Function of the Trithorax-like gene during Drosophila development

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Abstract

Maintenance of homeotic gene expression during Drosophila development relies on the Polycomb and the trithorax groups of genes. Classically, the Polycomb proteins act as repressors of homeotic gene function, whereas trithorax proteins function as activators. However, recent investigation has indicated that some of these maintenance genes may act both as repressors and activators. One of those is the Drosophila Trithorax-like gene that codes for the GAGA factor. To investigate its dual activator/repressor role, we have studied the function of the Trithorax-like throughout Drosophila development. Embryos lacking both the maternal and the zygotic Trithorax-like function do not develop suggesting that Trithorax-like might be required in oogenesis. Homozygous Trithorax-like null mutant embryos show reduced expression levels of some of the homeotic proteins. Trithorax-like mutant larval clones, however, do not show phenotypes indicative of either activation or repression of homeotic gene function. These results suggest that Trithorax-like is required during embryogenesis but not throughout larval development for the regulation of homeotic gene expression. Moreover, this temporal requirement seems also to regulate MCP-mediated silencing. Finally, lack of Trithorax-like function modulates the gain of function phenotypes caused by over-expression of homeotic genes. To explain Trithorax-like gene function, we propose a model where very early in development, GAGA factor probably establishes a chromatin ground state for transcription. The differential “on/off” transcriptional state of the homeotic genes is then established and propagated by the action of the specific regulatory proteins independently of the GAGA factor. We also suggest that GAGA factor may not have a dual activator/repressor function. Rather, Trithorax-like mutations may produce dual loss of activation and loss of repression effects.

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Introduction

The restricted pattern of homeotic genes expression is established early in development through the action of the transiently acting segmentation genes (Qian et al., 1991, 1993; Zhang et al., 1991). Once the domains of expression are defined, their maintenance throughout development becomes dependent on the Polycomb group (PcG) and trithorax group (trxG) of genes (Kennison, 1995). The PcG genes control the silencing of homeotic genes outside of their domains of expression, whereas the trxG genes maintain the expression of the homeotic genes activated within their restricted domains. PcG and trxG proteins contain structural motifs that are found in chromatin-associated proteins, and that are involved in protein–protein interactions (Paro and Harte, 1996). They are ubiquitously expressed in the embryo and imaginal cells and, thus far, only two of them, the Pleiohomeotic protein (PHO) and the Trithorax-like protein (TRL or GAGA factor or GAF) have been shown to bind DNA sequence specifically (Brown et al., 1998a; Pedone et al., 1995). Therefore, mechanisms must function to maintain a restricted domain of protein expression, in the absence of restrictedly expressed DNA-binding proteins, throughout proliferation and development.

Genetic experiments have predicted the existence of as many as 40 PcG proteins (Jürgens, 1985) that can act together through the formation of multimeric protein complexes that repress transcription of the homeotic genes. To date, only 15 PcG have been isolated and molecularly characterized and, indeed, some PcG proteins have been shown to interact in vitro (Kyba and Brock, 1998; Peterson et al., 1997) and in vivo, they are associated with large protein complexes (Franke et al., 1992; Furuyma et al., 2003; Shao et al., 1999; Tie et al., 2001, 2003). Biochem-
rical experiments have identified two principal large PcG multicomponent complexes: the PRC1 (Polycomb Repressive Complex 1, containing among others, the Polycomb protein itself) (Shao et al., 1999), and the ESC-EZ (extra sex comb-Enhancer of zeste) complexes (Furuyama et al., 2003; Tie et al., 2001). These complexes differ in their PcG protein components, and they apparently function at different but overlapping times in development (Poux et al., 2001). PHO being the only PcG protein with sequence-specific DNA-binding activity, has been proposed to initiate the recruitment of other PcG complex proteins to specific DNA sequences (Brown et al., 1998b). However, PHO-binding sites alone are not sufficient to silence the homeotic gene expression (Busturia et al., 2001; Mishra et al., 2001; Mohd-Sarip et al., 2002). Many trxG proteins have been characterized and also found to form different multi-component complexes. These proteins form a more heterogenous group that also associates with chromatin and are involved in maintenance of transcriptional activation (Francis and Kingston, 2001 for a review). The trithorax (TRX) protein is a component of Trithorax Acetylation Complex 1 (TAC1) (Petruk et al., 2001). Several other large protein complexes containing one or more trxG proteins have been identified and shown to interact with TRX. Examples include the ASH1 complex (Rozovskaia et al., 1999) and the Brahma complex (Rozenblatt-Rosen et al., 1998).

Maintenance proteins act on DNA elements known as Polycomb Response Elements (PREs) (Simon et al., 1993) and Trithorax Response Elements (TREs) (Tillib et al., 1999) to repress and activate, respectively, homeotic gene function. Although it is not known whether PREs and TREs need to be physically associated to function, it has often been found that DNA fragments containing PRE activity also function as TREs. This may mean that the action of the activator and repressor is closely related and that proper maintenance of homeotic gene expression involves a critical balance between activators and repressors. PRE and TRE sequences are, with few exceptions, poorly defined. The exceptions are the binding sites for PHO and GAF, which are present in most, but not all, of the PREs so far identified (Mihaly et al., 1998).

The GAF, encoded by the Trithorax-like gene in Drosophila has been classically included in the trxG of proteins (Farkas et al., 1994). This protein contains two distinct domains: a zinc finger DNA-binding domain that binds to elements that contain repeats of GAGAG (Pedone et al., 1995), and a conserved Broad-complex, tramtrack, bric à brac (BTB) or poxvirus and zing finger (POZ) domain, which mediates protein–protein interactions (Espinás et al., 1999). The GAF was first identified as a sequence-specific DNA-binding protein which stimulates the transcription of the engrailed and the Ultrabithorax promoters in vitro (Biggin and Tjian, 1988; Soeller et al., 1988). Since then, many Drosophila gene promoters have been shown to contain binding sites for GAF, which can been found within and outside the PREs (e.g., in the promoter regions) (Biggin and Tjian, 1988, Ringrose et al., 2003; Soeller et al., 1988, Strutt et al., 1997, van Stenness et al., 2003). Also, many studies have suggested that GAF does not function as a “typical” transcription factor. Rather, it regulates transcriptional activation by facilitating the formation and/or maintenance of nucleosome-free regions of the chromatin, thereby allowing access of other factors to the DNA (Lu et al., 1993). As the GAF is the only trxG protein thus far shown to bind DNA in a sequence specific manner, it is proposed that it recruits other trxG and PcG complexes to DNA, although GAF-binding sites are not sufficient to exert that function (Horard et al., 2000). GAF is included in the trxG activating proteins because Trl mutations enhance the haplo-insufficient phenotype observed in mutations of the homeotic gene Ultrabithorax (Horard et al., 2000). However, studies of the function of Trl in the regulation of homeotic genes have indicated that GAF may influence the repression as well as the activation of the homeotic genes (Gildea et al., 2000) and it has lately been included in the enhancers of TrxG and PcