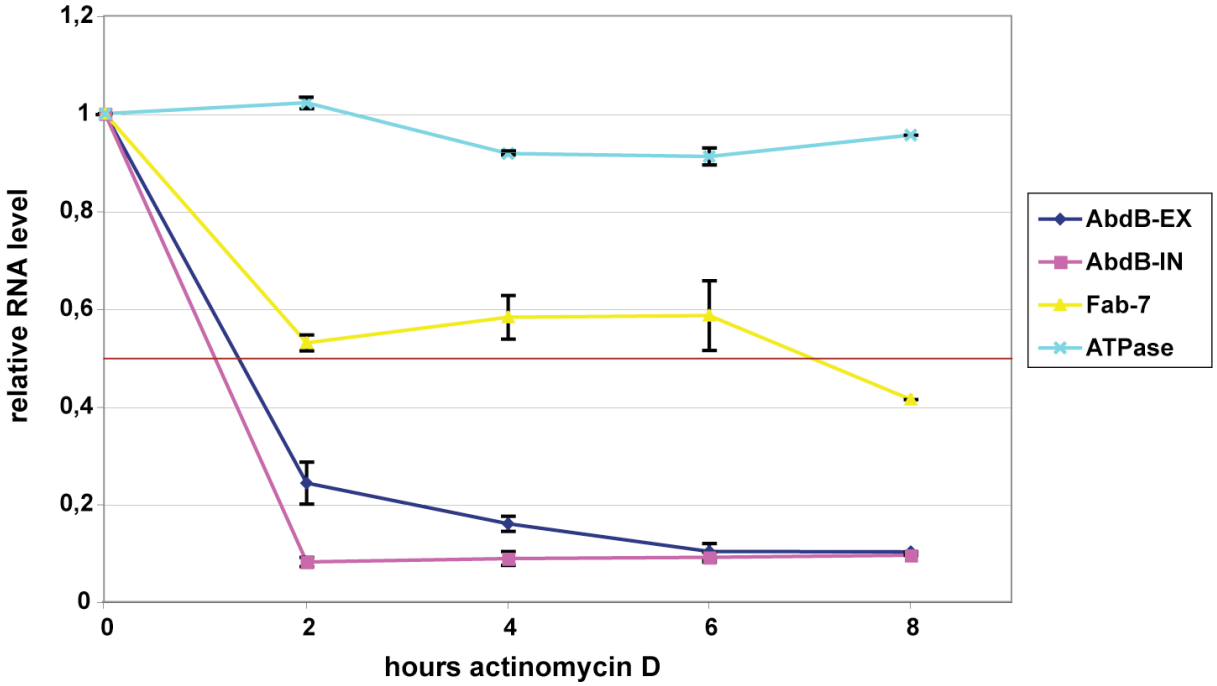

Figure 2.21: Relative RNA levels following transcriptional arrest by Actinomycin D. Plotted are the percentages of steady-state RNA levels in relation to the starting amount before addition of Actinomycin D. Red line indicates 50% of starting levels. Half-lives of *AbdB* exonic and intronic fragments, as well as of the non-coding *Fab-7* RNA were estimated by determining the time point at which approximately 50% of starting amounts of each RNA were left.

As expected, the *AbdB* intron fragment was the least stable with a half-life of approximately 1.1h. The *AbdB* exon fragment was only slightly more stable and had a half-life of around 1.3h. As the *AbdB* intron and exon fragments should be derived from identical transcripts, their rates of transcription should also be identical. This means that the 59fold higher steady-state levels of mature *AbdB* mRNA compared with the intronic *AbdB* fragment (see Fig. 2.19) are due to a relatively low difference in stability.

In relation to this, the non-coding *Fab-7* RNA with a half-life of approximately 2.5h (Fig. 2.21) was 2.3fold more stable than the *AbdB* intron. This demonstrates that the 9fold higher level of *Fab-7* RNA compared with the *AbdB* intron fragment was at least partly caused by the higher stability of the non-coding RNA. However, although the *Fab-7* RNA fragment tested was more stable than the mature *AbdB* mRNA, it was approximately 6fold less abundant. Thus, the observed differences in steady state levels are presumably caused by a combination of an increased stability of non-coding *Fab-7* RNA with differences in the rates of transcription. It is interesting to note that the kinetics of *Fab-7* RNA degradation differ from the kinetics of *AbdB* mRNA degradation. The level of *Fab-7* RNA decreases dramatically during the first two hours, but shows only a moderate decline during the rest of the time-course, wheras the level of *AbdB* mRNA decreases continually. Thus, the non-coding

Fab-7 RNA might exist in two distinct pools; one that degrades very fast, and a second pool that seems to be more stable.

These results suggest that intergenic transcripts spanning the *Fab-7* PRE are not immediately degraded following their synthesis, which is consistent with the notion that the non-coding *Fab-7* RNA might be functionally involved in counteracting PcG-mediated silencing.

2.3.3 Non-coding transcripts spanning PREs are localized to discrete spots within the nucleus

If the intergenic RNAs generated by transcription through PREs have a function in this anti-silencing mechanism, one would predict that they would stay associated with the chromatin. Indeed, RNA *in situ* hybridizations in embryos and third instar larvae indicated that in contrast to the homogeneously distributed mRNAs, the intergenic transcripts appeared in a weaker, „dot-like“ pattern (Fig. 2.11 and 2.12; data not shown). To be able to visualize the localization of non-coding transcripts with a higher resolution, a fluorescent RNA *in situ* hybridization (RNA FISH) protocol was established. As expected, *AbdB* mRNA showed a diffuse cytoplasmic localization in PS10-14 of stage 10 wildtype embryos, with increasing levels toward the posterior-most segment (Fig. 2.22A-A’’’).

In contrast, *Fab-7* sense RNA was restricted to PS12-14, the only region in the embryo in which this PRE is in the epigenetically active state and thus does not function as a silencer. Moreover, the RNA signals indeed appeared in a dotted pattern, and close inspection showed that these dots were restricted to the cell nuclei (Fig. 2.22B-B’’’).

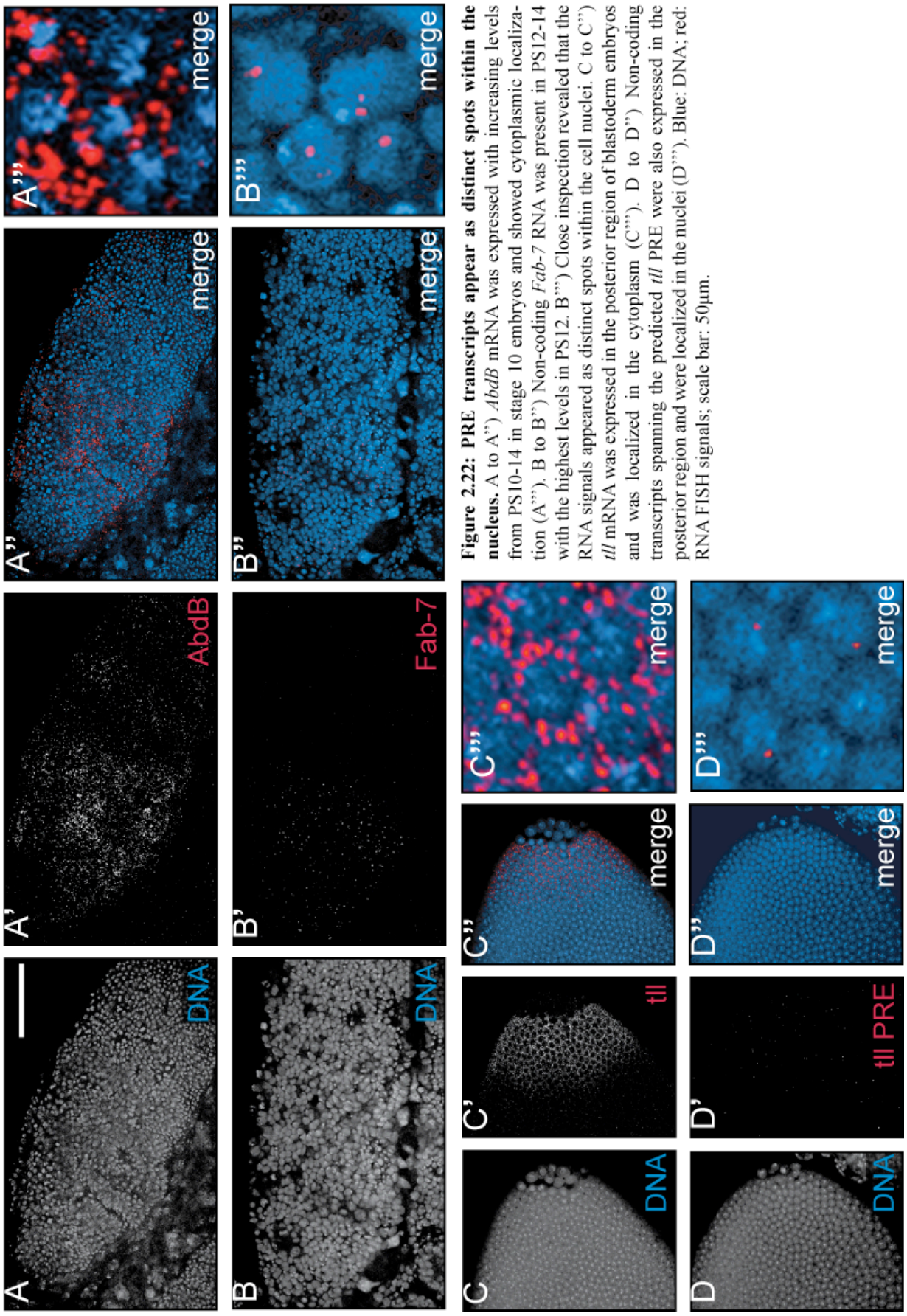

Figure 2.22: PRE transcripts appear as distinct spots within the nucleus. A to A''') *AbdB* mRNA was expressed with increasing levels from PS10-14 in stage 10 embryos and showed cytoplasmic localization (A'''). B to B''') Non-coding *Fab-7* RNA was present in PS12-14 with the highest levels in PS12. B''') Close inspection revealed that the RNA signals appeared as distinct spots within the cell nuclei. C to C''') *tll* mRNA was expressed in the posterior region of blastoderm embryos and was localized in the cytoplasm (C'''). D to D''') Non-coding transcripts spanning the predicted *tll* PRE were also expressed in the posterior region and were localized in the nuclei (D'''). Blue: DNA; red: RNA FISH signals; scale bar: 50µm.

A similar result was obtained when assessing the localization of non-coding *bxd* and *Mcp* RNAs (data not shown). Consistent with the idea that anti-silencing by transcription through PREs might be a fundamental aspect of the PcG/TrxG memory system, intergenic antisense transcripts spanning the predicted *tll* PRE also appeared as distinct spots within the nuclei (Fig. 2.22D-D'''), while the *tll* mRNA was evenly distributed in the cytoplasm (Fig. 2.22C-C'''). Although it is entirely feasible that the nuclear RNA signals represent only nascent transcripts, these results do not exclude the possibility that non-coding PRE transcripts remain associated with the chromatin following their production.

2.3.4 Fab-7 sense and antisense transcripts can be found within the same nucleus

Previous results and data from the present study showed that while in late embryos and during the third larval stage, sense transcription through the *Fab-7* PRE prevails (Fig. 2.11), both sense and antisense transcription occurs during early embryogenesis (Fig. 2.23A and B; Rank 2002). As both sense and antisense transcripts are found in overlapping domains within the embryo, this raised the question whether this reflects a true simultaneous bi-directional transcription, or if sense and antisense transcription are spatially separated among the two homologous chromosomes or even occur in different cells.

In order to test this, DIG- and fluorescein-labeled RNA probes were generated to simultaneously detect the sense and antisense strand of the *Fab-7* RNA in adjacent fragments. Fig. 2.23C-C'' and D-D'' show the expected RNA FISH signals obtained from each of the sense and antisense RNA probes, respectively. In general, these signals were weaker than the *Fab-7* RNA FISH signals in the previous experiments, in which the templates used for the generation of RNA probes were considerably longer (3.6kb as opposed to 1.5 and 1kb).

In the double RNA FISH analysis, sense and antisense *Fab-7* transcripts were detected simultaneously only within a low fraction of nuclei (25 nuclei in 8 embryos). However, it has to be taken into consideration that these pictures represent single confocal sections. In most of the cases in which *Fab-7* transcripts in both orientations were present, these signals were found to be localized in different subnuclear domains (23/25; Fig. 2.23E and F). Co-localization of sense and antisense *Fab-7* RNA occurred only in 2/25 nuclei, and these signals were very weak (Fig. 2.23G).

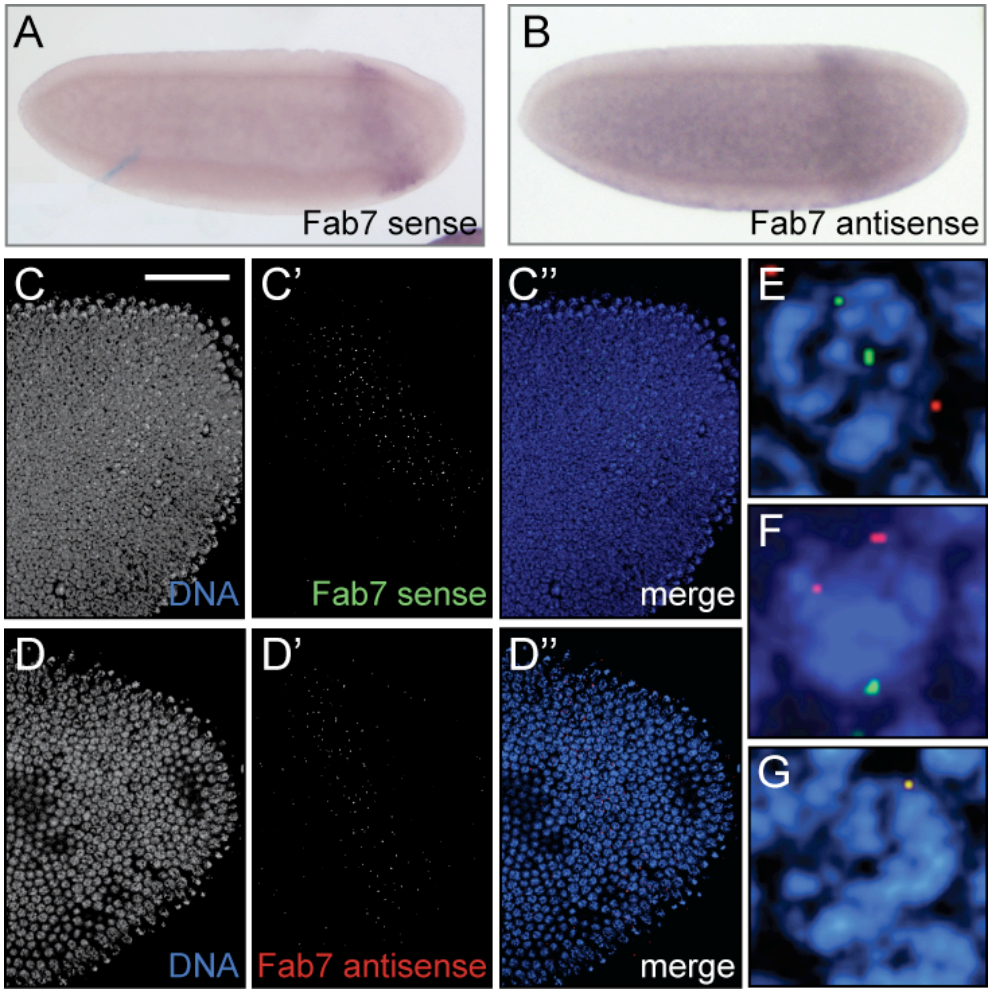

Figure 2.23: *Fab-7* sense and antisense transcripts can be found simultaneously in single nuclei. A) and B) RNA *in situ* hybridization showed that *Fab-7* sense and antisense RNAs, respectively, were expressed in overlapping domains in early embryos. C) to C'') show the fluorescent RNA signal obtained when detecting the *Fab-7* sense strand by RNA FISH. D) to D'') RNA FISH signals obtained using a probe detecting *Fab-7* antisense RNA. E) and F) show examples in which *Fab-7* sense (green) and antisense (red) RNA signals were observed in different subnuclear localizations within single nuclei. G) In rare cases, *Fab-7* sense and antisense transcripts were found to co-localize within a single nucleus. DNA is depicted in blue. Scale bar: 50µm.

As the half-life of at least the sense *Fab-7* RNA is approximately 2.5h (Fig. 2.21), it is possible that these rare events of sense and antisense RNA co-localization represent transcripts which have been synthesized at different times, but stay associated with their site of production long enough to allow their simultaneous detection. Taken together, these results suggest that the Fab-7 PRE is not simultaneously transcribed in both directions.

2.3.5 Non-coding Fab-7 RNA can be detected in mitotic nuclei

The previous RNA in situ hybridization experiments demonstrated that PREs are transcribed in the brains of third instar larvae (see Fig. 2.11). Together with the observation that limiting the time frame of transcription through the transgenic *Fab-7* PRE led to a re-silencing of the *miniwhite* gene in rare cases, this suggests that transcription through PREs may be required

throughout development to maintain the chromatin in a transcriptionally competent state. One hypothesis arising from this is that the transcription through a PRE may be required at least once every cell cycle, which would result in the epigenetic „bookmarking“ of the chromatin structure, thus ensuring the mitotic transmission of the activated state.

One approach to test this is to assess the pattern of non-coding transcription through a PRE and relate it to different cell cycle phases. One critical cell cycle stage through which the epigenetic state of a PRE has to be transmitted is mitosis. It has been shown that during the pro- and metaphases of mitosis, RNA polymerase II transcription is largely shut down, but processive transcription at a few loci becomes detectable again at the anaphase-telophase transition (Chen et al. 2005).

To determine whether non-coding *Fab-7* RNA is present on mitotic chromatin, *Fab-7* RNA FISH was combined with α-tubulin immunostaining, which should allow the identification of mitotic nuclei in wildtype *Drosophila* embryos (Foe 1989).

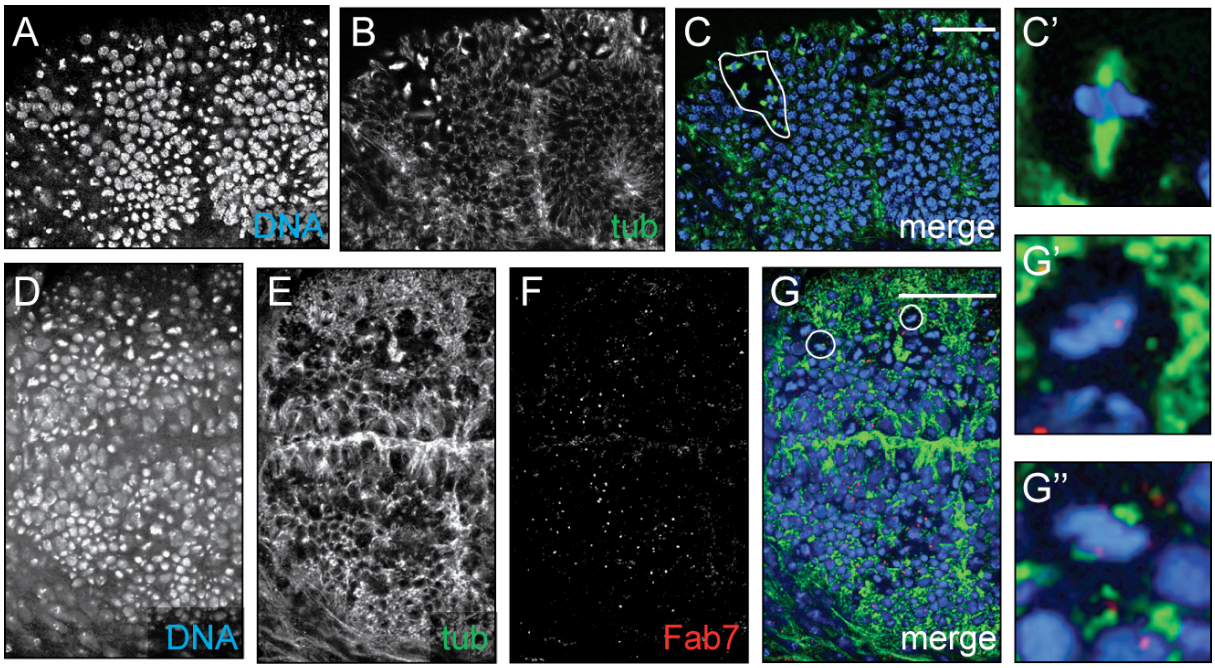

Figure 2.24: *Fab-7* RNA can be found associated with mitotic chromatin. A) to C) α-tubulin immunostaining identified mitotic domains in posterior body segments of stage 9-10 embryos. The magnification (C') shows that mitotic nuclei could easily be distinguished by their condensed appearance and association with spindle microtubuli visualized by the immunostaining. D) to G) Combining the α-tubulin immunolocalization with *Fab-7* RNA FISH allowed the simultaneous detection of non-coding *Fab-7* RNA and mitotic nuclei. G') and G'') show magnifications of two examples of mitotic nuclei, in which *Fab-7* RNA could be detected. Blue: DNA; green: α-tubulin; red: *Fab-7* RNA. Scale bars: 50µm.

Fig. 2.24A-C' show that mitotic nuclei, in which the condensed chromatin is associated with the mitotic spindle microtubuli, can be faithfully detected by α-tubulin immunostaining. Due to the limited preservation of proteins throughout the RNA FISH procedure, the simultaneous

detection of *Fab-7* RNA and identification of mitotic nuclei by α-tubulin immunostaining proved to be more difficult. Fig. 2.24D-G show that although in general, α-tubulin was detectable following the *Fab-7* RNA FISH, mitotic spindle tubuli were not stable enough to survive the procedure in most cases. However, mitotic nuclei could still be identified due to the compaction of the chromatin and the lack of homogeneous cytoplasmic α-tubulin distribution (compare Fig. 2.24C with G). In most of the thus identified mitotic nuclei, there was no *Fab-7* RNA detectable. However, in a very few cases, weak *Fab-7* RNA FISH signals were present on mitotic chromosomes (Fig. 2.24 G' and G'').

These results raise the intriguing possibility that the non-coding *Fab-7* RNA might remain associated with mitotic chromatin, and that this may be required for the transmission of the activated epigenetic state at the *Fab-7* PRE through cell division.

Discussion

3. Discussion

PREs are a key component of the cellular memory system required for the maintenance of determined cell fates throughout development. Through the targeting of PcG and TrxG complexes to the chromatin, PREs mediate the epigenetic inheritance of both repressed and active transcriptional states of target genes, respectively. The default state of a PRE is to function as a silencer. As a consequence, the inheritance of active chromatin states requires a mechanism that counteracts this repression.

In this thesis, the non-coding transcription through PREs themselves has been identified to form the basis of such an anti-silencing mechanism. The continuous transcription through the *Fab-7* PRE on a transgene prevents the establishment of PcG-mediated repression, and renders the chromatin in a conformation permissive for the expression of target genes. In the context of the transgene, the PcG proteins PC and PHO, and the TrxG protein TRX associate with the repressed as well as with the transcribed *Fab-7* PRE. The epigenetic state of a PRE may therefore primarily be regulated through the modulation of PcG/TrxG activites, rather than by their differential association with the chromatin.

To get insight into the mechanism behind the anti-silencing function of transcription through PREs, the non-coding RNAs generated during this process were analyzed. The results show that *Fab-7* RNA is fairly stable and can even be found associated with mitotic chromatin. This raises the possibility that non-coding RNAs generated at PREs might be an intergal part of the mechanism that counteracts the silencing functions of PcG complexes.

3.1 Transcription through PREs functions as an anti-silencing mechanism

3.1.1 The transgenic reporter system reveals a novel function of non-coding transcription

PREs have been initially identified through genetic analysis in the homeotic BX-C. In conjunction with nearby boundary elements, PREs separate adjacent regulatory domains from one another and are responsible for maintaining the expression patterns of homeotic genes established by a given PS-specific regulatory domain throughout development (Mihaly et al.

1998). Previous studies have shown that regulatory regions spanning PREs are transcribed in the same colinear fashion as the homeotic genes they regulate: More proximal domains in the BX-C are transcribed in more anterior cells within the embryo (Sanchez-Herrero and Akam 1989; Cumberledge et al. 1990; Rank et al. 2002; Bae et al. 2002). The main objective of this PhD project was to determine the functional significance of this non-coding transcription. Is it causally related with the switch of a PRE into the activated mode, or does it reflect fortuitous transcription as a consequence of the activation of the chromatin structure?

To address this question, a transgenic reporter system was established. The presence of a PRE on a transgene typically results in the repression of adjacent reporter genes through the targeting of PcG complexes. If the transcription through a PRE induces the epigenetic activation of this element, it should no longer be able to function as a silencer. The degree of epigenetic silencing imposed by a PRE depends on its chromosomal environment. To circumvent such position effects, a strategy was used that allowed the analysis of reporter gene expression when the nearby *Fab-7* PRE was transcribed, and compare it to the situation when the PRE was not transcribed, at the identical insertion site of the transgene. As a read-out of the epigenetic state of the *Fab-7* PRE, the well-established *miniwhite* transformation marker was used. The level of *miniwhite* expression is very sensitive to its chromosomal environment, and the degree of eye pigmentation can be measured over a wide range of intensities, making this gene a very suitable indicator for epigenetic transcriptional control.

To determine if the constitutive transcription through *Fab-7* leads to the epigenetic activation of this PRE, the *actin5C* promoter was used. In the absence of transcription, the *Fab-7* PRE strongly repressed *miniwhite* expression, and as expected, the degree of silencing varied considerably depending on the insertion site of the transgene (see Fig. 2.3). Forced transcription through *Fab-7* from the constitutive *actin5C* promoter completely reverted this repression. The level of eye pigmentation in this situation was almost identical to the eye colour of transgenic flies carrying no PRE on the transgene. This shows that transcription through *Fab-7* resulted in a relief of silencing, without directly activating the expression of *miniwhite* above the level that was typical for the insertion site of the transgene. The analysis of the pFTA and pFLA transgenic lines (see Fig. 2.5) showed that this relief of silencing requires the transcriptional machinery to pass through the *Fab-7* PRE, and that the recruitment of transcriptional activators into its vicinity is not sufficient for this. This is in contrast to the stochastic repression of reporter genes caused by their integration into the vicinity of centromeric or telomeric heterochromatin (termed position-effect variegation, or

PEV). Silencing in this situation can be reverted simply through the recruitment of the GAL4 transcription factor to the transgene (Ahmad and Henikoff 2001).

Taken together, the non-coding transcription through PREs is not a consequence of gene activation, but forms the basis of a novel anti-silencing mechanism. Presumably, the spatially and temporally regulated transcription of non-coding RNAs in the BX-C described previously induces a remodeling of the chromatin structure, thereby rendering it refractory to epigenetic silencing by PcG proteins. The consequence of this is the PS-specific activation of *Hox* genes along the A-P body axis.

One prediction of such a model is that ectopic transcription through PREs should result in the activation of *Hox* gene expression outside their normal domains. Previous studies show that this is indeed the case: The insertion of promoters into the BX-C leads to homeotic gain-of-function phenotypes which are correlated with the appearance of intergenic transcripts in body segments, in which the corresponding regulatory regions are normally repressed (Bender and Fitzgerald 2002; Hogga and Karch 2002).

The non-coding transcription through PREs appears to be a central requirement for counteracting PcG silencing. With this in mind, the finding that a subset of TrxG components are involved in the global regulation of transcription can be reconciled with their specific genetic interactions with PcG genes (Kennison and Tamkun 1988). The Brahma (BRM) nucleosome remodeling complex, for example, largely overlaps with RNA polymerase II on polytene chromosomes, and its activity is required for the efficient recruitment of the transcriptional machinery to the chromatin (Armstrong et al. 2002). Similarly, the Kothalo (KTO) and Skuld (SKD) proteins are components of the Mediator complex, which stimulates basal transcription initiation by linking specific transcription factors to the core RNA polymerase II machinery (Boube et al. 2000; Treisman 2001). At PREs, the prevention of PcG-mediated silencing vitally depends on the efficiency of processive transcription through these elements themselves (Schmitt et al. 2005). As a consequence, all factors stimulating this process will antagonize the activities of PcG proteins. The PRE-specific functions of the BRM complex are presumably due to its direct interaction with the DNA-binding protein Zeste (Kal et al. 2000), while the factors responsible for KTO and SKD recruitment to PREs are not known.

3.1.2 Anti-silencing by transcription may be required throughout development

Intergenic transcription spanning PRE sequences in the BX-C is initiated early in nuclear division cycle 14, and thus shortly precedes the activation of homeotic gene expression

(Sanchez-Herrero and Akam 1989). This raised the hypothesis that non-coding transcription at this early stage might be sufficient to switch a PRE into the epigenetically activated mode. The analysis of the pFHs and pFHas transgenic lines has shown that this is not the case, since transcription through the *Fab-7* PRE from the zygotic *hunchback* promoter does not impair silencing of the miniwhite transformation marker (see Fig. 2.7). This suggests that although the transcription through the PRE at early stages is critical, it may be required for a longer time period. Indeed, when transcription through the *Fab-7* PRE was allowed to proceed until the end of embryogenesis, this was sufficient to prevent the re-establishment of PcG silencing until adulthood in most cases (see Fig. 2.10). In contrast to this, endogenous PREs in the BX-C including *Fab-7* are still transcribed in the brains of third instar larvae where homeotic genes are also expressed (see Fig. 2.11). Within the context of the BX-C, transcription through PREs may therefore be required continuously to counteract the establishment of PcG silencing in cells where transcriptionally active states have to be maintained.

3.1.3 Transcription through PREs – a general aspect of the cellular memory?

The PcG/TrxG memory system is known to control the expression of a large number of target genes. This raised the question whether the transcription through PREs also plays a role in the epigenetic regulation of genes outside the homeotic gene complexes. To answer this question, a few of the 167 potential PREs recently predicted *in silico* (Ringrose et al. 2003), were analyzed. There are several indications that these candidates are indeed true PREs: Their scores in the prediction are well above the cut-off of statistical significance, they map to known PcG binding sites in polytene chromosomes, and for *en* and *salm*, genetic interactions with PcG genes have been described (Landecker et al. 1994; Americo et al. 2002). As the assignment of PREs to target genes in the prediction was based on linear distance within the genome, low-complexity regions were chosen. The RNA *in situ* hybridization experiments revealed that the predicted PREs tested were indeed all transcribed in a regulated manner that reflected the embryonic expression patterns of their presumptive target genes (see Fig. 2.12). This illustrates that the anti-silencing function of transcription through PREs initially discovered in the BX-C may be a general aspect of the cellular memory system, rather than having evolved especially for the regulation of complex gene clusters.

The fact that transcripts spanning the predicted *tll* PRE were detected in the brain of third instar larvae suggests that also at this locus, transcription may be required constantly to prevent the establishment of PcG silencing at the PRE. Transcription through the known PRE at the *en* locus and the predicted *salm* PRE was not detected at this stage, although the associated target genes were strongly transcribed in imaginal discs (data not shown).

Similarly, although transcription through the *bxd* PRE paralleled the expression of the *Ubx* gene in the central nervous system of third instar larvae, no *bxd* transcripts were detected in the haltere and third leg imaginal discs, where *Ubx* is active at high levels (data not shown). The reason for this presumably is that the *bxd* regulatory region of the BX-C is mainly responsible for the specification of the first abdominal segment (A1), since *bxd* mutations result in the transformation of A1 into the metathoracic segment (T3). The haltere and third leg discs are however derived from the T3 segment, whose specification depends on the *abx/bx* regulatory regions. *abx/bx* mutations lead to a loss of *Ubx* expression in haltere and third leg imaginal discs, which ultimately results in a T3→T2 homeotic transformation (Lewis 1978; Bender et al. 1983; Little et al. 1990). Therefore, the maintenance of *Ubx* expression in haltere and third leg imaginal discs likely depends on the *bx* PRE located within the *abx/bx* region (Chiang et al. 1995). Supporting this, PcG/TrxG proteins have been shown to bind to the *bx* PRE (Chiang et al. 1995; Strutt et al. 1997; Orlando et al. 1998), and *abx* mutations enhance the *trx* mutant phenotype (Castelli-Gair and Garcia-Bellido 1990). Therefore, the *bx* PRE, rather than *bxd*, was expected to be transcribed in the haltere and third leg discs. However, the *bx* region resides within the *Ubx* gene, thus precluding the analysis of regulatory transcription through this PRE separately from *Ubx* gene transcription.

Similar to the *Ubx* locus, additional PREs with lower scores have been predicted in the *en* and *salm* gene regions, whereas a single PRE was predicted at the *tll* locus (Ringrose et al. 2003). Therefore, the expression of *en* and *salm* might be regulated by several PREs which are differentially deployed in different tissues, in a fashion analogous to the PS-specific functions of PREs in the BX-C.

Alternatively, the regulation of the epigenetic state of the *en* and *salm* PREs may be subject to different regulatory mechanisms depending on the tissue. In this respect, it is interesting to note that the expression of *en* and *salm* in imaginal discs is very dynamic. These dynamic expression patterns rely on morphogen gradients within the imaginal disc tissue. Signaling by morphogens is ultimately converted into changes in gene expression through the action of downstream transcription factors. In contrast to the expression of homeotic genes or *tll*, whose expression has to be maintained fairly constant within their original domains in the absence of transcription factors, the PREs regulating *en* and *salm* in imaginal discs have to integrate and respond to incoming signals. A similar situation is found at the *hedgehog (hh)* locus. HH expression in wing imaginal discs is induced by the Engrailed (EN) transcription factor (Tabata et al. 1992; Zecca et al. 1995). The subsequent maintenance of *hh* expression through a phase of proliferation is independent of EN and relies on a PRE. This indicates that the *hh*

PRE becomes switched into the epigenetically activated mode in response to EN during larval development, and as a consequence, maintains *hh* transcriptionally active during subsequent cell divisions (Maurange and Paro 2002).

Taken together, these results suggest that during embryogenesis, transcription through PREs may be universally employed to counteract the default silencing by PcG proteins. At later stages, the requirement for anti-silencing by transcription might depend on the tissue and on the locus. If the long-term commitment of a cell depends on the constant expression of a given gene, transcription through the corresponding PRE may be required permanently to prevent epigenetic silencing by PcG proteins. In contrast, if the expression of target genes (like *en, salm,* or *hh*) has to be modulated in response to incoming signals, transcription through PREs might not be required throughout development.

3.2 What are the epigenetic changes induced by transcription through Fab-7?

One hypothesis of how the transcription through PREs prevents silencing is that it might induce the specific displacement of PcG proteins from the chromatin. This appeared as an attractive possibility, since intergenic transcription in budding yeast can prevent the binding of transcription factors to their cognate binding sites (Martens et al. 2004; Schmitt and Paro 2004).

3.2.1 PC, PHO, and TRX are constitutively bound to the Fab-7 PRE

In a first approach, this hypothesis was tested by comparing the association of PC, PHO, and TRX with the pFAs-1 transgene in polytene chromosomes before and after excision of the *actin5C* promoter. In similar studies, PC, PSC, and PH were found to remain bound to a transgenic *Fab-7* PRE when switched into the epigenetically activated mode (Cavalli and Paro 1999). In that study, the epigenetic activation of the *Fab-7* PRE depended on the efficiency of the transiently induced expression of the GAL4 transactivator by heat-shock. Generally, this heat-shock dependent activation was not achieved in all cells, which is reflected by the variegated expression of the *miniwhite* reporter in adult eyes, rather than a homogeneous dark eye pigmentation (Cavalli and Paro 1998; Cavalli and Paro 1999). In contrast, the pFAs-1 line established in the present study served as a more homogeneous starting material for such an analysis, since the epigenetic activation of the PRE in this case

was caused by the ubiquitous transcription from the *actin5C* promoter. Surprisingly, both PC and PHO remained strongly bound to the *Fab-7* PRE on the pFAs-1 transgene when it was transcribed (see Figs. 2.14 and 2.15). Consistent with the previous study (Cavalli and Paro 1999), this implies that the recruitment of PcG proteins to the chromatin does not inevitably entail silencing. Similarly, TRX was also efficiently recruited to the *Fab-7* PRE in the absence of the *actin5C* promoter, suggesting that the association of this HMTase does not automatically result in the activation of the chromatin structure (see Fig. 2.16).

In accordance with this, the tethering of different PcG proteins or TRX to a reporter gene via fusions with the LexA DNA-binding domain did not automatically result in epigenetic silencing, nor direct transcriptional activation, respectively. Instead, the activities of these proteins were sensitive to the transcriptional state of the reporter gene during embryogenesis (Poux et al. 2001; Poux et al. 2002). Hence, it is presumably not the mere presence or absence of PcG/TrxG proteins that decides whether a target gene becomes silenced or remains transcriptionally active.

In another study, Déjardin and Cavalli (2004) described the identification of a minimal *Fab-7* PRE fragment. This 219bp minimal PRE was able to silence adjacent reporter genes in a PcG-dependent manner. In addition, this PRE could be switched into the epigenetically activated mode by providing a transient GAL4 pulse during embryogenesis. In this case, the epigenetic activation of the „core“ PRE has been correlated with the displacement of the PcG protein Polyhomeotic (PH) (Déjardin and Cavalli 2004). At the full-length *Fab-7* element, such a decrease in PH binding has not been observed upon epigenetic activation (Cavalli and Paro 1999). One important difference is that the switch of the minimal *Fab-7* PRE into the activated mode was not accompanied by non-coding transcription (Déjardin and Cavalli 2004). Results from our own group showed that an intermediate *Fab-7* fragment of 870bp containing more PcG binding sites, could not be activated under similar conditions. This suggests that over a certain threshold level of silencing, transcription through a PRE is required to efficiently counteract the activities of PcG complexes (Rank et al. 2002; Schmitt et al. 2005).

The major disadvantage of polytene chromosome analysis is the relatively high variability of the immunostaining procedure. Another fact that should not be neglected is that polytene chromosomes are special in that they are generated by multiple cycles of endoreduplication, resulting in up to 2048 copies. It cannot be excluded that individual copies may behave differently, i.e. it is possible that on some DNA strands, the *actin5C* promoter is actively transcribing, whereas on others it is not. As a consequence, one protein band at the site of

transgene insertion might actually represent a mixture of epigenetically repressed and activated copies of *Fab-7*, aligned with each other in polytene chromosomes.

To circumvent this problem, and to be able to quantify the association of PC, PHO, and TRX with the *Fab-7* PRE in a diploid tissue, ChIP analyses were performed, using embryos as starting material. The central premise to this experiment was that transcription from the *actin5C* promoter should be sufficiently high to prevent PcG-mediated silencing in all cells. The second assumption was that the transgenic *Fab-7* PRE acted as a silencer in all cells. The RNA *in situ* hybridizations showed that transcription through *Fab-7* from the *actin5C* promoter indeed occurred in all cells (see Fig. 2.2). In addition, the eye pigmentation of adult flies was evenly dark, indicating that transcription was sufficiently high in all cells. As to the second assumption, the only indicator for this was the homogeneous silencing of the *miniwhite* gene in the eyes of adult flies (see Fig. 2.3).

The quantification of immunoprecipitated material showed that the amounts of PC, PHO, and TRX bound to the transgene did not change upon transcription through the *Fab-7* PRE. This was true for both the boundary and the „core“ PRE fragment within the *Fab-7* element, as well as for the *miniwhite* promoter (see Fig. 2.17). The only fragment for which a slight decrease in PHO binding was observed mapped near the promoter of the *lacZ* gene. This is most likely due to an increased distance between the *lacZ* gene and the *Fab-7* PRE in the presence of the *actin5C* promoter, rather than reflecting the epigenetic activation of the associated PRE (see Fig. 2.17). Consistent with the results obtained from the analysis of polytene chromosomes, this suggests that the transcription through the *Fab-7* PRE neither leads to a displacement of PC or PHO, nor results in a specific recruitment of TRX to the chromatin. This partly contrasts with results obtained from the analysis of epigenetic changes at the endogenous BX-C in tissue culture cells. For instance, the level of PC bound at a homeotic gene promoter is significantly lower when this promoter is transcriptionally active than when it is repressed (Breiling et al. 2004). In addition, in a recent ChIP-on-CHIP approach in our own group, the association of PC, PHO, and TRX with the chromatin was compared in Kc and SF4 tissue culture cells. In these experiments, PC was highly enriched at repressed PREs and the corresponding target gene promoters, whereas the binding levels were significantly decreased at active loci. Consistent with the results described here, the association of PHO and TRX with PREs remained equal, irrespective of the epigenetic state. However, at activated loci, PHO spread over extensive regions, covering entire transcriptionally active „domains“ (Christian Beisel, personal communication). Such a spreading was not observed during the ChIP analysis described here. However, only a limited

number of PCR fragments were analyzed in the present study, whereas in the ChIP-on-CHIP approach, tiling arrays covering the entire ANT-C and BX-C were used. It is therefore possible that PHO might also display this spreading behaviour on the transgene, but was not detected due to the low number of fragments analyzed. In support of this, the immunosignal of PHO at the pFAs-1 transgene was considerably stronger when the *Fab-7* PRE was transcribed from the *actin5C* promoter (see Fig. 2.15). In addition, PHO was enriched over entire regions of decondensed chromatin in polytene chromosomes, termed puffs, which are indicative of high transcriptional activity. PHO might therefore have quite different functions in addition to its repressive role at PREs. Consistent with this, the mammalian homologue YY1 has been described to function both as a transcriptional repressor and activator (Thomas and Seto 1999).

Another explanation for the results may be that in the experiments described here, the association of PcG/TrxG proteins with the *Fab-7* PRE was analyzed in tissues, in which the associated *miniwhite* promoter is normally not active. Although the chromatin in the vicinity of the transcribed *Fab-7* PRE should be competent for transcription in all cells, transcription from the *miniwhite* promoter is only expected to occur within its normal expression domains in the eye tissue. Since the function of a PRE depends on its interaction with the promoter it regulates, it is possible that major changes in the binding of PcG/TrxG proteins will only be induced in tissues, in which the target gene promoter is transcriptionally active.

Alternatively, the *Fab-7* PRE may behave differently when isolated on a transgene. At their endogenous loci, PREs presumably do not function in isolation, but co-operate with other PREs in the vicinity. First, when on a transgene, silencing imposed by a PRE is stronger in the homozygous compared with the heterozygous situation, termed pairing-sensitive silencing (Kassis 2002). Second, the degree of PcG-mediated repression depends on the chromosomal environment of the PRE. Third, the genome-wide prediction has shown that with the exception of small genes (<3.5kb), several PREs are clustered at a given locus (Ringrose et al. 2003). Finally, the results from several studies suggest that the efficient silencing of a target gene promoter by a PRE depends on the interaction between different PcG complexes, which would bring regulatory sequences into close proximity. Presumably, such interactions occur frequently between different regulatory elements clustered within the BX-C. As a consequence, a decrease in the level of PcG proteins at a given PRE observed in the context of the BX-C might reflect a weaker interaction between this PRE and other regulatory sequences in addition to a loss of PcG binding. In contrast, the *Fab-7* PRE on the transgene is isolated, and since the experiments were done in the $Fab\text{-}7^{1}$ deletion background, no specific

interaction between the transgenic *Fab-7* PRE and the endogenous BX-C was expected. This might be one of the reasons why no changes in the binding of PC to the the pFAs-1 transgene were detected upon transcription through the *Fab-7* PRE from the *actin5C* promoter. In addition, the age of embryos collected for the experiment ranged from 4-20h. Even if the *Fab-7* PRE was homogeneously repressed or activated in the two sets of embryos, PC, PHO, and TRX might still associate with the *Fab-7* PRE in both situations, but may do so at different stages of development, depending on the phase in the establishment of epigenetic memory.
In summary, the epigenetic state at the transgenic *Fab-7* PRE is not primarily regulated by the differential association of PC, PHO, and TRX with the chromatin. Instead, the transcription through *Fab-7* may cause a modulation of PcG/TrxG enzymatic activities, thereby rendering the chromatin structure permissive for the transcription of target genes.

3.2.2 The histone variant H2Av is not specifically incorporated at PREs

The role of histone variants in the regulation of the chromatin structure is more and more appreciated. Recent studies have shown that different isoforms of the canonical H2A are intimately connected to processes of epigenetic gene regulation.
While the mammalian genome encodes four distinct H2A isoforms, only one variant is found in *Drosophila*, H2Av (Leach et al. 2000). H2Av is essential for viability, and its critical functions reside in a C-terminal extended domain, which maps to the region contacting the surface of histone H4 within the nucleosome. Structural analysis suggests that a stretch of acidic amino acid residues within this C-terminal domain of H2Av might lead to a destabilization of the nucleosome, which in turn potentially influences the folding of the chromatin into a higher order structure (Clarkson et al. 1999).
Previous studies have shown that H2Av behaves genetically as a PcG gene (Leach et al. 2000; Swaminathan et al. 2005), and mutations result in a reduced binding of PC to polytene chromosomes. In addition, the homeotic *Antennapedia (Antp)* gene shows ectopic expression in the brains of H2Av mutant larvae (Swaminathan et al. 2005). Taken together, this suggested that H2Av might be specifically incorporated at repressed PREs and thus be involved in the inheritance of epigenetic silencing. However, the analysis of polytene chromosomes showed that a H2Av-GFP fusion protein is not bound to the pFAs-1 transgene, irrespective of its epigenetic state (see Fig. 2.18). This is presumably not due to a problem of sensitivity, because even at the BX-C, in which several PREs are clustered, H2Av-GFP was not detectable. In addition, there was almost no overlap between H2Av-GFP and PC binding on whole chromosome spreads, further supporting the notion that H2Av-GFP is not incorporated at PREs at all. In these experiments, antibodies directed against an H2Av-GFP

fusion protein instead of the endogenous H2Av were used. However, the H2Av-GFP transgene has been shown to rescue H2Av mutations, strongly suggesting that the fusion protein behaves as the wildtype protein (Clarkson and Saint 1999). Moreover, the H2Av-GFP fusion protein showed a similarly strong association with centromeric heterochromatin and the heterochromatinized fourth chromosome as the wildtype protein, supporting a suggested role of H2Av and its mammalian homologue H2AZ in the formation of constitutive heterochromatin (Rangasamy et al. 2003; Swaminathan et al. 2005).

If H2Av-GFP is not incorporated into the chromatin at PREs at all, how can its genetic interaction with PcG genes and the loss of *Antp* repression in H2Av mutants be explained? The H2Av homologue Htz1 in budding yeast has been reported to function as a boundary, preventing the spread of silent heterochromatin into adjacent euchromatic regions (Meneghini et al. 2003). If, in addition to being involved in the formation of constitutive heterochromatin, H2Av has a similar role in *Drosophila*, the observed interaction with PcG silencing might well be due to indirect effects. Supposed that one function of H2Av is to separate PcG-regulated domains from adjacent regions, the loss of H2Av might lead to a redistribution of PcG proteins along the chromosomes. As PcG silencing has been shown to be sensitive to dosage effects (Kennison and Tamkun 1988), a redistribution of PcG proteins would consequently lead to a decrease in the levels of PcG proteins bound to repressed PREs. Eventually, this might result in the loss of PcG target gene repression. However to date, such a boundary function of H2Av has not been described in *Drosophila*, and further experiments are needed to clarify this.

3.3 Anti-silencing at PREs – Transcription- versus RNA-based models

The results from the analysis of the transgenic reporter system have shown that non-coding transcription through PREs triggers the activation of the chromatin structure. Does this mechanism depend on the process of transcription, the non-coding RNAs generated, or a combination of both?

On the transgene, transcription of either sense or antisense *Fab-7* RNA had the same activating effect (see Fig. 2.3). This suggests that the anti-silencing function of transcription through PREs may mainly rely on the process of transcription, rather than the sequence of the non-coding RNA generated. In addition, endogenous *Fab-7* transcription occurs in both orientations in early embryos, whereas at later stages, only the sense transcript is generated.

The predicted PREs at the *en, slou,* and *salm* loci are transcribed bi-directionally (Fig. 2.12), whereas the *bxd* and *Mcp* PREs, as well as the predicted *tll* PRE are transcribed in one direction only (Figs. 2.11 and 2.12; Rank et al. 2002). Thus, at some PREs, transcription of only one strand is sufficient to prevent silencing, whereas at other loci, transcription of both strands may be required.

PREs are defined by the increased clustering of DNA binding motifs for the transcription factors GAF, Zeste, and PHO (Ringrose et al. 2003). However, the comparison between different *Drosophila* species suggests that PREs behave very dynamically: The composition of motifs within a single PRE and its position relative to its predicted target gene show considerable evolutionary plasticity (Leonie Ringrose, personal communication). It is hard to imagine that in all of these cases, the non-coding RNAs adopt sequence-specific secondary structures which are sufficiently similar to mediate interactions with, for instance, PcG or TrxG proteins. Nevertheless, the generation of a sufficient amount of non-coding RNA at a PRE *per se* may lead to non-specific interactions with PcG/TrxG proteins, thereby influencing the efficiency of PcG-mediated repression.

3.3.1 The stability of Fab-7 RNA is consistent with a possible molecular function

If the non-coding RNAs generated during the transcription through PREs are structurally involved in counteracting PcG silencing, they might be protected from their rapid degradation. Consistent with this, the non-coding *Fab-7* RNA was considerably more stable than an intronic fragment of the *AbdB* pre-mRNA, which is presumably degraded immediately after splicing (see Fig. 2.21). The half-life of *Fab-7* was even longer than that of the mature *AbdB* mRNA, which is processed, exported into the cytoplasm, and translated. Concerning the kinetics of *Fab-7* RNA degradation, it is worth noting that the RNA levels decreased rapidly at the beginning of the time-course, but showed only a moderate decline at later time points. This might reflect the presence of two distinct pools of *Fab-7* RNA: One that is free and prone to rapid degradation, whereas the other may be protected by bound proteins. Interestingly, the PcG protein E(Z) and the TrxG protein TRX have recently been reported to interact with single-stranded DNA and RNA *in vitro* (Krajewski et al. 2005). It is therefore tempting to speculate that non-coding *Fab-7* RNA might be bound by proteins such as E(Z) or TRX, and would thus be shielded from degradation.

Alternatively, the moderate half-life of *Fab-7* RNA might simply be caused by the intrinsic stability of this transcript. Although attempts to map the precise 5' and 3' ends of the *Fab-7* RNA have failed, it presumably originates from very large transcripts spanning several kb (Rank et al. 2002 and G. Rank, personal communication). As nuclear RNAs are

predominantly degraded by exonucleases (Vasudevan and Peltz 2003), the primary determinant of its stability might be the length of the *Fab-7* RNA.

A second prediction to be inferred from a possible structural function of non-coding RNAs spanning PREs is that they should remain associated with the chromatin after their synthesis. The RNA FISH analysis showed that the non-coding RNAs are localized to discrete nuclear domains (see Figs. 2.22 and 2.23). At this point, it is not clear whether these signals represent only nascent transcripts or if the non-coding RNAs accumulate at the chromatin. Further experiments are required to distinguish between these possibilities.

3.3.2 RNAi is presumably not involved in the epigenetic regulation at PREs

The RNA *in situ* hybridizations have shown that some PREs are transcribed uni-directionally, whereas at other loci, transcription occurs in both sense and antisense directions (see Fig. 2.12 and Schmitt et al. 2005). This was at first surprising, because bi-directional transcription potentially leads to the generation of double-stranded (ds) RNA. In fission yeast, the simultaneous sense and antisense transcription of pericentromeric repeats and the generation of dsRNA has been linked with the formation of repressive heterochromatin (reviewed in Wassenegger 2005). In this scenario, long primary dsRNAs are processed by the RNAse III-type endonuclease Dicer into small interfering RNAs (siRNAs). siRNAs are incorporated into the RNA-induced initiator of transcriptional gene silencing (RITS) complex, guiding RITS to complementary sites of the genome. As a consequence, the Clr4 histone methyltransferase becomes recruited to the chromatin and methylates histone H3K9, which ultimately results in the formation of heterochromatin and transcriptional silencing.

In contrast, the bi-directional transcription through PREs in spatially overlapping domains within the embryo is associated with epigenetic activation, rather than silencing. The double-label RNA FISH experiments showed that both sense and antisense *Fab-7* RNAs can sometimes be detected within single cells (see Fig. 2.23). In most of these cases, they are however located in separate subnuclear domains. In addition, previous attempts in our lab to detect siRNAs generated from *Fab-7* RNA precursors were unsuccessful (Stefan Schönfelder, personal communication), and no genetic interaction was found between genes involved in the RNAi pathway and PcG genes (Nathalie Aulner, personal communication). Taken together, this suggests that the bi-directional transcription through PREs does not evoke an RNAi response, presumably because sense and antisense RNA transcription are spatially separated.

3.3.3 A role for non-coding transcription in mitotic inheritance?

Transcription through endogenous PREs can be detected in the brains of third instar larvae, suggesting that at least in this tissue, persistent non-coding transcription is required to efficiently counteract PcG silencing throughout development (Schmitt et al. 2005). In such a scenario, the problem of epigenetic inheritance is moved to another level: What prevents the PcG proteins from silencing intergenic transcription? The assumption that the promoters of intergenic transcripts are not sensitive to PcG silencing is probably not valid. The restriction of non-coding transcription in the BX-C to defined spatial domains depends on the same set of early segmentation genes as the expression of the protein encoding mRNAs (Casares and Sanchez-Herrero 1995). As such, their subsequent regulation might be subjected to the same regiment of factors as the coding transcripts. However, the transcription through PREs begins slightly earlier during development than *Hox* gene expression. This suggests that although the spatial limitation of intergenic transcription and *Hox* gene expression might be controlled by the identical transcriptional repressors, their initial activation presumably depends on distinct mechanisms.

With the processive transcription as the central issue, it is crucial to understand how this process is initially activated and maintained throughout development. It has been suggested that GAF might be involved in the formation of a chromatin ground state for transcription during early embryogenesis (Bejarano and Busturia 2004). As such, the transcriptional activation of intergenic promoters might be triggered by a general mechanism, whereas transcription factors encoded by the gap and pair-rule genes function as repressors to define its spatial limitations.

At later stages, the persistence of non-coding transcription through PREs might depend on its timing with respect to the cell cycle. It has recently been shown that low-level transcription is resumed at the anaphase-telophase transition of mitosis (Chen et al. 2005). Interestingly, non-coding *Fab-7* RNA can be detected on mitotic chromosomes (see Fig. 2.24), raising the hypothesis that the transcription through PREs might re-initiate even before cell division is completed. Alternatively, the *Fab-7* signals observed might represent RNA that had been synthesized in the previous cell cylce, but remained associated with the chromatin throughout mitosis. In such a scenario, the presence of non-coding RNA *per se* might serve as a signal to trigger the re-initiation of transcription through the PRE early in the next cell cycle.

In principle, one round of transcription per cell cycle might be sufficient to maintain the chromatin in the active state. At least *in vitro*, the PRC1 complex efficiently blocks nucleosome remodeling by the BRM complex and transcription only when pre-bound to the

chromatin (Francis et al. 2001). Since PcG proteins dissociate from mitotic chromatin (Buchenau et al. 1998), at least one early round of transcription through a PRE, preceding the re-association of PcG proteins, might be sufficient to prevent silencing for the next cell cycle. In the RNA FISH analysis, *Fab-7* appeared as single or doublet spots within the nuclei, but occasionally, three or even four spots were observed (see Figs. 2.22, 2.23, and data not shown). This suggests that non-coding RNA is present on the chromatin both before and after DNA replication. However, it is well known that PREs on homologous chromosomes tend to interact with each other, so it is impossible to infer the cell cycle stage simply by counting the number of signals. Therefore, a detailed analysis of this in combination with cell cycle markers has to be done to determine whether transcription through PREs occurs throughout the cell cycle or at specific stages.

3.4 Transcription through PREs shifts the balance from epigenetic silencing to activation

The epigenetic inheritance of transcriptional states requires the presence of functional TrxG and PcG proteins throughout development. Changing the dosage of either trxG or PcG genes at any time results in the misregulation of target genes. This predicts that the epigenetic state of a PRE is dynamic and depends critically on the balance between the antagonizing activities of activating TrxG and repressive PcG complexes. The function of transcription may be to shift this balance toward TrxG activities, which will ultimately result in the activation of the chromatin structure.

Figure 3.1 represents a possible model how the transcription through a PRE might prevent PcG-mediated silencing. During early embryogenesis, transcription through PREs is activated by an unknown signal. Its restriction to appropriate body segments depends on the action of transcription factors encoded by the segmentation genes (Casares and Sanchez-Herrero 1995). Subsequently, PcG and TrxG proteins become recruited to the chromatin and presumably bind to both repressed and transcribed PREs (Fig. 3.1). At a repressed PRE, the activities of PcG proteins predominate, leading to an enrichment of repressive chromatin modifications, such as methylated H3K27 and mono-ubiquitylated H2AK119 (Fig. 3.1A). Whereas the function of modified H2AK119 is not known, methylated H3K27 might strengthen the association of PcG complexes with the chromatin via its interaction with the chromodomain of PC (Fischle et al. 2003; Min et al. 2003). On the one hand, this might decrease the mobility of PcG complexes (Ringrose et al. 2004) and on the other hand, methylated H3K27 might

serve as a signal for efficient re-association of PcG complexes following mitosis. In addition, PcG complexes might directly inhibit TrxG activities such as nucleosome remodeling by BRM complexes and the initiation of transcription (Francis et al. 2001). The outcome would be epigenetic silencing through the formation of a chromatin structure refractory to transcription.

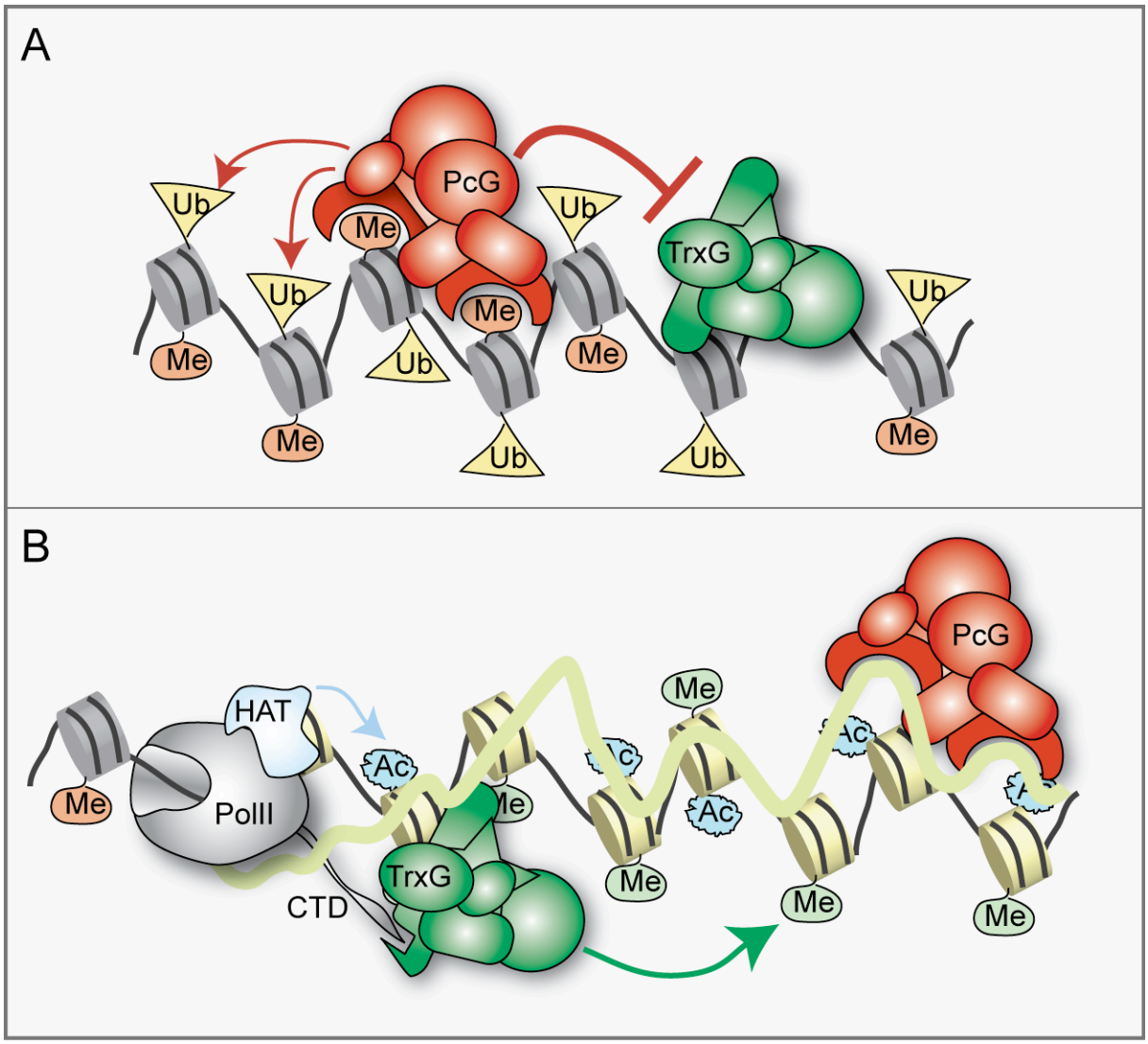

Figure 3.1: Model: Transcription through a PRE shifts the balance of PcG/TrxG activities toward epigenetic activation. A) At a non-transcribed PRE, PcG protein (red) activities prevail, leading to an enrichment of repressive epigenetic marks, like methylated H3K27 (Me, orange) and mono-ubiquitylated H2AK119 (Ub, yellow). In addition, PcG complexes directly inhibit TrxG (dark green) activities, such as nucleosome remodeling by the BRM complex. The result is the formation of a repressive chromatin structure. B) At a transcribed PRE, TrxG protein activities might be stimulated via their direct interaction with the C-terminal domain (CTD, grey) of the RNA polymerase II machinery (PolII, grey) and/or the non-coding RNA (green line) generated, favouring the enrichment of methylated H3K4 (Me, light green). Histone acetyltransferases (HAT, blue) might become recruited to a transcribed PRE through their interaction with PolII and/or TrxG proteins and catalyze the acetylation of histones (Ac, blue). In addition to the enrichment of active histone modifications, transcription through a PRE might directly interfere with PcG activities by promoting the removal of repressive chromatin marks by histone exchange. The non-coding RNA might interact with PcG proteins, thereby decreasing their activities. The outcome is the formation of a transcriptionally competent chromatin structure.

In contrast, if transcription through a PRE has been initiated during embryogenesis before PcG proteins first associate with the chromatin, the situation is different (Fig. 3.1B). The act of transcription alone or in combination with the RNA generated may counteract silencing by several mechanisms. For example TRX, like its mammalian homologue MLL (Milne et al. 2005), might interact with the transcribing RNA polymerase II and/or with the nascent non-coding RNA (Krajewski et al. 2005), which in turn might stimulate its histone methyltransferase (HMTase) activity (Petruk et al. 2001). This would then lead to the enrichment of methylated H3K4 at the PRE, which may serve as a bookmark to transmit the epigenetically active state through mitotic divisions (Kouskouti and Talianidis 2005). Histone acetyltransferases (HATs), either directly associated with the RNA polymerase II machinery (Wittschieben et al. 1999), or via the interaction with TRX (Dou et al. 2005), might become recruited to a transcribed PRE and catalyze the acetylation of histones.

In addition to favouring the enrichment of active chromatin modifications, transcription through a PRE might directly counteract the establishment of repressive chromatin structures. On the one hand, repressive epigenetic marks such as methylation of H3K27 might be removed via the transcription-coupled exchange of H3 by the H3.3 variant (Schwartz and Ahmad 2005). On the other hand, the non-coding RNA generated at a PRE might directly interact with PcG proteins, thereby interfering with their activities. For example, the H3K27 HMTase E(Z) has been shown to interact with RNA *in vitro* (Krajewski et al. 2005). In addition, PC might interact with RNA via its chromodomain in a similar fashion as MSL3 and MOF bind to *roX2* RNA in the dosage compensation complex (Akhtar et al. 2000). This, in turn might influence the integrity of PC-containing PcG complexes and impair their repressive activities.

Taken together, processive transcription through a PRE may counteract PcG silencing by directly inhibiting PcG activities and stimulating TrxG activities. By simultaneously increasing the constant turnover of histone modifications by transcription-coupled histone variant exchange, this will shift the balance toward the enrichment of active epigenetic marks, thereby rendering the chromatin permissive for gene expression. The mitotic inheritance of the active chromatin state might depend on epigenetic marks such as histone modifications alone or in combination with associated non-coding RNA.

3.5 Perspectives

The fundamental function of non-coding transcription in the regulation of the cellular memory might have important implications for the understanding of developmental decisions in vertebrates. Although mammalian PREs have not been identified so far, it is well established that the regulation of *Hox* gene expression depends on functional PcG and TrxG proteins. In contrast to the regulatory hierarchy of segmentation genes in *Drosophila*, the initial transcriptional activation of mammalian *Hox* genes occurs in response to a gradient of retinoic acid along the A-P body axis (Lufkin 1996). Retinoic acid has been shown to induce the transcription of overlapping antisense RNAs along with the expression of protein-coding genes on human chromosomes 21 and 22 (Cawley et al. 2004). Strikingly, there is evidence that such a co-regulation of intergenic antisense transcription and gene expression in response to retinoic acid treatment also occurs in the human *HoxA* cluster (Achim Breiling, personal communication). An important challenge for the future will be to determine whether this intergenic transcription has an analogous anti-silencing function as the transcription through PREs in *Drosophila*.

Apart from the epigenetic regulation of gene expression in response to developmental cues, the transcription of non-coding RNAs has been shown to play an important role in controlling mono-allelic gene expression in mammals. The most prominent examples here are X chromosome inactivation (XCI) during dosage compensation and genomic imprinting. At the *Igf2r* and *Kcnq1* loci, the mono-allelic repression of imprinted genes requires the selective transcription of non-coding *Air (Antisense Igf2r RNA)* and *Lit1* RNAs, respectively, on the paternally inherited allele (Fitzpatrick et al. 2002; Sleutels et al. 2002; Mancini-DiNardo et al. 2003). For efficient silencing, non-coding transcription has to occur in *cis*. This led to a model in which the non-coding RNAs spread along the chromatin of the imprinted gene cluster and mediate silencing by recruiting chromatin-modifying activities (Sleutels et al. 2003; Thakur et al. 2004). Interestingly, imprinted silencing at the *Kcnq1* locus has been shown to depend on functional EZH2, the mammalian homologue of Drosophila E(Z) (Mager et al. 2003).

During XCI, silencing of one of the female X chromosomes is triggered by the transcription of non-coding *Xist (X inactivation-specific transcript)* RNA, which coats the complete chromosome in *cis* and mediates the recruitment of *trans*-acting silencing factors (reviewed in Heard 2005). The choice which of the two homologous chromosomes will undergo XCI depends on distinct mechanisms at different stages of development. In pre-implantation embryos, the transcription of *Xist* RNA is imprinted, thereby leading to the selective inactivation of the paternally inherited X chromosome. After the blastocyst stage however,

XCI is random, and choice depends on the ratio between *Xist* RNA expression and the transcription of the overlapping *Tsix* antisense transcript (see Fig. 3.2 and Introduction 1.5.3; Shibata and Lee 2004).

Both imprinted and random XCI require the activities of PRC2 and PRC1 proteins, which mediate the enrichment of methylated H3K27 and mono-ubiquitylated H2A on the future X_i (Plath et al. 2003; de Napoles et al. 2004; Plath et al. 2004). The targeting of both PRC2 and PCR1 components to the future X_i has been proposed to depend on the transcription of *Xist* RNA (Plath et al. 2004). How can this be unified with the anti-silencing function of non-coding transcription through PREs described here? It is possible that during X inactivation and imprinting, epigenetic repression is achieved via several pathways, acting in parallel. For example, a mutated *Xist* allele lacking the region required for its silencing function, is able to coat the X chromosome in *cis,* and PRC2 and PRC1 components are still recruited to the chromatin (Plath et al. 2004). Thus, the silencing functions of *Xist* RNA and PcG complexes are presumably not directly linked with each other. Moreover, a direct interaction between PcG proteins and *Xist* RNA has not been shown so far. It is therefore possible that the targeting of PcG complexes to the future X_i might in fact not depend on *Xist* RNA transcription at all, but rather on the absence of antisense transcription from *Tsix* and/or *Xite* (see Fig. 3.2). In analogy to the situation at PREs, transcription from *Xite* might counteract the silencing of downstream located *Tsix* promoters during the initiation of XCI. The upregulation of *Tsix* in turn, promotes the downregulation of *Xist* and at the same time, might prevent PcG-mediated repression.

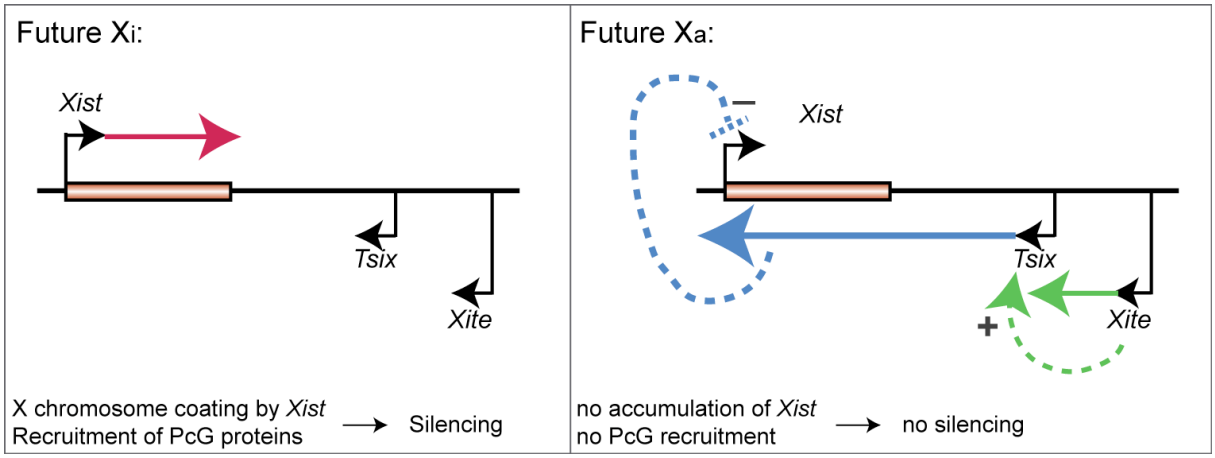

Figure 3.2: The choice of XCI is regulated by antagonizing intergenic transcripts. At the future inactive X chromosome (X_i, left), *Xist* RNA (red) is transcribed at a high level and coats the chromosome in *cis*. In addition, PcG proteins become recruited by an unknown mechanism. The result is the formation of a repressive chromatin structure. At the future active X chromosome (X_a, right), transcription from *Xite* (green) stimulates *Tsix* (blue) transcription, which in turn inhibits *Xist* (red) expression. In addition to downregulating *Xist*, transcription from *Xite* and/or *Tsix* might prevent silencing by PcG proteins. Modified from Shibata and Lee 2004.

During imprinted X inactivation, transcription from *Tsix* and *Xite* is not involved. Likewise, no activating non-coding transcripts have been identified at somatic imprinted loci. In these situations, the expression of non-coding RNAs required for silencing is controlled by the parent-of-origin specifc DNA methylation in their promoters (Zuccotti and Monk 1995; Mancini-DiNardo et al. 2003), raising the possibility that the differential recruitment of PcG proteins to imprinted loci might rely on other mechanisms.

Interestingly, the two homologous X chromosomes have recently been shown to associate transiently with each other at the onset of XCI. This pairing is essential for the initiation of XCI and requires the functions of *Tsix* and *Xite* (Xu et al. 2006). Similarly, the function of PREs critically depends on the pairing of homologous chromosomes, although the interaction in this situation is maintained throughout development.

In summary, the results presented in this thesis might have implications for our understanding of epigenetic gene regulatory mechanisms in mammals. Concerning the regulation of developmentally important genes by the PcG/TrxG memory system, the anti-silencing function of transcription through PREs might be conserved, although mammalian PREs have not been identified yet. Anti-silencing by non-coding transcription might also play a role in the regulation of mono-allelic gene expression during random XCI, but is presumably not involved in genomic imprinting.

Open questions

To understand the precise function of intergenic transcription in the regulation of epigenetic states at PREs, three main questions should be addressed in the future:

1. How does transcription through a PRE influence the activities of PcG and TrxG proteins?

   It is possible that the interactions of a subset of PcG and TrxG proteins with ssDNA and RNA described *in vitro* might also be involved in modulating their activities *in vivo*. Alternatively, the balance between epigenetic silencing and activation may depend on direct interactions of PcG and TrxG proteins with the transcribing RNA polymerase II machinery. To distinguish between these possibilities, it is essential to clarify whether the non-coding RNAs themselves are critical for the anti-silencing function of transcription through a PRE or not.

2. How is the epigenetically activated state of a PRE transmitted through mitosis?

   The observation that non-coding *Fab-7* RNA can be detected on mitotic chromosomes suggests that the association of non-coding RNA with the chromatin might act as a

signal that triggers the re-initiation of transcription through PREs early in the cell cycle. Alternatively, epigenetic marks on histones, histone variants, and/or proteins bound to the chromatin may act as bookmarks targeting the transriptional machinery to PREs during late stages of mitosis. To address this question, one would have to analyze the dynamic behaviour of non-coding RNA transcription with respect to the cell cycle.

3. What are the differences between PREs that are only transcribed during embryogenesis and those transcribed also at later stages?
   A possible reason for this is that the continuous transcription may be required for the maintenance of long-term decisions, whereas transient transcription would be sufficient to sustain epigenetically activated states for short-term periods. Ultimately, the difference between PREs acting as long-term or short-term epigenetic memories must be buried within their sequences. Thus, comparative analysis might reveal common features shared by one group of PREs, but not by the other.

Finding answers to these questions will provide a more detailed picture of the mechanism underlying the epigenetic regulation of the cellular memory in *Drosophila*, and will undoubtedly have implications on our understanding of epigenetic gene regulatory processes in mammals.

Material

4. Material

4.1 Antibodies

Antibody	Source	Obtained from	Dilution	
			Embryos	Polytenes
a-DIG-AP	sheep	Roche (No. 11093274910)	1:2000	
a-DIG	mouse	Roche (No. 11333062910)	1:400	1:200
a-DIG-Rhodamine	sheep	Roche (No. 11207750910)		1:100
a-fluorescein	rabbit	Molecular Probes (A-889)	1:300	
a-GFP	mouse	Roche (No. 11814460001)		1:50
a-mouse-Alexa488	goat	Molecular Probes	1:200	1:200
a-mouse-Cy3	rabbit	JacksonImmunoResearch	1:200	1:200
a-Pleiohomeotic	rabbit	Jürg Müller (EMBL)		1:100
a-Polycomb	rabbit	Britta Koch		1:80
a-Trithorax	rabbit	Inhua Chen-Muyrers		1:20
a-rabbit-Alexa488	goat	Molecular Probes	1:200	1:200
a-rabbit-Cy3	goat	JacksonImmunoResearch	1:200	1:200
a-tubulin	mouse	Sigma		1:50

4.2 Molecular weight markers

1 kb DNA ladder	NEB
100 bp DNA ladder	NEB
DNA molecular weight marker II	Roche

4.3 Enzymes

Calf intestinal alkaline phosphatase (CIP)	Merck
DNAse (RNase-free)	Roche
DNAfree™ DNase kit	Ambion
Klenow enzyme	Roche
M-MLV reverse transcriptase	Promega
Proteinase K	Roche
Pwo-Polymerase	Roche
Restriction enzymes	NEB

RNase (DNase-free)	Roche
RNase H	Invitrogen
RNaseOUT Ribonuclease inhibitor	Invitrogen
RNasin Ribonuclease inhibitor	Promega
Shrimp alkaline phosphatase (SAP)	Roche
SuperscriptIII™ reverse transcriptase	Invitrogen
Taq Polymerase	Qiagen
T4 DNA ligase	NEB / Roche
T3 RNA polymerase	Roche
T7 RNA polymerase	Roche

4.4 Oligonucleotides

All primer were purchased from MWG Biotech or SIGMA.

4.4.1 Primer used for cloning

Amplification of the hsp70 3'UTR from genomic DNA (restriction sites in italics)

hsp70-HindIII-up	5'-CCC*AAGCTT*GGTCGACTAAGGCCAAAGAG–3'
hsp70-BamHI-down	5'-CGC*GGATCC*GGAATTCGTCGTTTATGATATGA–3'

Amplification of lambda control DNA (restriction sites in italics)

lambda-HindIII-up	5'-CCC*AAGCTT*GGGCGGCGACCTCGCGGGTT–3'
lambda-BamHI-down	5'-GCG*GGATCC*GATGGCCTCATCCACACGCAG–3'

Amplification of the hsp70 3'UTR and the lambda control DNA flanked by loxP sites from the pSV-paX1 vector (restriction sites in italics)

loxP-5'-AscI	5'-TT*GGCGCGCC*AATACTTCACTAACAACCGGTACAG-3'
loxP-3'-AscI	5'-TT*GGCGCGCC*TGCCAAGCTACTCGCGACCA-3'

Amplification of en, slou, salm, and tll gene and PRE sequences from genomic DNA for subsequent cloning into the pCRII-TOPO and pCR4-TOPO vectors (orientations are always with respect to the orientation of the associated gene)

pCR4-encDNA-4.8 (en gene fragment in sense orientation)

en-UP	5'-GCGAGCGAGAGAGCGCTCTG-3'
en-LOW	5'-TTTACAGAGCGGTTGCAAGCGC–3'

pCRII-enPRE-1 (antisense orientation) and pCRII-enPRE-3 (sense orientation)

enPRE-UP	5'-GAGTTGTATCCTGTGATTACGTC-3'
enPRE-DOWN	5'-GCCATCTCTTTCCACAGACACTT-3'

pCR4-slou-5 (sense orientation) and pCR4-slouas-2 (antisense orientation)

slou-UP	5'-ACCACCAGAAGCAGAAGCAGCA–3'
slou-LOW	5'-TGTGCGATCCATCGTTGCTGTC–3'

pCR4-slouPRErev-7.5 (antisense orientation) and pCR4-slouPRE-7.7 (sense orientation)

slouPRE-UP	5'-GTGTGTGAGACCCATCCTTCG–3'
slouPRE-LOW	5'-CCTTCAGCGAATGGTCAATGAG-3'

pCR4-salm-2.1 (antisense) and pCR4-salm-2.2 (sense)

salm-UP	5'-CGGTAGCGATCAGGAGGAGAAC–3'
salm-LOW	5'-GGTGATCCAGGTGGGATTGCGA-3'

pCR4-salmPREas-3.7 (antisense) and pCR4-salmPRE-3.5 (sense)

salmPRE-UP	5'-CATTCGTGCCCTCTCTTTCTCAG–3'
salmPRE-LOW	5'-TACATCCTGACAATCACTATCAC–3'

pCR4-tll-4.8 (antisense)

tll-UP	5'-CCAGGTCGCATTCTATACCATGT–3'
tll-LOW	5'-GCAAAGTTGAAACTTTTGCGAATG–3'

pCR4-tllPREas-5.4 (antisense) and pCR4-tllPRE-5.3 (sense)

tllPRE-UP	5'-TGTATTGGCCCTTGGAAACTGTTT–3'
tllPRE-LOW	5'-CCTTAGGTAGACAAACTCCGCG–3'

pCR4-ry-1 (sense orientation)

ryTOPO-UP	5'-AGTGTCGCCTGATCCGGAGTG-3'
ryTOPO-LOW	5'-CAACGATATACTGGTCCGGAGT-3'

pCR4-ryprom (sense orientation)

rypromTOPO-UP	5'-GCCACTCGTAGGGAATTAATTATG-3'
rypromTOPO-LOW	5'-TGTGGTCAGCTAGGAAAGCAATC-3'

4.4.2 Primer used for sequencing or to test Cre/loxP and Flp/FRT recombination by genomic PCR

Fab-5'-Seq	5'-GCGTGTGGATTGAATACTTGC-3'
Fab-3'-Seq	5'-TACAGTTTAATGCCCCATCATGC-3'
Seq-pUZmod-hb-3'	5'-ATTGAATTGTCGCTCCGTAG-3'
Seq-pUZmod-hb-5'	5'-GCATGTCCGTGGGGTTTGAA-3'
Flp/Cre-test	5'-CGGTGCGGGCCTCTTCGCT–3'
FLP1	5'-GTGGGGTTTGAATTAACTCATAA-3'
FLP4	5'-ACCCAGTTGCGGAGGAAATTC–3'
FLP5	5'-TCGCAATCATACGCACTGAGC–3'

4.4.3 Primer used for RT-PCR

SalmRT_low	5'-CTCGGAACTGGGCTTTGTCATC-3'
SalmRT_UP	5'-CCTGTCCAACGTTCTGTGTGCG-3'
SalmPRERT_low	5'-CTCACCATTCGCCATCTCGCT-3'
SalmPRERT_UP	5'-TGCCTAGACTTGAAATGCTAAAAAT-3'
SlouRT_low	5'-TCGCTCCTGCATTTCCTCGTC-3'
SlouRT_UP	5'-CACCTCGCGTCAAATTGCAGCA-3'
SlouPRERT_low	5'-GGCGTGGCCAGCGATGGGTTT-3'
SlouPRERT_UP	5'-ACCCGCACACCCAAATGCAGA-3'
TllRT_low	5'-TTCATCAGAATTTCGGCGGGTAT-3'
TllRT_UP	5'-CAGTGCCAAGTGAAATTCAATTTG-3'

TllPRERT_low	5’-TCAGATTGTACAGATTTCTTGAGC-3’
TllPRERT_UP	5’-TGAACCTTTGATTTTAACAACTCCG-3’
EnRT_low	5’-TGCACGCCCCCGTCAGAAGG-3’
EnRT_UP	5’-ACCAACGACGAGAAGCGTCCA-3’
EnPRERT_low	5’-CCACCTTTAGGCCATGTGTAGA-3’
EnPRERT_UP	5’-CCCACATCTATACACTATTTGCAG-3’

4.4.4 Primer used for real-time RT-PCR

Fab-7 PRE

Fab7-IX-UP	5’-GAGTGGCGAGCAGAGCAGCAT-3’
Fab7-IX-LOW	5’-ATGATGGCCGAGCTGAAAATGAA-3’

AbdB intron fragment

AbdB-IN-UP	5’-GTCGTCGTATCGGTTGAAATAGCA-3’
AbdB-IN-LOW	5’-CAATGACTTTCGCACCCACATAAA-3’

AbdB exon fragment

AbdB-EX-UP	5’-GGAGCAAGGATGTGGATGAGCAA-3’
AbdB-EX-LOW	5’-CGAACTAAGCTGCATTATCGTGTTG-3’

ATPasecf6

ATPcf6F and ATPcf6R, kindly provided by Ian M. Roustan-Espinosa (Roustan-Espinosa 2005)

4.4.5 Primer used for ChIP analysis

Fab-7 prox

Fab7-II-UP	5’-TTGTCTCTGCTTCTGGACCTATGCT-3’
Fab7-II-LOW	5’-ATCGGTCCATACCCTAAAAGGCAA-3’

Fab-7 bound

Fab7-V-UP	5’-GCTGTCACGGGGAAGCACAGA-3’
Fab7-V-LOW	5’-TGTGCGTGCGGTTCTCTTATCAC-3’

Fab-7 PRE

Fab7-IX-UP and –LOW, see above

bxd

bxd-UP	5’-GACGTGCGTAAGAGCGAGATACAG-3’
bxd-LOW	5’-GCACTTAAAACGGCCATTACGAA-3’

lacZ promoter

lacZFOR and lacZREV, Rank 2002

white promoter

MwupFor (gift from A. Monqaut)

white prom LOW NEW	5’-AGTGCAACTGAAGGCGGACATT-3’

4.5 Plasmids

pBS SK-/3.6Fab7#5	pBS SK- containing the full-length 3.6kb *HindIII Fab-7* fragment in distal-proximal orientation, (Prestel 2003)
pBS SK-/3.6Fab7#6	pBS SK- containing the full-length 3.6kb *HindIII Fab-7* fragment in proximal-distal orientation, (Prestel 2003)
pBS SK II 2293 pd (C3)	pBS SK II containing the *bxd* PRE in proximal-distal orientation, (Prestel 2003)
pBS SK II 2293 dp (A1)	pBS SK II containing the *bxd* PRE in distal-proximal orientation, (Prestel 2003)
pBS SK II 4479 pd (CM1)	pBS SK II containing the *Mcp* PRE in proximal-distal orientation, (Prestel 2003)
pBS SK II 4479 dp (AM1)	pBS SK II containing the *Mcp* PRE in distal-proximal orientation, (Prestel 2003)
pBS669	plasmid containing the *hsp70* 3'UTR, kindly provided by K. Basler; (Struhl and Basler 1993)
pCRII®TOPO	TOPO TA cloning vector, containing SP6 and T7 promoter sequences; Invitrogen
pCRII-encDNA-4.8	pCRII vector containing a fragment of the *engrailed* gene in sense orientation; this work
pCRII-enPRE-1 and -3	pCRII vector containing the *engrailed* PRE in antisense and sense orientation, respectively; this work
pCR4®TOPO	TOPO TA cloning vector, containing T3 and T7 promoter sequences; Invitrogen
pCR4-ry-1	pCR4 vector containing a fragment of the *rosy* gene in sense orientation; this work
pCR4-ryprom	pCR4 vector containing a fragment of the *rosy* promoter in sense orientation; this work
pCR4-salm-2.1 and -2.2	pCR4 vector containing a fragment of the *spalt major* gene in antisense and sense orientation, respectively; this work
pCR4-salmPREas-3.7 and -3.5	pCR4 vector containing the predicted *spalt major* PRE in antisense and sense orientation, respectively; this work
pCR4-slou-5 and –as-2	pCR4 vector containing a fragment of the *slouch* gene in sense and antisense orientation, respectively; this work

pCR4-slouPRErev-7.5 and -7.7	pCR4 vector containing the predicted *slouch* PRE in antisense and sense orientation, respectively; this work
pCR4-tll-4.8	pCR4 vector containing a fragment of the *tailless* gene in antisense orientation; this work
pCR4-tllPREas-5.4 and -5.3	pCR4 vector containing the predicted *tailless* PRE in antisense and sense orientation, respectively; this work
pFAs	*Fab-7* reporter construct: *Fab-7* PRE cloned downstream of the *actin5C* promoter; sense transcription (also: pUZmod-actin-Fab(pd)); (Schmitt 2002)
pFAas	*Fab-7* reporter construct: *Fab-7* PRE cloned downstream of the *actin5C* promoter; antisense transcription (also: pUZmod-actin-Fab(dp)); (Schmitt 2002)
pFHs	*Fab-7* reporter construct: *Fab-7* PRE cloned downstream of the zygotic *hunchback* promoter; sense transcription (also: pUZmod-hb-Fab(pd)); (Schmitt 2002)
pFHas	*Fab-7* reporter construct: *Fab-7* PRE cloned downstream of the zygotic *hunchback* promoter; antisense transcription (also: pUZmod-hb-Fab(dp)); (Schmitt 2002)
pFLA	*Fab-7* reporter construct: *Fab-7* PRE cloned downstream of the *actin5C* promoter and lambda control DNA; this work (map see appendix)
pFTA	*Fab-7* reporter construct: *Fab-7* PRE cloned downstream of the *actin5C* promoter and *hsp70* 3'UTR transcription terminator; this work (map see appendix)
pMK26/ACTSV40BS	expression vector for *Drosophila* tissue culture cells, containing the *actin5C* promoter, kindly provided by M. Koelle
pSV-paX1	vector containin loxP recombination sites; (Buchholz et al. 1996)

4.6 Bacterial cell lines

TOP10	*E.coli F-mcr*A Δ(*mrr-hs*ΔRMS-*mcr*BC) Φ*lacZ*dM15 Δ*lac*X74 *rec*A1 *arad*139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G; Invitrogen
XL1-Blue	*E.coli recA – (recA1 lac – endA1 gyrA96 thi hsdR17 supE44 relA1 {F' proAB lacIq lacZDM15Tn10});* Stratagene

4.7 Cell culture lines

SF4	diploid derivative of SL-2 tissue culture cells (Schneider 1972); kindly provided by P. Becker, Munich

4.8 Fly lines

4.8.1 General fly lines

Genotype	*Donor*
w[1118]	Paro lab stock collection
w[1118]; BcGla/CyO	Paro lab stock collection
w[1118]; TM6B,Tb[1]/TM3,Sb[1]	Paro lab stock collection
w[1118]; CyO/Sp; TM2,Ubx/MKRS	Paro lab stock collection

4.8.2 GAL4 drivers, lacZ, and GFP transgenic lines

Genotype	*Donor*
yw; P(eyGAL4 EGFP)47	S. Schönfelder, (Schönfelder 2005)
b pr cn Df vgD/CyO,hb-lacZ[ry^{+}]	C. Maurange
w[1118], P{w[+mC]=His2Av[T:Avic/GFP-S65T]}62A	Bloomington Stock Center, No. 5941

4.8.3 Mutations

Genotype	*Donor*
Fab7[1]	Paro lab stock collection

4.8.4 Transgenic lines expressing site-specific recombinases

Genotype	*Donor*
y[1]w[67c23]; noc[Sco]/CyO, P{w[+mC]=Crew}DH1	Bloomington Stock Center, No. 1092
P{ry[+t7.2]=hsFLP}12, y[1] w[*]; noc[Sco]/CyO	Bloomington Stock Center, No. 1929
UASP-Cre EBD 304 II.6	C. Lehner, No. 1043 (Heidmann and Lehner 2001)
UASP-Cre EBD 304 II.6; MKRS/TM2,Ubx	This work

4.8.5 Generated fly lines

pFAs-1	is identical to *pUZmod-actin5c-Fab(pd) 32.1 (II),* (Schmitt 2002)
pFAs-1 Cre	*pFAs-1,* after Cre/*loxP* recombination, this work
pFAs-1 Flp	*pFAs-1*, after Flp/*FRT* recombination, this work
pFAs-1 Flp Cre	*pFAs-1*, after Flp/*FRT* and Cre/*loxP* recombination, this work
pFAs-1; Fab7[1]	*pFAs-1,* in *Fab7[1]* deletion background, this work
pFAs-1 Cre; Fab7[1]	*pFAs-1 Cre,* in *Fab7[1]* deletion background, this work

pFAs-2	is identical to *pUZmod-actin5c-Fab(pd) 23.1 (III),* (Schmitt 2002)
pFAs-2 Cre	*pFAs-2,* after Cre/*loxP* recombination, this work
pFAs-2 Flp	*pFAs-2*, after Flp/*FRT* recombination, this work
pFAs-2 Flp Cre	*pFAs-2*, after Flp/*FRT* and Cre/*loxP* recombination, this work
pFAas-1	is identical to *pUZmod-actin5c-Fab(dp) 14.1 (III),* (Schmitt 2002)
pFAas-1 Cre	*pFAas-1*, after Cre/*loxP* recombination, this work
pFAas-1 Flp	*pFAas-1*, after Flp/*FRT* recombination, this work
pFAas-1 Flp Cre	*pFAas-1*, after Flp/*FRT* and Cre/*loxP* recombination, this work
pFAas-2	is identical to *pUZmod-actin5c-Fab(dp) 23.1 (III)*, (Schmitt 2002)
pFAas-2 Cre	*pFAas-2,* after Cre/*loxP* recombination, this work
pFAas-2 Flp	*pFAas-2*, after Flp/*FRT* recombination, this work
pFAas-2 Flp Cre	*pFAas-2*, after Flp/*FRT* and Cre/*loxP* recombination, this work
pFTA-1	is identical to *pFTA B2 III*, carries the pFTA construct on the third chromosome (pairing-sensitive silencing, PSS), this work
pFTA-1 Cre	*pFTA-1*, after Cre/*loxP* recombination, this work
pFTA 1 II/CyO	carries the pFTA construct on chromosome II (PSS), this work
pFTA S1 X	carries the pFTA construct on the X chromosome, this work
pFTA S1 X Cre	*pFTA S1 X,* after Cre/*loxP* recombination, this work
pFTA 2.1 III	carries the pFTA construct on chromosome III, this work
pFTA A1 III	carries the pFTA construct on chromosome III, this work
pFLA-1	is identical to *pFLA 11.2 III*, carries the pFLA construct on chromosome III, this work
pFLA-1 Cre	*pFLA-1,* after Cre/*loxP* recombination, this work
pFLA 9.1 III	carries the pFLA construct on chromosome III, this work
pFLA 9.1 III Cre	*pFLA 9.1 III*, after Cre/*loxP* recombination, this work
pFLA 2.3 III	carries the pFLA construct on chromosome III, this work
pFLA 2.3 III Cre	*pFLA 2.3 III,* after Cre/*loxP* recombination, this work
pFLA 3.1 II	carries the pFLA construct on chromosome II, this work
pFLA 3.1 II Cre	*pFLA 3.1 II*, after Cre/*loxP* recombination, this work
pFLA 4.2 II	carries the pFLA construct on chromosome II, this work
pFHs-1	is identical to *pUZmod-hb-Fab(pd) 18.2 (II),* (Schmitt 2002)
pFHs-1 Cre	*pFHs-1*, after Cre/*loxP* recombination, this work
pFHs-1 Flp	*pFHs-1*, after Flp/*FRT* recombination, this work
pFHs-1 Flp Cre	*pFHs-1*, after Flp/*FRT* and Cre/*loxP* recombination, this work
pFHas-1	is identical to *pUZmod-hb-Fab(dp) 37.1 (III),* (Schmitt 2002)
pFHas-1 Cre	*pFHas-1*, after Cre/*loxP* recombination, this work
pFHas-1 Flp	*pFHas-1*, after Flp/*FRT* recombination, this work
pFHas-1 Flp Cre	*pFHas-1,* after Flp/*FRT* and Cre/*loxP* recombination, this work

4.9 Technical devices

4.9.1 Microscopy

Microscopes	Olympus BX60
	Leica TCS SP2
	Leica DMRXA

Stereomicroscopes	Leica MS5 Leica MS7,5 Leica MZFLIII
Camera	Olympus DP50 Hamamatsu C4742-95
Lamp	Leica KL1500 LCD
Software	OpenLab 1.7.8 Studio Lite 1.0

4.9.2 Microinjection

Femtotips	Eppendorf
Microloader tips	Eppendorf
Microinjector	FemtoJet®, Eppendorf
Micromanipulator	Leitz
Microscope	Leitz Labovert

4.9.3 Agarose gel electrophoresis

Gel electrophoresis chamber	ZMBH fine mechanics workshop
Voltage source	LKB/Bromena 2301 Pharmacia EPS 500/400

4.9.4 Data processing

Computer	Apple G4
Software	Adobe Illustrator CS2 Adobe Photoshop CS2 Endnote 9.0 Microsoft Excel Microsoft Powerpoint Microsoft Word

4.9.5 Further devices and materials

Canula	ecoLab
Coverslips	Menzel
DABCO	Merck
DAPI	Sigma
Double-sided sticky tape	Scotch 3M
Filters (0.2 µm, 0.45 µm)	Schleicher & Schuell
Filter paper	Whatman, 3 mm
Fly cages	ZMBH fine mechanics workshop
Forceps	A. Dumont & Fils
GeneAmp PCR System 9700	Applied Biosystems
Highspeed Plasmid Midi Kit	Qiagen
Horizontal shaker	GFL – Gesellschaft für Labortechnik
Magnetic stirrer	Ikamag
Micropestle	Eppendorf
Microwave	Panasonic Dimension

Nylon membrane	Roche
Oligotex mRNA isolation kit	Qiagen
Pipetman	IBS Integra Biosciences
Pipettes (1 ml, 200 µl, 100 µl, 2 µl)	Gilson
Petri dishes	Greiner
pH meter	inoLab
Phosphorimager	Fujifilm FLA-3000
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Rotator	Heidolph
Slides	Menzel
Syringes	BD Biosciences
Table top centrifuge	Eppendorf
Thermomixer	Eppendorf
Vortex Genie	Bender & Hobein AG
Waterbath	Julabo EcoTemp EW, Julabo
Centrifuge	Beckmann J2-MC

4.10 Chemicals

Acetic acid	AppliChem
Actinomycin D	Sigma
Agarose ultra pure	GibcoBRL
BCIP/NBT stock solution	Roche
Bioruptor UCD-200	Diagenode
Bromophenol blue	Serva
DEPC	Sigma
DMSO	AppliChem
dNTPs	Peqlab
DTT	Merck
EDTA	Merck
EGTA	E. A. Thomas
Estrogen	Sigma
Ethanol	AppliChem
Ethidium bromide	AppliChem
Formamide	AppliChem
37% Formaldehyde solution	E. A. Thomas
25% Glutardialdehyde	Merck
Glycerol	AppliChem
Glycine	AppliChem
Hepes	AppliChem
Isopropanol	AppliChem
KCl	Merck
$K_4(Fe(CN)_6)$	Serva
$K_3(Fe(CN)_6)$	Fluka
KH_2PO_4	Merck
LiCl	Sigma
Maleic acid	Fluka
Methanol	AppliChem
Milk powder	Humana Anfangsmilch PRE

$MgCl_2$	Merck
NaCl	Merck
Na-Deoxycholate	Sigma
Na_2HPO_4	Merck
Na-hypochlorite	E. A. Thomas
n-heptane	AppliChem
Nipagin	Merck
NP-40	Sigma
Orange G	Sigma
p-Formaldehyde	Sigma
PMSF	Serva
Poly(L)lysine	Sigma
Propionic acid	Merck
SDS	Merck
Sodium citrate	AppliChem
Sucrose	Merck
Tris	AppliChem
Triton X-100	Merck
TRIzol® Reagent	Invitrogen
Tween-20	Sigma
Voltalef-10S oil	elf aquitaine
X-Gal	AppliGene

Methods

5. Methods

5.1 Molecular methods

5.1.1 Phenol-chloroform extraction of DNA

An equivalent volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to a DNA solution and vortexed thoroughly for at least 1min. The emulsion was centrifuged for 5min at 13.000 rpm at RT (Eppendorf table top centrifuge). The upper aqueous phase containing the DNA was carefully transferred into a new Eppendorf tube. One volume of chloroform was added, vortexed for 1min, and centrifuged for 5 min at 13.000 rpm at RT. The upper aqueous phase was transferred to a new tube. The procedure was repeated once, followed by precipitation of the DNA with ethanol.

5.1.2 Ethanol precipitation of DNA

1/10 volume of 3.5 M sodium acetate pH 5.2 and 2.5 volumes 100% ethanol (-20°C) were added to the DNA sample, mixed, and incubated for at least 1 h at -20°C. If small amounts of DNA were to be precipitated, 1 µl glycogen (20 mg/ml, Roche) was added as a carrier. The precipitated material was centrifuged for 45 min at 13.000 rpm at 4°C. The supernatant was removed and the preciptated DNA was vacuum-dried and resuspended in TE-buffer or ddH_2O.

TE-buffer pH 8.0: 10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0

5.1.3 Analysis of DNA fragments by agarose gel electrophoresis

Depending on the size of the DNA fragments to be separated, 0.5-2% (w/v) agarose gels were used. Ethidium bromide was added to a final concentration of 0.5 µg/ml. Samples supplemented with DNA sample buffer were loaded and electrophoresis was conducted at 100-250V for 30 min – 2 h at 4°C. After separation, the gels were analyzed using a transilluminator with UV light, photographed, and printed with a RAYTEST IDA (Image and Documentation Analysis) gel documentation device. For preparative gels, the fragment of interest was cut out under UV light and the DNA was purified.

Buffers:	1x TAE buffer:	40 mM Tris-acetate
		1 mM EDTA pH 8.0
	Ethidium bromide stock:	10 mg/ml
	6x DNA sample buffer:	0.25% (w/v) Bromophenol-blue or OrangeG
		30% (w/v) glycerol
		0.1 mM EDTA

5.1.4 PCR purification / gel extraction

For the purification of DNA following PCR reaction or prior to restriction endonuclease digestion, the QIAquick PCR purification kit (Qiagen) was used according to the manufacturer's protocol. The DNA was eluted in 30-50 µl ddH_2O. Alternatively, the MiniElute PCR purification kit (Qiagen) was used which allows the concentration of DNA in 10 µl ddH_2O.

To purifiy DNA from agarose gels, the QIAquick gel extraction kit (Qiagen) was applied as described in the manufacturer's manual. The DNA was eluted in 30-50 µl ddH2O.

5.1.5 Restriction endonuclease digestion of DNA

For analytical digests, 100-300 ng DNA were digested with 1-10 U of restriction enzyme (New England Biolabs) with its appropriate buffer according to the manufacturer's protocol in a volume of 20 µl. In general, the digests were incubated for 1 h at 37°C.

For preparative digests, 5-10 µg DNA were used with 10-40 U of restriction enzyme. Digest were incubated for 1-4 h up to overnight at 37°C. For some reactions, a heat inactivation step at 65°C for 20 min was necessary to inactivate the restriction enzyme, or a phosphatase treatment to prevent re-ligation. Afterwards, the digested DNA was purified using the QIAquick PCR purification kit or isolated by preparative agarose gel electrophoresis.

Digestions with two different restriction enzymes were performed either in parallel or sequentially with one purification step using the QIAquick PCR purification kit in between.

5.1.6 Phosphatase treatment of DNA

To prevent re-circularization of a digested vector DNA in a ligation reaction, the DNA was treated either with Shrimp Alkaline Phosphatase (SAP, New England Biolabs) wor Calf Intestine Phosphatase (CIP, New England Biolabs) to remove the 5' end phosphate group of the DNA. Phosphatase treatment was typically performed directly following a restriction digest in the same buffer. For the SAP reaction, 0.5 U/µl enzyme were added to the digestion

reaction, incubated for an additional hour at 37°C prior to heat inactivation at 65°C for 20 min. For the CIP reaction, 0.5 U/µl enzyme were added, incubated for 1 h at 37°C, and the DNA was isolated by agarose gel electrophoresis or spin column purification using the PCR purification kit.

5.1.7 Ligation of DNA fragments

Ligation reactions were prepared with 300 ng vector DNA and a 3fold molar excess of insert DNA in 10µl with 1 U T4 DNA ligase (Roche) according to the manufacturer's instructions. The reaction was incubated either for 2 h at RT or overnight at 16°C.

5.1.8 End-filling of DNA single strands

1 µl of Klenow fragment (2 U/µl; Roche) and 5 µl 2.5 mM dNTP mix (dATP, dTTP, dCTP, dGTP) were added to 50µl restriction endonuclease digestion reactions, followed by a 15 min incubation at 30°C. Subsequently, the DNA was purified using the QIAquick PCR purification kit (Qiagen).

5.1.9 Preparation of LB agar plates

For agar plates, 1,5% Bacto-Agar was added to the LB medium. After boiling in a microwave, the appropriate antibiotic was supplemented to a final concentration of 100 µg/ml for Ampicillin, and 34 µg/ml for Chloramphenicol.

LB (Luria-Bertani) medium:	1% (w/v) Bacto-tryptone
	0.5% (w/v) yeast extract
	1% (w/v) NaCl
	pH 7
Ampicillin stock:	100 mg/ml
Chloramphenicol stock:	34 mg/ml

5.1.10 Freezing of bacteria

Sterile glycerol (240 µl) was added to 760 µl of an overnight bacterial culture, which was then immediately vortexed, shock-frozen in liquid nitrogen, and stored at -80°C.

5.1.11 Production of electro-competent XL1-blue E.coli cells

A single colony of XL1-blue *E.coli* cells was inoculated into 10 ml SOC medium and incubated overnight at 37°C under vigorous shaking. The next day, 4 ml of this culture were transferred into 1 l prewarmed SOC medium and incubated at 37°C until a density of $OD_{600} \approx$

0.6 was reached. The following steps were conducted at 4°C using precooled material. The culture was centrifuged for 12 min at 5000 g. The sedimented cells were resuspended in a large volume of ddH_2O and re-centrifuged. This step was repeated twice. Finally, cells were resuspended in 5 ml of freshly prepared 10% glycerol, aliquoted into sterile 1.5 ml Eppendorf tubes, and shock-frozen in liquid nitrogen. Competent cells were stored at -80°C.

SOB medium: 2% (w/v) Bacto-tryptone
0.5% (w/v) yeast extract
0.05% (w/v) NaCl
2.5 mM KCl
10 mM $MgCl_2$
pH 7.5

SOC medium: SOB medium
20 mM glucose (sterile)

5.1.12 Transformation of E.coli by electroporation

Electro-competent cells were thawed on ice. The DNA (50-100 ng for a re-transformation, or 2-10 µl of a ligation reaction) was added to a 50 µl cell aliquot and transferred to a precooled cuvette (Equibio). Electroporation was performed at 1250 V with an Eppendorf Electroporator 2510. Afterwards, 1 ml LB medium was added and cells were incubated for 1 h at 37°C on a roller shaker. Different aliquots of transformed cells (50 µl – 1 ml) were plated on LB agar plates supplemented with the appropriate antibiotic and incubated overnight at 37°C.

5.1.13 TOPO TA cloning

TOPO TA cloning (Invitrogen) allows efficient cloning of Taq polymerase-amplified PCR products into various TOPO TA vectors. For PCR amplification, either the Taq polymerase (Qiagen) or the Expand High Fidelity PCR System (Roche) was used. Usually, the ligation reaction was set up with half the volumes recommended and was incubated for 15-20 min at RT:

0.5 µl salt solution (TOPO kit)
0.5 µl TOPO TA cloning vector
2 µl PCR product

2 µl of the ligation reaction were added to an aliquot of TOP10 chemically competent cells (Invitrogen) and mixed gently. After a 15-20 min incubation on ice, the cells were transformed by a 30 sec heat-shock in a 42°C waterbath without shaking. The transformed

cells were immediately transferred onto ice. Subsequently, 250 µl SOC medium were added and the reaction tubes were incubated on a horizontal shaker (200 rpm) at 37°C for 1 h. 50µl and 200 µl aliquots were spread onto prewarmed LB agar plates containing the appropriate selection antibiotic and incubated at 37°C overnight.

5.1.14 Isolation of Plasmid DNA from bacteria

For DNA isolation in general, alkaline lysis was applied using the buffers supplied by Qiagen.

Small scale DNA preparation (Mini Prep)

A single colony was inoculated into 3 ml LB medium supplemented with the appropriate antibiotic and incubated overnight at 37°C under vigorous shaking. 1.5 ml of the overnight culture was transferred into a 1.5 ml Eppendorf tube and centrifuged for 1 min at 13.000 rpm (Eppendorf table top centrifuge). The supernatant was discarded and another 1.5 ml of overnight culture were added, and the centrifugation step was repeated. The cell pellet was resuspended in 150 µl buffer P1, and then 150 µl of buffer P2 were added. After gentle mixing, cell lysis was allowed to proceed for 5 min at RT. Then 300 µl buffer P3 were added and after gentle mixing, the tubes were incubated on ice for 10 min. The bacterial lysate was centrifuged for 30 min at 13.000 rpm at 4°C. The supernatant was transferred into a new tube and the DNA was precipitated by adding 0.7 volume of 100% isopropanol. The precipitated DNA was pelleted by centrifugation at 13.000 rpm for 45 min at 4°C. To wash the DNA pellet, 1 ml 70% ethanol was added and the samples were centrifuged for 10 min at 13.000 rpm and 4°C. The DNA pellet was vacuum-dried and resuspended in 30-50 µl ddH_2O.

Buffers:	P1:	50 mM Tris-HCl pH 8.0
		10 mM EDTA
		100 µg/ml RNase A
	P2:	200 mM NaOH
		1% SDS
	P3:	3 M potassium acetate pH 5.5

Large scale DNA preparation (Midi Prep)

The Highspeed Midi kit from Qiagen was used to isolate larger quantities of pure DNA according to the manufacturer's protocol. The DNA was eluted in 500 µl ddH_2O.

5.1.15 Polymerase Chain Reaction (PCR)

The template DNA was amplified by PCR using Taq- (Qiagen) or High Fidelity Taq/Pwo-polymerase (Roche) as appropriate. Specific sense and antisense oligonucleotide primer flanking the desired target sequence were used.

A typical PCR reaction was prepared as follows:

0.1 – 100 ng	DNA
5 µl	10x appropriate reaction buffer
0.5 µl	25 mM dNTP-mix (dATP, dTTP, dGTP, dCTP; Peqlab)
0.5 µl	sense primer (100 pmol/µl)
0.5 µl	antisense primer (100 pmol/µl)
1U	DNA polymerase
ad. 50 µl	H_2O

For PCR reactions using genomic DNA, 50-100 ng were used as a template. The following parameters were adjusted for each PCR reaction, depending on the template, the size of the fragment to be amplified, and the melting temperatures of the primer:

5 min	94°C	
30-45 sec	94°C	
30-45 sec	55-62°C	25-35 cycles
0.5 – 3 min	72°C	
10 min	72°C	

5.1.16 Isolation of genomic DNA from adult flies (Quick Fly Genomic DNA Prep)

In a standard procedure, 30 flies were anesthesized, collected, and frozen at -80°C. The flies were homogenized with a micropestle (Eppendorf) in 100 µl Buffer A. An additional 100 µl of Buffer A were added and grinding continued. After addition of another 200 µl Buffer A, grinding was continued until only cuticles remained. The homogenate was incubated at 65°C for 30 min. 800 µl of freshly prepared LiCl / KAc solution were added, mixed, and left on ice for at least 10 min. After centrifugation at 13.000 rpm for 15 min at RT, 1 ml of the supernatant was transferred into a new tube, avoiding floating crud. Following another centrifugation and supernatant transfer step, DNA was precipitated by addition of 600 µl 100% isopropanol, and centrifugation at 13.000 rpm for 15 min at RT. After two washing steps with 70% ethanol, the DNA pellet was vacuum-dried. To avoid shearing of the DNA, 150 µl ddH_2O were added to the dry pellet, and the solution was left to dissolve at 4°C

overnight. On the next day, the DNA was resuspended carefully by pipetting up and down, followed by a 15 min incubation at 65°C. The DNA was then stored at -20°C.
If more or less flies were used (min. 5 flies), all volumes were adjusted accordingly.

Buffer A: 100 mM Tris-HCl pH 7.5
100 mM EDTA
100 mM NaCl
0.5% (w/v) SDS

Lithium cloride (LiCl) / potassium acetate (KAc):
Mix 1 part of 5 M KAc with 2.5 parts of 6 M LiCl immediately before use

5.1.17 Isolation of genomic DNA from single flies

Single flies were squashed in 50 µl Gloor and Engel's extraction buffer (Gloor et al. 1993) in a 1.5 ml Eppendorf tube using a 200 µl pipette tip. To isolate genomic DNA from single fly heads, flies were transferred into 1.5 ml Eppendorf tubes and flash-frozen in liquid nitrogen. To isolate the heads, the Eppendorf tubes containing frozen flies were tapped onto the table. Using a brush, single heads were transferred into fresh Eppendorf tubes and homogenized as above, using 20 µl of Gloor and Engel's extraction buffer per sample. The homogenate was incubated for 20 min at 37°C, and then for 2 min at 80°C to inactivate the proteinase K. The DNA was stored at 4°C. Typically, 4µl of DNA isolated from whole flies, or 5µl of DNA isolated from single heads were used in a standard PCR reaction.

Gloor and Engel's extraction buffer: 10 mM Tris pH 8.2
1 mM EDTA
25 mM NaCl
200 µg/ml proteinase K (20 mg/ml stock, added freshly)

5.1.18 Southern Blot

Synthesis of DIG-labeled DNA probe and test hybridization

For probe production, the DIG High Prime DIG labeling kit (Roche) was used according to the manufacturer's instructions. Briefly, 1 µg of template DNA in a 18 µl volume were denatured by boiling for 10 min, then quickly chilled on ice. 4 µl of DIG High Prime mix were added. The reaction was mixed, centrifuged briefly, and then incubated at 37°C

overnight. The next day, the labeled DNA probe was purified using the QIAquick PCR purification kit, and eluted in 50 µl ddH_2O.

The efficiency of probe synthesis and labeling was tested by spotting 1 µl of serial dilutions of the probe, starting from 1:50 to up to 1:100.000, onto a positively charged nylon membrane (Roche). The DNA was cross-linked to the membrane twice at 1200µJ using an UV Stratalinker (Stratagene). Detection was performed as described below.

Preparation of DNA, restriction endonuclease digestion, and gel electrophoresis

Genomic DNA was isolated from flies as described above. 20 or 30 µl of DNA were used which corresponds to 8 or 12 flies, respectively. The DNA was digested with EcoRI or EcoRI/HindIII for at least 4 h at 37°C in 50 µl volume. The DNA was ethanol-precipitated for at least 1 h at -20°C as described, with 1 µl glycogen (Roche, 20 mg/ml) added as a carrier, and resuspended in 20 µl ddH_2O. The DNA samples supplemented with loading buffer were loaded on a 0.8% agarose gel and run overnight at 30-40 V and 4°C.

Denaturation, neutralization, and transfer

To prepare the separated DNA in the agarose gel for the transfer to a nylon membrane, the gel was submerged in 0.25 M HCl for 10 min on a shaker at RT and rinsed with ddH_2O prior to the incubation for 2x 15 min in denaturation buffer. After shortly rinsing in ddH_2O, the gel was incubated for 2x 15 min in neutralization buffer, followed by an additional rinse step and a 10 min incubation in 20x SSC buffer. For the capillary transfer to the membrane, the gel was placed upside down on Whatman paper (Schleicher & Schuell) on a glass plate, with its left and right ends hanging in 20x SSC buffer. The positively charged nylon membrane (Roche) was moistened with 20x SSC buffer and put on the upper side of the gel. Three layers of Whatman paper and a layer of approximately 10 cm of apura paper were put on top of it and fixed with a weighted glass plate. Transfer was allowed to proceed overnight. The next day, the positions of the gel and the lanes were marked on the membrane using a pencil. Then the membrane was washed for 5 min in ddH_2O prior cross-linking of the DNA with UV light. Cross-linking of the wet membrane was first performed twice on the upper nylon membrane side, then twice on the lower side at 1200µJ. The blot was either used directly for hybridization or sealed in a nylon bag and stored at 4°C.

Solutions:	Denaturation buffer:	0.5 M NaOH
		1.5 M NaCl
	Neutralization buffer:	0.5 M Tris-HCl pH 7.5
		3 M NaCl

	20x SSC:	3 M NaCl
		300 mM Sodium citrate pH 7.0

Hybridization

The DIG Easy Hybridization solution (Roche) was prepared according to the manufacturer's instructions and prewarmed to 40°C. The nylon membrane was blocked by pre-hybridizing it with the solution for 30 min at 40°C. The DIG-labeled DNA probe was denatured by boiling at 95°C for 5 min and immediately put on ice to prevent renaturation. After addition of denatured probe (in general 50 µl) to 8 ml DIG Easy Hybridization solution, the blot was hybridized at 40°C overnight with gentle agitation. The membrane was first washed twice for 5 min in a large volume of wash buffer 1 at RT, then twice for 15 min in wash buffer 2 at 68°C, both steps under constant agitation.

Buffers:	Maleic acid buffer:	0.1 M Maleic acid
		0.15 M NaCl
		adjust to pH 7.5 with NaOH pellets
	Wash buffer 1:	1x SSC
		0.1% (w/v) SDS
	Wash buffer 2:	0.1x SSC
		0.1% (w/v) SDS

Detection

After transferring the membrane into a flat container, it was rinsed 2-5 min in washing buffer and incubated for 30 min in 100 ml freshly prepared blocking solution (1% (w/v) blocking reagent, Roche, in washing buffer) according to the manufacturer's instructions. The α-DIG-AP conjugate was diluted to 75 mU/ml (1:10.000) in 20 ml blocking solution. Antibody incubation was done for 30 min at RT, then the membrane was washed twice for 15 min in 100 ml washing buffer and equilibrated for 2-5 min with 20 ml detection buffer. For detection, the membrane was transferred into a hybridization bag, 1 ml CSPD (Roche) was added and left for 5 min at RT. To activate the CSPD, the hybridization bag was sealed and incubated for 10 min at 37°C. The membrane was then exposed to a film (Roche) for 15-30 min.

Buffers:	Washing buffer:	0.1 M Maleic acid
		0.15 M NaCl
		0.3% (v/v) Tween-20
		adjust to pH 7.5

Detection buffer: 0.1 M Tris-HCl pH 9.5
0.1 M NaCl
50 mM $MgCl_2$

5.1.19 RNA isolation

For isolation of total RNA from *Drosophila* embryos, embryos were collected on acetic acid agar plates (see 5.3.2). Using a brush and tap water, embryos on agar plates were transferred into self-made washing sieves (using a 74 µm mesh size sieve, Neolab) and washed extensively with tap water. Excessive liquid was removed and embryos were dechorionated by incubating them for 2.5-3 min in a 3% Na-hypochlorite / PBS solution. After thorough washing in tap water, excessive liquid was removed. Using a spatula, embryos were transferred into a 15 ml Falcon tube and weighed. Per 50 mg of embryos, 1 ml TRIzol® Reagent (GibcoBRL) was added and the embryos were homogenized mechanically using a Polytron UltraTurrax homogenizer (generally 60-90 sec). For RNA isolation from SF4 tissue culture cells, 5x 10^6 cells were pelleted by centrifugation, resuspended in 0.5 ml TRIzol® Reagent, and homogenized by pipetting up and down. At this step, RNA was either directly isolated or the material was flash-frozen in liquid nitrogen and stored at -80°C.

To isolate RNA, the homogenized material was thawed, transferred into Eppendorf tubes, and centrifuged for 10 min at 12.000 rpm (Eppendorf table top centrifuge) at 4°C. The supernatant was transferred into a new tube and left for 5 min at RT. Then 0.2 ml chloroform per 1 ml TRIzol® Reagent were added and the mixture shaken gently for 15 sec. After a 2-3 min incubation at RT, the samples were centrifuged for 15 min at 12.000 rpm at 4°C. The aqueous upper phase was transferred into a fresh Eppendorf tube and the RNA was precipitated by adding 0.25 ml isopropanol and 0.25 ml sodium citrate/NaCl per 1 ml TRIzol® Reagent and incubation for 10 min at RT. To precipitate low amounts of RNA, 1 µl glycogen (20 mg/ml stock, Roche) was added as carrier. The samples were centrifuged for 10 min at 12.000 rpm at 4°C, and the pellets were washed twice in 70% ethanol. Between the washing steps, the samples were centrifuged for 5 min at 7.500 rpm at 4°C. The RNA pellets were air-dried for approximately 1 min, and excess liquid was removed using a pipet tip (avoid prolongued drying, otherwise the pellet will not solubilize well). RNA was resuspended in DEPC-treated ddH_2O, flash-frozen in liquid nitrogen, and stored at -80°C.

Solutions: TRIzol® Reagent (GibcoBRL)
chloroform
100% Isopropanol

0.8 M Sodium citrate / 1.2 M NaCl

70% Ethanol

DEPC-treated ddH_2O

5.1.20 Purification of poly(A)$^+$ RNA from total RNA

To isolate poly(A)$^+$ RNA from total RNA, the Oligotex mRNA mini kit (Qiagen) was used according to the manufacturer's protocol (Oligotex mRNA Spin-Column Protocol).

5.1.21 DNase treatment of RNA

To remove DNA contamination from RNA preparations, the samples were subjected to DNase treatment. To do this, two alternative procedures were applied.

Digestion of DNA with DNase I:

Typically, the following reactions were set up in 250µl PCR tubes:

10 µg RNA

5 µl 10x NEB2 buffer (New England Biolabs)

2.5 µl RNasin (40 U/µl, Promega)

2.5 µl DNase I, RNase –free (10 U/µl, Roche)

ad. 50 µl DEPC-treated ddH_2O

The reactions were incubated for 1 h at 37°C in a thermocycler, followed by a 5 min incubation at 75°C to inactivate the enzyme. Subsequently, the RNA was purified by phenol-chloroform extraction and ethanol precipitation (see under 5.1.1 and 5.1.2).

DNase treatment using the Ambion DNA-free™ kit

Up to 20 µg of total RNA were digested using the DNA-free™ kit (Ambion) according to the manufacturer's instructions.
DNase-treated RNA samples were flash-frozen in liquid nitrogen and stored until use at -80°C.

5.1.22 Reverse transcription

cDNA synthesis for subsequent amplification by PCR

Generally, cDNA synthesis using M-MLV reverse transcriptase (Promega) was performed as described in the manual. Briefly, reactions were set up in 250 µl PCR tubes as follows:

1 µg total DNA-free RNA or up to 1 µg poly(A)$^+$ RNA

250 ng random hexamer primer (Roche) or 2 pmol gene-specific primer

ad. 13 µl DEPC-treated ddH_2O

The samples were denatured for 5 min at 70°C in a thermocycler, and immediately transferred onto ice to prevent renaturation. Then

1 µl RNasin (40 U/µl, Promega)

5 µl 5x reaction buffer (Promega)

5 µl 10 mM dNTP-mix (dATP, dTTP, dCTP, dGTP; Peqlab)

1 µl M-MLV reverse transcriptase (200 U/µl, RNase H minus, Promega)

were added and the samples were mixed. The cDNA synthesis was carried out in a thermocycler under the following conditions:

10 min 25°C

50 min 50 °C

10 min 55°C

15 min 75 °C

The reactions were either used directly or stored at -20°C. Generally, 2-5 µl of cDNA were used as template for the following PCR analysis.

cDNA synthesis for subsequent analysis by real-time PCR

For the subsequent analysis by real-time PCR, cDNA was synthesized using the SuperScriptIII™ first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. Briefly, the follwing reactions were set up in 250 µl PCR tubes:

1 µg DNA-free RNA

1.5 µl random hexamers (50 ng/µl)

1.5 µl 10 mM dNTP mix (dATP, dTTP, dCTP, dGTP; Peqlab)

ad. 15 µl DEPC-treated ddH_2O

The samples were denatured for 5 min at 65°C in a thermocycler and then quickly transferred onto ice. After > 1 min incubation,

3 µl 10x reaction buffer

6 µl 25 mM $MgCl_2$

3 µl 0.1 M DTT

1.5 µl RNaseOUT (40 U/µl)

1 µl SuperScriptIII™ reverse transcriptase (200 U/µl)

ad. 30 µl DEPC-treated ddH_2O

were added and the cDNA synthesis reaction was carried out in a thermocycler using the following conditions:

10 min 25°C

10 min 42°C
50 min 55°C
5 min 85°C

Subsequently, 0.75 µl *E. coli* RNase H (2 U/µl) were added and the samples were incubated for 30 min at 37°C to remove RNA templates. cDNA samples were stored at -20°C.

5.1.23 Real-time PCR

Primer for real-time PCR were designed to match the following criteria:

1) Avoid low complexity regions, expecially runs of 6 same nucleotides, or 4 G's
2) The length should vary between 19-23 bases, ideally 21nt
3) The melting temperature according to the nearest neighbor method should be 70-73°C
4) The GC content should be 35-65%
5) The last 5 bases (3') should have 2 or less GC. The ΔG of these 5 bases should be at least -9cal/mol, i.e. they should be unstable.
6) The primer should not form hairpins and should not self-anneal, especially via the last four bases. Internal self-annealing up to four bp is allowed.
7) The PCR product should be 100-250bp long.
8) The forward and reverse primer should not anneal to each other.
9) BLAST search should not yield other sequences in which a stretch of 15bp or more are matching.

Real-time PCR was conducted in a ABIPrism Gene Amp®5700 thermocycler. Single reactions contained 5 µl template (varying amounts), 5 µl primer mix (1200 nM or 600 nM each sense and antisense primer), and 10 µl master mix (to prepare mix, add 37.6 µl ROX reference dye to 940 µl SYBR green mix (SYBR green JumpStart™ Taq Ready™ Mix, Sigma). The PCR conditions were the following:

20 sec 25°C	
2 min 50°C	
2 min 95°C	
25 sec 95°C	
30 sec 60°C	50 cycles
30 sec 72°C	
dissociation protocol starting at 60°C	

To test the efficiency and dynamic ranges of primer pairs, serial dilutions of genomic DNA from 800 ng to 0.08 ng) were used as templates in real-time PCR reactions. The thus obtained standard curves (means of three independent experiments) were used to determine the relative amounts of templates in real-time RT-PCR reactions.
For real-time RT-PCR reactions, serial dilutions of template, starting at 5 µl of standard 30 µl cDNA reactions (see above), were used. As a negative control (no template), 5 µl of reactions from which the reverse transcriptase had been omitted were used.
For the determination of relative amounts of *AbdB* exon fragment, *AbdB* intron fragment, and *Fab-7* RNA in SF4 cells, three independent real-time RT-PCR experiments were carried out. For each individual experiment, the relative amounts of starting material were determined using the standard curves obtained from genomic DNA. To determine the relative levels of RNA following transcriptional arrest by Actinomycin D treatment, standard curves for each primer pair were generated using serial dilutions of cDNA templates. Here, two independent experiments were carried out.

5.1.24 Chromatin Immunoprecipitation (ChIP) from Drosophila embryos

Chromatin preparation

4-20 h old embryos were collected on acetic acid agar plates and dechorionated (see 5.1.19). After extensive washes in eggwash, excessive liquid was removed and up to 1 g of embryos was transferred into a 50 ml Falcon tube. After washing once in 0.01% Triton X-100 / PBS, the embryos were cross-linked for 15 min in 10 ml cross-linking solution in the presence of 30 ml n-heptane. During the incubation time, the samples were shaken vigorously on a roller. Cross-linked embryos were spun down for 1 min at 500g, and the reaction was stopped by exchanging the supernatant with 125 mM glycine / 0.01% Triton X-100 / PBS. After the embryos had sunk to the bottom, the supernatant was discarded and exchanged by 10 ml Wash A buffer. After transferring the embryos into 15 ml Falcon tubes, they were incubated in Wash A buffer for 10 min on a roller. To remove the Wash A buffer, the embryos were again centrifuged for 1 min at 500g. The embryos were resuspended in 10 ml Wash B buffer and incubated another 10 min on a roller. After centrifugation, the supernatant was discarded and approximately 300 µl of embryos per 15 ml Falcon tube were resuspended in 2 ml final volume of sonication buffer. To shear the chromatin to an average size of 200-1000bp, the Falcon tubes were transferred into a pre-cooled ultrasound sonifier (Bioruptor UCD-200, Diagenode) and sonified for 15 cycles 30 sec ON, 30 sec OFF at maximum power (H). As the efficiency of shearing varied with the number of Falcon tubes used for each sonification, the

tray was always loaded with 6 Falcon tubes, even when less samples were to be sheared. After shearing, the samples were adjusted to 0.5% N-lauroylarcosine and rotated for 10 min on a roller. Debris was removed by centrifuging the samples for 5 min at 13.000 rpm and 4°C, and transferring the supernatant into fresh Eppendorf tubes. The chromatin was flash-frozen in liquid nitrogen and stored at -80°C.

To check the quality of the chromatin, a 50 µl aliquot of each sample was extracted and analyzed by agarose gel electrophoresis: To digest the RNA, DNase-free RNase was added to each aliquot at a final concentration of 50 µg/ml, followed by a 30 min incubation at 37°C. Then, proteinase K was added to a final concentration of 500 µg/ml, and SDS to a final concentration of 1 % (w/v). To partially reverse-cross-link the samples, they were incubated for 1 h at 56°C, before the DNA was extracted with phenol / chloroform, and precipitated with ethanol, using glycogen as a carrier (see 5.1.1 and 5.1.2). The DNA was resuspended in ddH_2O, and 3-5 µg were loaded on an agarose gel.

Buffers:

PBS:
137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
2 mM KH_2PO_4

Eggwash:
0.03% Triton X-100
0.4% (w/v) NaCl
0.01% (v/v) Triton X-100 / PBS

Cross-linking solution:
50 mM Hepes pH 8
1 mM EDTA
0.5 mM EGTA
100 mM NaCl
1.8% formaldehyde, add just before cross-linking
125 mM glycine / 0.01% (v/v) Triton X-100 / PBS

Wash A:
10 mM Hepes pH 7.6
10 mM EDTA
0.5 mM EGTA
0.25% (v/v) Triton X-100

Wash B:
10 mM Hepes pH 7.6
200 mM NaCl
1 mM EDTA

	0.5 mM EGTA
	0.01% (v/v) Triton X-100
Sonication buffer:	10 mM Hepes pH 7.6
	1 mM EDTA
	0.5 mM EGTA
0.5% N-lauroylsarcosine:	prepare 20% (v/v) stock and store at RT

Chromatin Immunoprecipitation (ChIP)

100 µl Protein A Separose (Amersham) slurry per sample were pre-blocked for 2-3 h at 4°C on a rotating wheel using 1 mg/ml BSA and 1 mg/ml herring sperm DNA (Invitrogen) diluted in RIPA buffer. To remove the blocking solution, the samples were centrifuged for 2 min at 5500 rpm (Eppendorf table top centrifuge) and 4°C. After 3x 5 min washes in RIPA buffer at 4°C, 100 µg of chromatin per sample were thawed on ice and adjusted to RIPA conditions:

x µl (=100 µg) chromatin
10 µl 1M Tris pH 8
28 µl 5 M NaCl
10 µl 10% Triton X-100
1 µl 10% SDS
1 µl 10% Na-Deoxycholate
1 µl 1M PMSF
ad. 1 ml ddH_2O

1 ml of RIPA-adjusted chromatin was added to 50 µl of pre-blocked Protein A Sepharose and incubated for 2 h at 4°C on a roller for pre-clearing. After centrifugation (2 min 5500 rpm at 4°C), the pre-cleared chromatin was transferred into a new tube. One aliquot per sample was saved as input control and stored at -20°C. 2-5 µg of antibody were added per IP (5 µl of each α-PC, α-PHO, or α-TRX antisera). As a negative control, the antibody was omitted in one sample (MOCK). The samples were incubated on a rotating wheel at 4°C overnight. To isolate the antibody-chromatin complexes, 50 µl pre-blocked Protein A Sepharose per sample were added and incubated for 3 h at 4°C on a roller. To eliminate non-specific interactions, the samples were then washed twice for 3 min at 4°C in RIPA buffer, twice for 3 min at 4°C in RIPA / high salt buffer, twice for 3 min at 4°C in IP2 buffer, and twice for 3 min at 4°C in TE buffer. Between the washing steps, the samples were centrifuged for 2 min at 5500 rpm at 4°C. To be able to remove the supernatants without loss of sample, 1 ml syringes with 25 gauge needles were used.

To extract the DNA, the Protein A Sepharose complexes were first resuspended in 100 µl TE buffer, then DNase-free RNase was added to each sample (including the input controls) to a final concentration of 50 µg/ml. RNA digestion was allowed to proceed for 30 min at 37°C, before the samples were adjusted to 0.5% SDS and proteinase K was added to a final concentration of 500 µg/ml. After overnight incubation at 37°C, the samples were heated to 65°C for 6 h to reverse the cross-link. Afterwards, the DNA was extracted using phenol / chloroform (see 5.1.1). The lower phenol pase was back-extracted using an equal volume of 50 mM Tris-HCl pH 8. The aqueous phases from the phenol extraction and the back-extraction were combined and extracted with chloroform. The DNA in the upper aqueous phase was ethanol-precipitated, using glycogen as a carrier (see 5.1.2). The DNA pellets were resuspended in 50 µl ddH_2O and stored at -20°C.

Buffers:	RIPA buffer:	10 mM Tris-HCl pH 8
		140 mM NaCl
		1 mM EDTA
		1% (v/v) Triton X-100
		0.1% (w/v) SDS
		0.1% (w/v) Na-Deoxycholate
		1 mM PMSF (add just before use)
	RIPA high salt:	10 mM Tris-HCl pH 8
		500 mM NaCl
		1 mM EDTA
		1% (v/v) Triton X-100
		0.1% (w/v) SDS
		0.1% (w/v) Na-Deoxycholate
		1 mM PMSF (add just before use)
	IP2:	10 mM Tris pH 8
		250 mM LiCl
		0.5% (v/v) NP-40
		0.5% (w/v) Na-Deoxycholate
		1 mM EDTA
	TE buffer:	10 mM Tris-HCl pH 7.4
		1 mM EDTA

Analysis of precipitated material by semi-quantitative PCR

For each sample, a serial dilution of input material and 5 µl of precipitated material was used in standard PCR reactions. The PCR was adjusted to conditions under which the reaction was still in the linear range of amplification (usually 30 cycles). To quantify the relative amounts of precipitated material, 20 µl of each 50 µl PCR reaction were loaded on a 1.5% agarose gel and scanned using a Fujifilm FLA-3000 phosphorimager. The intensities of PCR bands were quantified using the AIDA imaging software.

5.2 Cloning strategies

5.2.1 Cloning of the pFAs and pFAas reporter constructs

See diploma thesis Schmitt 2003.

5.2.2 Cloning of the pFHs and pFHas reporter constructs

See diploma thesis Schmitt 2003.

5.2.3 Cloning of the pFTA and pFLA constructs

The pBS669 vector containing the *hsp70* 3'UTR transcriptional terminator was kindly provided by K. Basler (Struhl and Basler 1993). The 2kb *hsp70* 3'UTR poly(A) signal (txterm) excluding the *scs'* insulator was amplified by PCR using the primer pair hsp70 BamHI-DOWN and hsp70 HindIII-UP. The 2kb *lambda* control DNA fragment was amplified with primer pair lambda HindIII-UP and lambda BamHI-DOWN, using the DNA maker II (Roche) as a template. The PCR products were isolated by agarose gel electrophoresis and purified using the Qiagen gel extraction kit. After digestion with BamHI and HindIII and purification, the DNA fragments were subcloned into the BamHI- and HindIII-digested pSV-paX1 vector (Buchholz et al. 1996). Using the primer pair loxP-5'Asc and loxP-3'Asc, the loxP-txterm-loxP and loxP-lambda-loxP fragments were amplified by PCR, isolated by agarose gel electrophoresis, purified, and digested with *AscI*. The pFAs vector was digested with *AscI* to remove the loxP sites and the *actin5C* promoter and subsequently re-ligated. To linearize the vector, it was digested with *BglII*, and single strands filled by a Klenow reaction (see 5.1.8). The pMK26 vector (kindly provided by M. Koelle) was digested with *Acc65I* and *HindIII*, and the *actin5C* promoter was isolated by agarose gel electrophoresis and single stranded ends were filled in a Klenow reaction (see 5.1.8). The resulting *actin5C* promoter fragment and the digested pFAs vector were subsequently blunt-

end ligated. The ligation products were linearized by digesting with *AscI*, and ligated to *AscI*-digested loxP-txterm-loxP and loxP-lambda-loxP PCR fragments to yield the pFTA and pFLA constructs. Both constructs were sequenced (GATC Biotech) prior to injection.

5.2.4 Cloning of templates for the generation of RNA probes

PCR fragments spanning PRE sequences associated with different genes or exon fragments located within these genes were amplified from genomic DNA (Primer list see appendix) and cloned into pCRII-TOPO or pCR4-TOPO vectors by TOPO TA cloning (see 5.1.13). Fragments were cloned in both sense and antisense directions with respect to the orientation of the respective genes.

5.3 Drosophila handling and genetic methods

5.3.1 Drosophila handling

Fly stocks were raised on standard fly food and crossed at 25°C with 60-70% relative humidity. Fly stocks were maintained at 18°C with 60-70% relative humidity.

Standard fly food:
- 10 l H2O
- 80 g Agar-agar
- 180 g dry yeast
- 100 g soy flour
- 220 g beet syrup
- 800 g corn meal
- 24 g nipagin (methyl-4-hydroxybenzoate, Merck)
- 62.5 ml propionic acid (Sigma)

5.3.2 Preparation of acetic acid agar plates for embryo collection

22.5 g Agar-agar in 1 l ddH_2O were boiled in a microwave and allowed to cool down to approximately 60°C. 25 g sucrose and 2.5 ml 100% acetic acid were added before the mixture was poured into 9 cm diameter petri dishes. After solidification, acetic agar plates were stored at 4°C. For embryo collection, adult flies were transferred into medium-sized (9 cm diameter, 12 cm height) cages, which were closed at the bottom with acetic acid agar plates. Fresh yeast paste was added to feed the flies.

5.3.3 Generation of transgenic flies by P-element mediated germ line transformation

Transgenic flies carrying the construct of interest were generated by P-element mediated germ line transformation (Rubin and Spradling 1982; Spradling and Rubin 1982). The constructs to be injected were prepared as follows: 9 µg of plasmid DNA together with 3 µg of pUChsΔ2-3 helper DNA encoding the P transposase were ethanol-precipitated and resuspended in 30 µl of injection buffer. Prior to injection, the DNA mixture was centrifuged for 5 min at 13.000 rpm (Eppendorf table top centrifuge) and 4°C, and 2.5 µl of the supernatant were loaded into a Femtotip needle (Eppendorf). The injection needle was submerged in oil to avoid clogging.

Adult w^{1118} flies were transferred to medium size cages (9 cm diameter, 12 cm height) and were allowed to lay eggs on acetic acid agar plates for 25min at 25°C. Using a brush and tap water, embryos were transferred into a sieve and washed extensively. After dechorionation by a 2.5 min incubation in bleach and extensive washing in tap water, 80-100 embryos were transferred onto a 1 cm x 3 cm block of acetic acid agar and aligned under a binocular. The aligned embryos were then transferred onto a double-sided sticky tape (3M, Scotch) mounted on a coverslip, with the posterior pole pointing toward the edge of the coverslip. Embryos were dehydrated in a closed chamber containing Silica gel for 7 min and covered with Voltalef 10S oil (Lehmann & Voss & Co.). Microinjection of DNA dissolved in injection buffer was performed under a stereomicroscope at 18°C with the Femtotip needle using an Eppendorf FemtoJet Microinjector at 200-600 hPa injection pressure. In general, about 300-400 embryos were injected per construct and were allowed to develop in oil in a humid chamber at 18°C. Only embryos in which the pole cells were not yet visible were injected, all embryos older than stage 2 (Campos-Ortega and Hartenstein 1997) were discarded. Freshly hatched first instar larvae were transferred into a vial containing standard fly food enriched with yeast paste, and were kept at 25°C until the founder G_0 generation hatched.

Injection buffer:	5 mM KCl
	0.1 mM NaH_2PO4, pH 6.8
PBS:	137 mM NaCl
	2.7 mM KCl
	10 mM Na_2HPO_4
	2 mM KH_2PO_4
	pH 7.4
Bleach:	3% Na-hypochlorite in PBS

5.3.4 Establishing transgenic lines and mapping of the integration site

The hatched founder G_0 generation flies were crossed to *w^{1118}; Bc Gla / CyO* virgins or males and progenies were then screened for the transformation marker *miniwhite*, i.e. pigmented eyes. Depending on the construct and the insertion site, the eye colour varied from almost white to orange. F_1 generation transformants were then back-crossed to *w^{1118}; Bc Gla / CyO* flies twice to map the chromosomal insertion site:

	pigmented eyes	white eyes
X chromosome	*w / P(w$^+$) ; + / Bc Gla* *w / P(w$^+$) ; + / CyO* *w / P(w$^+$) ; Bc Gla / CyO*	*w / - ; + / Bc Gla* *w / - ; + / CyO* *w / - ; Bc Gla / CyO*
2nd chromosome	*w ; P(w$^+$) / Bc Gla* *w ; P(w$^+$) / CyO*	*w ; Bc Gla / CyO*
3rd chromosome	*w ; + / Bc Gla ; P(w$^+$) / +* *w ; + / CyO ; P(w$^+$) / +* *w ; Bc Gla / CyO ; P(w$^+$) / +*	*w ; + / Bc Gla ; +* *w ; + / CyO ; +* *w ; Bc Gla / CyO ; +*

After the chromosomal insertion site was mapped, homozygous fly lines were established. To do this, heterozygous flies were either crossed directly with one another and homozygous individuals identified by virtue of their eye colour. Alternatively, heterozygous flies were first crossed to appropriate Balancer flies and homozygous flies then identified by the absence of the dominant marker of the respective Balancer. For the pFAs, pFAas, pFHs, and pFHas lines, single insertions were verified by Southern Blotting.

5.3.5 Flp/FRT site-specific germ line recombination

To excise the *Fab-7* PRE from the pFAs, pFAas, pFHs, and pFHas transgenes *(P(w$^+$)),* the transgenic lines were crossed with a line expressing the Flp recombinase under the control of a heat-shock promoter at 18°C (G_0) (Fig. 5.1; (Chou and Perrimon 1996):

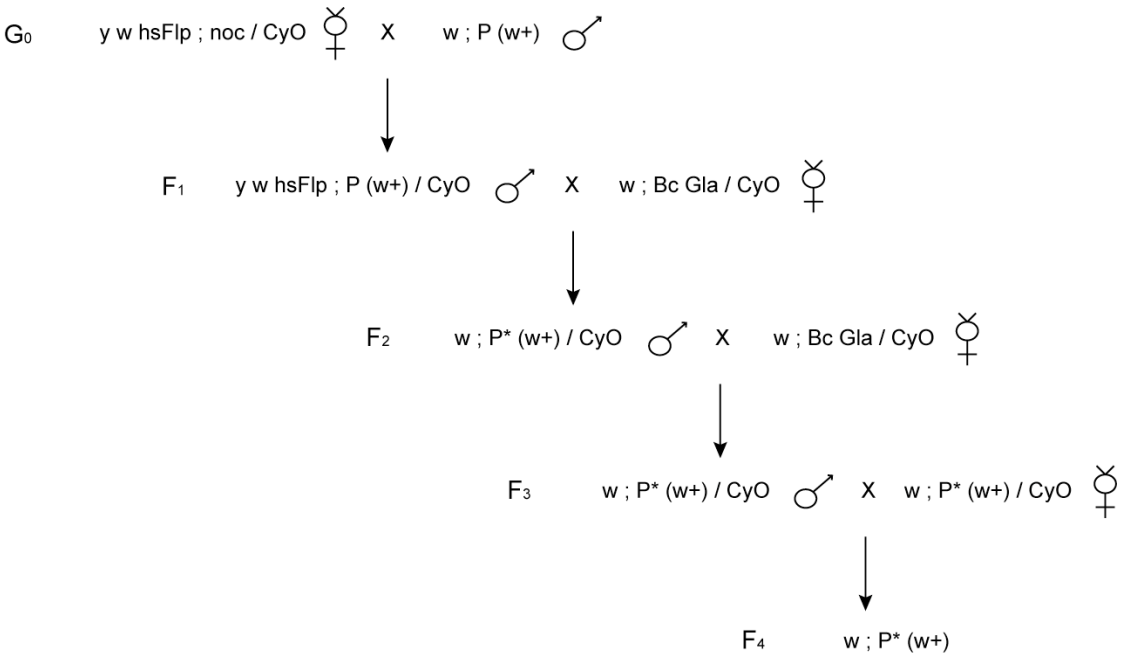

Figure 5.1: Crossing scheme to remove the *Fab-7* PRE by Flp/*FRT* recombination (see text for details).

During the first 64 h of development, the Flp recombinase was induced in the F_1 progeny by heat-shocking for 2 h at 37°C. 24 h later, a second heat-shock was applied, and the embryos were transferred to 25°C until adulthood. Males of the hatched progeny, carrying the hsFlp transgene as well as the reporter construct *(P (w+))* were crossed to *Bc Gla / CyO* balancer virgins. The males hatching in the next generation (F_2, *P* (w+)*) were again crossed to *Bc Gla / CyO* balancer virgins in single pair matings. Individuals of the F_3 generation were tested for successful recombination by single fly PCR. Recombinants *(P* (w+))* were crossed with each other to establish homozygous stocks (F_4).

5.3.6 Cre/loxP site-specific germ line recombination

To excise the *actin5C* or the zygotic *hunchback* promoter from the pFAs, pFAas, pFHs, or pFHas transgenes, transgenic males were crossed to virgins expressing the Cre recombinase (G_0):

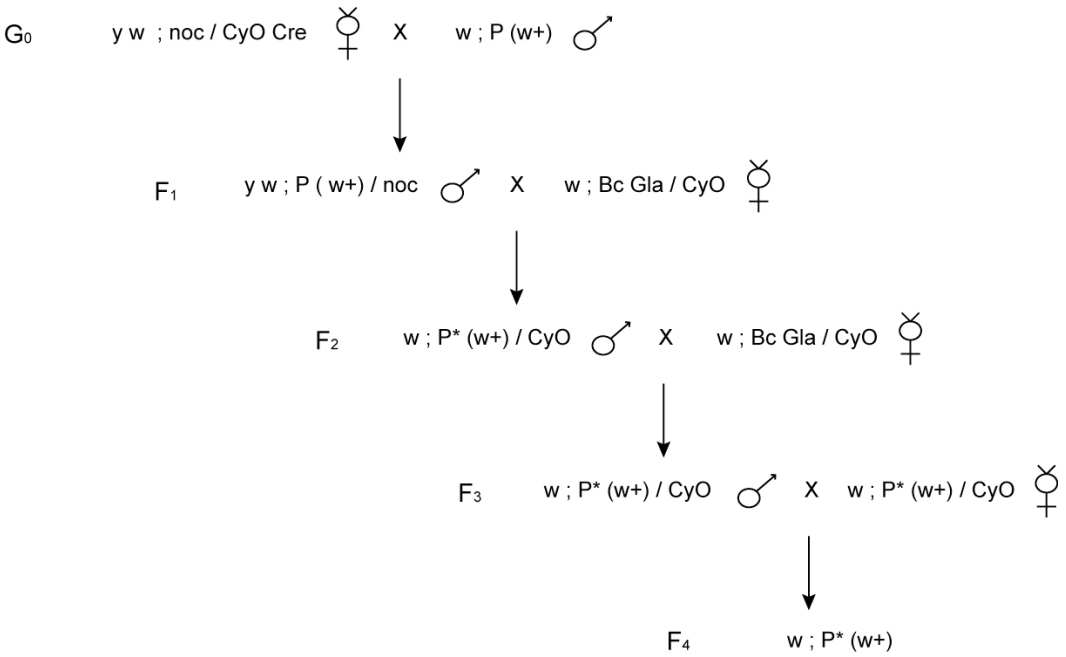

Figure 5.2: Crossing scheme to excise the *actin5C* promoter or the zygotic *hunchback* promoter by Cre/*loxP* site-specific recombination (see text for details).

The *hsp70-Mos1* promoter driving the expression of the Cre recombinase is active in both somatic and germline tissues (Siegal and Hartl 1996). Due to the strong maternal effect of Cre recombinase expression, males hatching in the next generation (F_1), bearing the transgene *(P (w+))* but not the *CyO*-marked Cre recombinase, were crossed to *Bc Gla / CyO* balancer virgins. Males hatching in the F_2 generation were again back-crossed to *Bc Gla / CyO* balancer virgins in single pair matings. In the F_3 generation, successful recombinants *(P* (w+))* were identified by single fly PCR and homozygous stocks (F_4) were established.

5.3.7 Induction of Cre/loxP recombination in first instar larvae

To excise the *actin5C* promoter from the pFAas transgene, the pFAas line as well as the *UASP-CreEBD304II.6* transgene (Heidmann and Lehner 2001) were first combined with the *eyGAL4* driver (Schönfelder 2005). Subsequently, these lines were crossed with each other in small size cages (5.3 cm diameter, 10 cm height) and embryos were collected on acetic acid agar plates. Freshly hatched first instar larvae (ca. 24h after egg laying at 25°C) were then transferred onto instant *Drosophila* medium (Sigma) lacking or containing estrogen. Estrogen concentrations varied from 0.03 mg/ml to 0.09 mg/ml. Freshly hatched flies were scored for changes in eye pigmentation and Cre recombinase induction was verified by PCR from single fly heads.

5.3.8 Determination of eye pigmentation in adult flies

To take photographs of adult fly eyes, freshly hatched female flies were left for additional 24 h at 25°C and then either photographed directly or stored at -20°C.

For quantitative pigment measurements, ten freshly hatched females were collected and left at 25°C to age for 24 h, then transferred into an Eppendorf tube. For decapitation, the flies were flash-frozen in liquid nitrogen and vortexed for about 20 sec. The heads were isolated and transferred into a fresh Eppendorf tube. For homogenization, 100 µl EPE buffer were added and the heads ground using an Eppendorf micropestle. An additional 150 µl EPE buffer were used to wash the micropestle, and extraction was allowed to proceed in the dark at 25°C for 1 h. To remove the head debris, the samples were centrifuged for 2 min at 13.000 rpm (Eppendorf table top centrifuge), and 150 µl of clear supernatant were transferred into a new tube. The centrifugation step was repeated and 120 µl of the supernatant transferred into a new tube. The relative levels of eye pigmentation were determined by measuring the absorbance at 480 nm in a photometer. As a negative control, heads of w^{1118} flies were used.

EPE buffer: 30% ethanol-HCl pH 2

5.4 Drosophila histological methods

5.4.1 Histochemical detection of β-galactosidase activity in embryos

Embryos were collected for 0-20 h on acetic acid agar plates and dechorionated (see 5.1.19). After washing with PBS, embryos were fixed in fix solution for 10 min at RT. The fix solution was removed by extensive washing with PBS, and excessive liquid was removed before the embryos were transferred into a 1.5 ml siliconized Eppendorf tube. 1 ml of staining solution was added and the β-galactosidase reaction was allowed to proceed at 37°C. To stop the reaction, the embryos were washed several times with PBS. Stained embryos were mounted in 80% glycerol / PBS and examined under a light microscope.

Fix solution: 5 ml n-heptane
0.9 ml 25% glutaraldehyde (microscopy grade)
0.1 ml 1 M phosphate buffer pH 7
-> mix well, use the upper phase for fixation

Staining solution: 10 mM phosphate buffer pH 7
150 mM NaCl
1 mM $MgCl_2$

6 mM $K_4(Fe(CN)_6)$
6 mM $K_3(Fe(CN)_6)$)
0.3% (v/v) Triton X-100
-> incubate 5 min at 37°C, then add X-Gal to a final concentration of 0.2%, and incubate another 5 min at 37°C. Before use, centrifuge 3 min at 13.000 rpm (Eppendorf table top centrifuge), and use the supernatant for staining.

5.4.2 RNA in situ hybridization of Drosophila embryos

Collection and fixation of embryos

0-12 h old embryos were collected on acetic acid agar plates in medium sized cages (see 5.3.3). After dechorionation and washing with tap water, the embryos were transferred into a glass scintillation vial containing 6 ml n-heptane (or a 1.5 ml Eppendorf tube containing 600 µl n-heptane). For fixation, 1 ml (or 100 µl) 4% p-formaldehyde / PBS was added and the embryos were shaken vigorously for 20 min at RT. To remove the vitelline membrane, 10 ml (or 700 µl) 100% methanol were added and the samples were vortexed for at least 1 min. After sedimentation of the devitellinized embryos, the supernatant was discarded and exchanged by 10 ml (or 1 ml) 100% methanol. This washing step was repeated at least 3 times until the supernatant remained clear. Using a cut pipette tip, the embryos were transferred into a fresh Eppendorf tube and stored at -20°C until used (up to several weeks).

Preparation of 4% p-formaldehyde: Dissolve 4% (w/v) p-formaldehyde powder in PBS by heating at 60°C, then adjust to pH 7 using KOH; freeze 1 ml aliquots at -80°C. Before using, thaw aliquot at 60°C, discard if precipitate forms.

Preparation of RNA probes

DIG- or fluorescein-labeled strand-specific RNA probes were synthesized by *in vitro* transcription:

1 µg linear DNA template (linearized plasmid DNA or PCR product containing T7 / T3 promoter sequence)
2 µl 10x DIG / fluorescein RNA labeling mix (Roche)
2 µl 10x transcription buffer (Roche)

20 U RNasin (Promega)
20 U T7 / T3 RNA polymerase (Roche)
ad. 20 µl RNase-free ddH_2O

After thorough mixing, the reactions were incubated for 4 h at 37°C. Then, 5 µl RNase-free ddH_2O and 1 µl RNase-free DNase (20 U/µl, Roche) were added, followed by an additional 20 min incubation at 37°C. The RNA was purified using the QIAquick PCR purification kit according to the manufacturer's instructions, and eluted in 50µl RNase-free ddH_2O. The efficiency of the *in vitro* transcription reaction was tested by loading 1/10 of the reaction on an agarose gel. DIG- or fluorescein-labeled RNA probes were flash-frozen in liquid nitrogen and stored at -80°C.

Pre-treatment of embryos and in situ hybridization

Approximately 30 µl of fixed embryos per sample were allowed to warm up to RT. To re-hydrate them, they were incubated sequentially for 5 min in 1 ml 70% and 30% methanol / PBT on a roller. After an additional 5 min incubation in 1 ml PBT, the embryos were re-fixed for 15 min at RT in 1 ml 4% p-formaldehyde / PBT on a roller. The fix solution was removed by rinsing 3x in 1 ml PBT and washing twice for 5 min in 1 ml PBT. In the meantime, a waterbath was heated to 60°C, and an aliquot of hybridization buffer was heated. After the PBT washes, the embryos were transferred into the 60°C waterbath and rinsed once in 300 µl pre-warmed hybridization buffer. The hybridization buffer was replaced by 300 µl fresh, pre-heated hybridization buffer, and the pre-hybridization was allowed to proceed for at least 1 h at 60°C.

For each sample, 3 µl of labeled RNA probe were added to 30 µl of hybridization buffer and denatured for 5 min at 70°C. The pre-hybridization solution was discarded and replaced quickly by 30 µl of denatured RNA probe. Hybridization was carried out at 60°C overnight.

The next morning, 500µl pre-heated hybridization buffer were added to each sample, followed by a 10 min incubation at 60°C. To remove excessive probe, the samples were washed 3x in 300 µl post-hybridization buffer for 10 min at 60°C. The samples were then transferred to RT, rinsed 3x with 1 ml PBT, followed by two more washes in 1 ml PBT for 10 min.

PBT:	PBS
	0.1% (v/v) Tween-20 (Sigma)
20x SSC:	175.3 g NaCl
	88.2 g sodium citrate
	ad. 1l ddH_2O

	pH 7
Hybridization buffer:	50% formamide
	5x SSC
	50 µg/ml heparin (50 mg/ml stock in 4xSSC)
	100 µg/ml herring sperm DNA (10 mg/ml stock, Invitrogen)
	0.1% (v/v) Tween-20
	pH 5; store at -20°C
Post-hybridization buffer:	50% formamide
	5x SSC
	pH 5; store at -20°C

Non-fluorescent signal detection

α-DIG Fab fragments (Roche) were diluted 1:200 in PBT and preabsorbed for 1.5 h on fixed embryos. Preabsorbed antibodies were stored at 4°C for several weeks. The preabsorbed α-DIG antibodies were diluted to a final concentration of 1:2000 in PBT / 5% goat serum, and 100 µl were added to each sample, followed by a 2 h incubation at RT on a roller. After removal of the supernatant, the samples were rinsed 3x in 1 ml PBT, followed by 4 washes for 10 min in 1ml PBT. The embryos were then rinsed 3x in 1 ml AP-buffer and equilibrated in 1 ml AP-buffer for 10 min. Using a cut pipette tip, the embryos were transferred into 24 well plates, and the AP-buffer was replaced by 500 µl developing solution. The alkaline phosphatase reaction was allowed to proceed until clear signals were visible, and then stopped by rinsing several times in PBT. The signal was fixed by washing twice for 20 min with 100% ethanol, followed by several rinses in PBT. Using a cut pipette tip, the embryos were transferred onto a glass slide and mounted in 80% glycerol / PBS. For permanent preservation, the coverslips were sealed with nail polish. Samples were examined under a light microscope.

AP-buffer:	20 mM Tris-HCl pH 9.5
	100 mM NaCl
	50 mM $MgCl_2$
Developing solution:	NBT / BCIP stock (Roche) diluted 1:50 in AP-buffer (prepare fresh every time)

Fluorescent signal detection (RNA FISH)

After hybridization and post-hybridization washes, the embryos were incubated for 2 h at RT on a roller in 100 µl mouse monoclonal α-DIG antibody (diluted 1:400 in PBT / 5% goat serum, Roche) or 100 µl preabsorbed polyclonal rabbit α-fluorescein antibody (diluted to a final concentration of 1:300 in PBT / 5% goat serum). After removal of the antibody solution, the samples were rinsed 3x in 1 ml PBT, followed by 4 washes for 10 min in 1 ml PBT. To prevent bleaching of the fluorophore, all following steps were carried out in the dark. The samples were incubated with secondary antibodies (100 µl of goat α-mouse-Cy3 (JacksonImmunoResearch; preabsorbed) or goat α-rabbit-Alexa 488 (Molecular Probes; preabsorbed)) diluted 1:200 in PBT / 5% goat serum for 1 h at RT on a roller. After removal of the antibody solution, the samples were rinsed 3x in 1 ml PBT and washed 4x for 10 min in 1 ml PBT. To stain the DNA, the embryos were incubated in 0.5 µg/ml DAPI / PBT for 10 min at RT. Following a 5 min wash in 1 ml PBT at RT, the embryos were mounted in Mowiol and the stainings were visualized using a confocal microscope.

DAPI stock solution	dissolve 1 mg DAPI (4'-6-Diamidino-2-phenylindole) in 1 ml PBS, store at -20°C
Mowiol:	add 2.4 g Mowiol 4-88 to 6 g glycerol. Add 6 ml ddH_2O and stir the mixture for 3 h at RT. Then add 12 ml 0.2 M Tris-HCl pH 8.5 and incubate for 10 min at 50°C. Remove undissolved particles by centrifuging 15 min at 5.000g. Add DABCO (1,4-diazobicycle-[2.2.2]-octane) anti-fading reagent to a final concentration of 2.5%. Store 1 ml aliquots at -20°C. After thawing, Mowiol aliquots can be stored at RT and used for several days before solidification.

5.4.3 Immunostaining of Drosophila embryos

0-12 h old embryos were collected, dechorionated, and fixed as described under 5.4.2. Stored embryos (around 30 µl) were warmed up to RT, re-hydrated in a decreasing methanol series (5 min in 1 ml 70%, 5 min in 1 ml 30% methanol / PBS), and then washed twice for 5 min in PBX. Then, the embryos were blocked for 6 h in 1 ml blocking buffer on a rotator at 4°C. Primary antibodies (mouse α-tubulin (Sigma), 1:50) were diluted in blocking buffer and 100 µl were pipetted to the embryos. The incubation with primary antibody was allowed to proceed overnight at 4°C on a rotating wheel. The next morning, the samples were rinsed 3x

in 1 ml blocking solution and then washed twice for 30 min in 1 ml blocking solution. Secondary antibodies (preabsorbed goat α-mouse-Cy3 (JacksonImmunoResearch), 1:200) were diluted in blocking buffer and 100 µl were added to the embryos. After a 2 h incubation on a roller at 4°C, the embryos were rinsed 3x and washed 2x for 30 min in 1 ml blocking buffer. Stained embryos were mounted in Mowiol and examined using a confocal microscope.

Buffers:	PBX:	PBS, 0.1% (v/v) Triton X-100
	Blocking buffer:	PBS
		1% (v/v) Triton X-100
		0.1% (w/v) BSA

5.4.4 RNA ImmunoFISH on Drosophila embryos

This protocol was adapted from a protocol obtained from Giacomo Cavalli (IGH Montpellier). To combine RNA FISH with immunostaining, the DNA staining at the end of the RNA FISH protocol was omitted and the embryos were dehydrated by passing them through an increasing ethanol series (5 min 1 ml 30% ethanol / PBT, 5 min 1 ml 70% ethanol / PBT, 5 min 1 ml 100% ethanol). The embryos were then re-hydrated by subsequent washings in a decreasing ethanol series (in PBT) and washed twice for 5 min in 1 ml PBT.
The samples were blocked for 1 h at RT in 1 ml blocking buffer on a rotator. The blocking solution was replaced with 100 µl of primary antibody solution (mouse α-tubulin (JacksonImmunoResearch), diluted 1:50 in blocking solution) and the samples were incubated overnight at 4°C on a rotator. After three rinses in 1 ml PBS-Tr, the samples were washed 3x for 30 min in 1 ml PBS-Tr. Prior to the incubation with secondary antibodies, the samples were blocked once again for 1 h at RT in 1 ml blocking buffer. Secondary antibodies (goat α-mouse-Cy3 (JacksonImmunoResearch; preabsorbed) 1:200, or goat α-mouse-Alexa488 (Molecular Probes; preabsorbed) 1:200) were diluted in blocking buffer and 100 µl were pipetted to the embryos. The samples were incubated for 1 h at RT on a roller, followed by three short and three long washes in PBS-Tr (see above). The DNA was counterstained by incubating the samples for 10 min at RT in 1 ml 0.5 µg/ml DAPI, followed by 5 min washing in 1 ml PBS. Using a cut pipette tip, the embryos were transferred onto a slide, mounted in Mowiol, and examined under a confocal microscope.

Buffers:	PBS-Tr:	PBS, 0.3% (v/v) Triton X-100
	Blocking buffer:	PBS-Tr
		10% goat serum

5.4.5 DNA FISH on larval polytene chromosomes

Chromosome squashes

This protocol was adapted from(Lavrov et al. 2004). Larvae were grown on standard *Drosophila* medium supplemented with yeast paste. Female wandering third instar larvae were collected in PBS on ice (generally, the salivary glands of females are bigger than those of males). Two pairs of salivary glands were dissected in solution 1, and fat body cells were removed as much as possible. Using forceps, the two pairs of salivary glands were transferred into a drop (approximately 40 µl) of solution 2 on a slide. While continuously stirring, the glands were homogeneously fixed for 10 sec. Subsequently, the glands were transferred into a drop of solution 3 on a 18 cm x 18 cm coverslip and left for 2 min 10 sec. Using a poly(L)lysine coated slide, the glands were taken up and then broken up to spread the chromosomes. To do this, a pencil (HB) was tapped onto the coverslip with moderate force and constant moving of the coverslip (1-2 mm). Excess liquid was removed by turning the slide upside down and pressing it onto Whatman paper. The quality of the preparation was examined immediately under phase contrast. Using a diamond pencil, the position of the coverslip was marked on the slide before it was flash-frozen in liquid nitrogen. Using a razorblade, the coverslip was removed and the slide was then stored in PBS on ice (for storage up to one week, slides were transferred into a jar containing 100% methanol at 4°C). The slides were washed twice in PBS for 15 min with constant rocking. Subsequently, the chromosomes were dehydrated by passing them through an increasing series of ethanol / PBS (2x 5 min 70%; 2x 5 min 96%). Finally, the slides were air-dried and stored flat at 4°C. This storage ideally lasted at least overnight, as this markedly increased the adhesion of the chromosomes to the slide, and thus the quality of the DNA FISH signals.

Buffers:	Solution 1:	0.1% (v/v) Triton X-100 / PBS
	Solution 2:	50 µl 10% (v/v) Triton X-100 400 µl PBS 50 µl 37% p-formaldehyde -> this solution must be prepared fresh and can be used for 2-3 h when kept on ice
	37% p-formaldehyde stock:	1.85 g p-formaldehyde was weighed into a final volume of 5 ml ddH2O, and 70 µl 1N KOH were added to adjust to pH 7.4. The powder was dissolved by heating in a

60-65°C waterbath. 100 µl aliquots were stored at -80°C. Before use, aliquots were thawed by heating at 60-65°C. If precipitates formed, the solution was discarded.

Solution 3: 50 µl 37% p-formaldehyde
200 µl ddH2O
250 µl 100% acetic acid
-> This solution also has to be prepared fresh every time and is stable for 2-3 h when kept on ice.

Preparation of the DIG-labeled DNA probe

1 µg DNA (linear or circular plasmid DNA) was used as a template in a standard labeling assay according to the manual provided with the DIG-High Prime labeling kit (Roche). Briefly, the template DNA was dissolved in a volume of 16 µl and denatured by boiling for 10 min. The DIG-High Prime labeling mix was mixed thoroughly and 4 µl added to the denatured DNA. After short centrifugation, the reaction was incubated overnight at 37°C. The reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8) or heating for 10 min at 65°C, the DIG-labeled DNA was ethanol-precipitated and resuspended in 20 µl TE buffer (pH 7.4). 200 µl hybridization buffer were added and the probe was stored for several months at -20°C.

Hybridization buffer: 50% formamide
2x SSC
10% dextran sulfate
400 µg/ml herring sperm DNA (Invitrogen)
pH 7; store at -20°C

Pre-treatment and Hybridization

Just before hybridization, the slides were put into a Coplin Jar containing 2x SSC at RT. The jar was then transferred into a 70°C waterbath and left there for 40 min. Subsequently, the slides were dehydrated by passing through an ethanol / PBS series (2x 5 min 70%; 2x 5 min 96%) and air-dried. To denature the chromosomes, they were incubated for in 0.1 M NaOH for 10 min and then washed three times (1 min, 1 min, 5 min) in 2x SSC, followed by dehydration and air-drying. One aliquot of DIG labeled DNA probe (in hybridization buffer, around 15 µl per sample) was denatured by heating for 5 min at 80°C, then quickly chilled on

ice, pre-warmed to 37°C, and pipetted onto a 22 mm x 22 mm coverslip. The slide covered with polytene chromosomes was turned upside down to take up the coverslip with the probe. To prevent liquid evaporation, the coverslips were sealed with Fixogum. The lides were hybridized overnight in a humid chamber in a 37°C waterbath.

20x SSC:	175.3 g NaCl
	88.2 g sodium citrate
	ad. 1l ddH_2O
	pH 7

Washing and detection

After hybridization, the slides were immersed in pre-warmed 2x SSC to remove the coverslips (during the post-hybridization washes, cooling down of the samples should be avoided). They were then transferred into a Coplin Jar and washed 3x 5 min in 2x SSC at 42°C, then once for 5 min in 2x SSC at RT with rocking. Next, the slides were washed 5 min in TNT buffer on a shaker, then 100 µl of TNB buffer were pipetted onto the slide which was then covered with a 24 mm x 44 mm coverslip and incubated for 30 min at RT. The coverslip was removed by immersing the slide in TNT buffer. Mouse α-DIG antibody (Roche) was diluted in TNB buffer to a final concentration of 1:200, and 20 µl were then pipetted onto a 22 mm x 22 mm coverslip. The slide was turned upside down to take up the coverslip, then laid down flat and incubated in a humid chamber for 90 min at RT or overnight at 4°C. After 3x 5 min washes in TNT buffer on a shaker, the secondary antibody (goat α-mouse-Cy3, JacksonImmunoResearch) was diluted 1:200 in TNB buffer, and 20 µl were pipetted onto a 22 mm x 22 mm coverslip. Turning the slide upside down, the coverslip was taken up, followed by a 60-90 min incubation in a humid chamber at RT in the dark. Alternatively, sheep α-DIG-Rhodamine coupled antibody (Roche, 1:100) was used to detect the DIG-labeled probe, omitting the secondary antibody incubation. To prevent bleaching of the fluorophore, all following steps were also carried out in the dark. After 3x 5 min washes with TNT buffer on a shaker, the DNA was counterstained by incubating the slides for 10 min at RT in 1 µg/ml DAPI diluted in TNT buffer. The slides were washed once for 5 min in TNT buffer, before the samples were mounted in Mowiol. The signals were visualized using an epifluorescence microscope.

Buffers:	TNT buffer:	100 mM Tris-HCl pH 7.4
		150 mM NaCl
		0.05% (v/v) Tween-20

TNB buffer:	100 mM Tris-HCl pH 7.4
	150 mM NaCl
	4% BSA

5.4.6 Immunostaining of polytene chromosomes (combined with DNA FISH)

Chromosome squashes

The polytene chromosomes were prepared exactly as described under 5.4.5.

Immunostaining

Stored slides were washed twice for 15 min in PBS on a shaker, then blocked for 1 h in blocking solution with vigorous shaking. Primary antibodies (rabbit α-PC 1:80; rabbit α-PHO 1:100; rabbit α-TRX 1:20; mouse α-GFP (Roche) 1:50) were diluted in blocking solution, and 20 µl were pipetted onto a 22 mm x 22 mm coverslip. The slides were turned upside down to take up the coverslips and incubated in a humid chamber for 2 h at RT or (preferably) overnight at 4°C. The coverslips were removed by immersing the lides in PBS, then transferred into a Coplin Jar and washed for 15 min at RT in Wash 1 buffer with rocking. Next, the slides were washed for 15 min at RT in Wash 2 buffer and rinsed once in PBS. Secondary antibodies (preabsorbed goat α-rabbit-Alexa488 (Molecular Probes) 1:200, or preabsorbed goat α-mouse-Cy3 (JacksonImmunoResearch) 1:200) were diluted in blocking solution and 20 µl were pipetted onto a 22 mm x 22 mm coverslip. The slides were turned upside down to take up the coverslips and incubated in a humid chamber for 1 h at RT. The coverslips were removed by immersing the slides in PBS, followed by 15 min washing in Wash 1 buffer in a Coplin Jar with rocking. The slides were then washed once for 15 min in Wash 2 buffer and rinsed once in PBS.

Buffers:	Blocking solution:	PBS
		3% (w/v) BSA
		10% (w/v) non fat dry milk powder
		0.2% (v/v) NP-40
		0.2% (v/v) Tween-20
		prepare 1 l, store 50 ml aliquots at -20°C
	Wash 1 buffer:	PBS / 300 mM NaCl
		0.2% (v/v) NP-40
		0.2% (v/v) Tween-20
	Wash 2 buffer:	PBS / 400 mM NaCl

0.2% (v/v) NP-40
0.2 (v/v) Tween-20

Cytology

To counterstain the DNA, the slides were incubated for 10 min in 1 µg/ml DAPI / PBS, followed by a 5 min wash in PBS. The slides were mounted in Mowiol and examined under an epifluorescence microscope.

Combining immunostaining with DNA FISH

If the immunostained slides were to be subjected to DNA FISH, the DNA staining step with DAPI was omitted. To preserve the immunosignals, the slides were fixed in 3.7% p-formaldehyde / PBS for 15 min at 37°C. Next, the slides were washed 3x for 5 min in PBS, then dehydrated by passing through 70% (twice for 5 min) and 96% (twice for 5 min) ethanol / PBS and air-dried. The slides were then stored flat in a dark chamber at 4°C overnight. Subsequently, the DNA FISH was carried out as described under 5.4.5.

5.5 Cell culture methods

5.5.1 Cultivation of SF4 cells

The cells were cultivated in 50 ml and 250 ml cell culture flasks (CellStar) with 5 ml and 25-30 ml growth medium, respectively. At 80-90% confluency, the semi-adherent SF4 cells detach and proliferate in suspension. Cells were resuspended and diluted in fresh medium (1:10) approximately every 4 days. Cells were cultivated at 25°C under normal atmospheric pressure.

Culture medium: Schneiders medium (GibcoBRL)
1% Penicillin / Streptomycin
10% fetal calf serum (FCS)

5.5.2 Freezing cells for long term storage

Cells at 70-90% confluency were resuspended and transferred into 15 ml Falcon tubes with 10 ml of fresh medium. The cells were sedimented at 300g for 5 min and resuspended in 3-4.5 ml 10% (v/v) DMSO in growth medium with 20% FCS. 1.5 ml aliquots were transferred into cryotubes (Nunc) and incubated on ice for 1-1.5 h. The vials were stored overnight at -80°C and then transferred into a liquid nitrogen tank for long-term storage.

5.5.3 Thawing of frozen cells

Cells frozen in liquid nitrogen were thawed quickly in a 37°C waterbath. Cells were transferred into a 15 ml Falcon tube with 10 ml of fresh medium and centrifuged for 5 min at 300g. The cells were resuspended in 5 ml normal growth medium and transferred to 9 cm culture dishes containing 10 ml growth medium.

5.5.4 Treatment of SF4 cells with Actinomycin D

Cells were seeded in 250 ml tissue culture flasks in 30 ml growth medium at a concentration of 6 x 10^5 cells/ml. For transcriptional arrest, Actinomycin D (stock: 1 mg/ml in ethanol, stored at 4°C) was added to a final concentration of 1 µM. 5 ml aliquots of cells were taken prior to and 2 h, 4 h, 6 h, and 8 h after addition of Actinomycin D. Cells were pelleted by centrifuging 3 min at 1200 rpm. The supernatant was discarded and the cells put immediately on ice. The cell pellet was resuspended in 500 µl TRIzol®Reagent, flash-frozen in liquid nitrogen, and stored at -80°C, until total RNA was isolated (see 5.1.17).

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Publications

7. Publications

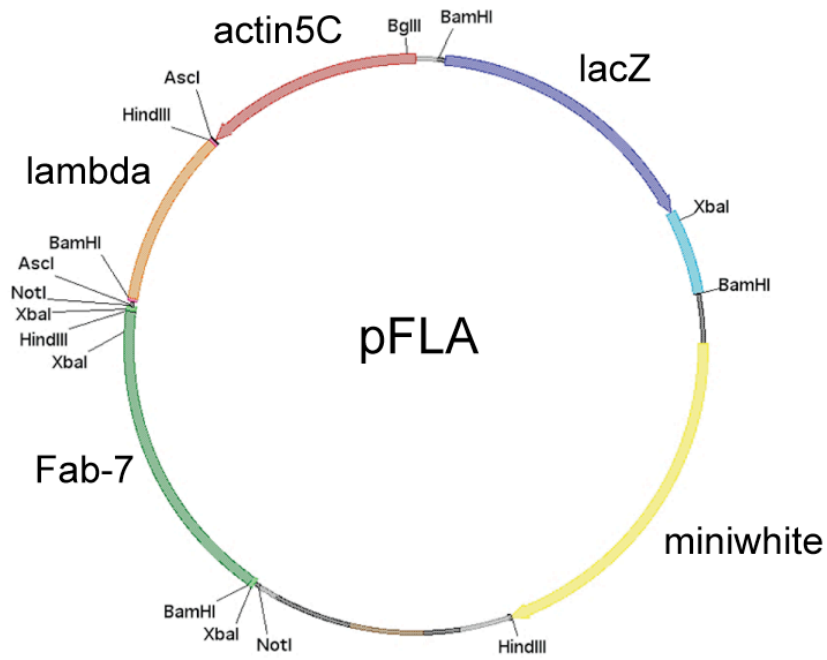
Schmitt, S. and Paro, R. 2004. Gene regulation: a reason for reading nonsense. *Nature* **429**(6991): 510-511.

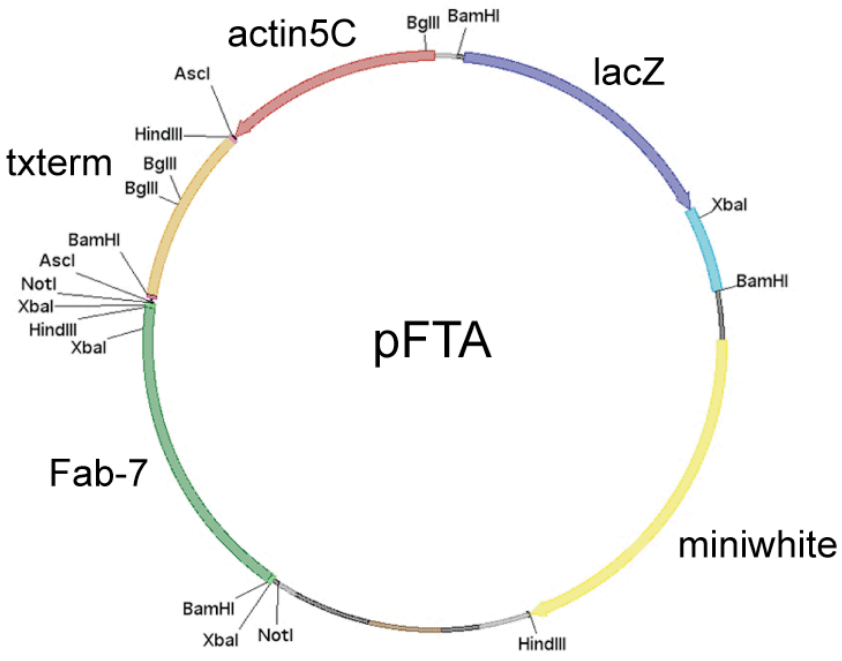
Schmitt, S., Prestel, M., and Paro, R. 2005. Intergenic transcription through a Polycomb group response element counteracts silencing. *Genes Dev* **19**(6): 697-708.

Appendix

8. Appendix

8.1 Vector maps





8.2 Abbreviations

8.2.1 Amino Acids

Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartate	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamate	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	lys
Mehionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophane	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

8.2.2 Genes, chromosomal markers, proteins, and protein domains

abdA	*abdominal-A*
AbdB	*Abdominal-B*
actin5C	*actin* gene at position 5C on cytogenetic map
ANT-C	Antennapedia Complex
Antp	*Antennapedia*
ASH1	Absent, small, and homeotic
Bc	Black cells
bcd	*bicoid*
Bc Gla	ln(2LR)*Gla*, Bc^{1} Gla^{1}, 2. chromosome balancer
BRM	Brahma
BX-C	Bithorax Complex
bx	*bithorax*
bxd	*bithoraxoid*
CHRASCH	Chromatin associated silencing complex for homeotics
dCBP	*Drosophila* CREB-binding protein
Cre	Causes recombination
DCC	dosage compensation complex
DSP1	Dorsal switch protein 1
dTAFII	*Drosophila* TFIID associated factor
EBD	estrogen-binding domain
en	*engrailed*
ESC	Extra sex combs
ey	*eyeless*
E(Z)	Enhancer of zeste
EZH2	Enhancer of zeste 2
Fab-7	*Frontoabdominal-7*

Flp	flipase
FRT	*Flp recombination target*
GAF	GAGA factor
GFP	Green fluorescent protein
HAT	histone acetyltransferase
HDAC	histone deacetylase
hb	*hunchback*
hh	*hedgehog*
HMGB2	high mobility group protein B2
HMTase	histone methyltransferase
Hox	*Homeobox*
HP1	Heterochromatin protein 1
hsp70	*heat-shock protein 70*
iab	*infraabdominal*
KTO	Kothalo
lacZ	*β-galactosidase*
LCR	locus control region
loxP	*locus of crossing over (x), P1*
Mcp	*Miscadestral pigmentation*
MLL	Mixed lineage leukemia
MOF	Males absent on the first
MOR	Moira
mw	*miniwhite*
nos	*nanos*
PC	Polycomb
PcG	Polycomb group
PH	Polyhomeotic
PHO	Pleiohomeotic
PRC	Polycomb group repressive complex
PRE	Polycomb group response element
PSC	Posterior sex combs
PSQ	Pipsqueak
RNAPII	RNA polymerase II
roX	*RNA on the X*
salm	*spalt major*
SKD	Skuld
slou	*slouch*
Su(Z)12	Suppressor of zeste 12
tll	*tailless*
TRAP	Thyroid hormone receptor-associated protein
TRX	Trithorax
UAS	*upstream activating sequence*
Ubx	*Ultrabithorax*
3'UTR	*3' untranslated region*
wg	*wingless*
XIC	X inactivation center
Xist	*X-inactivation specific transcript*
Xite	*X-inactivation intergenic transcription elements*
Z	Zeste

8.2.3 Others

A-P	anterior-posterior
aa	amino acid
Amp	ampicillin
AP	alkaline phosphatase
ATP	adenosine-5'-triphosphate
bp	basepair
BSA	bovine serum albumine
CIP	calf intestinal phosphatase
cm	centimeter
E.coli	*Escherichia coli*
EDTA	Ethylendiaminotetraacetic acid
Fig	Figure
h	hour
hPa	hectoPascal
nt	nucleotide
D-V	dorsal-ventral
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double-stranded
FISH	fluorescent *in situ* hybridization
g	gravitation
Hepes	N-(2-Hydroxyethyl)-piperazin-N'(2-ethansulfonic acid)
kb	kilobase
kDa	kiloDalton
LB	Luria Bertani
M	Molar
µg	microgram
min	minute
ml	milliliter
mm	millimeter
µm	micrometer
mM	millimolar
µM	micromolar
NBT	Nitro-Blue-Tetrazoliumchloride
NP-40	Nonidet P-40
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMSF	Phenylmethylsulfonylfluoride
PS	parasegment
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcription – polymerase chain reaction
SAP	shrimp alkaline phosphatase
sec	second
ss	single-stranded

TAE	Tris-acetate-EDTA
TE	Tris-EDTA
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfsmittel durchgeführt habe.

Heidelberg, 20.02.2006

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Sabine Schmitt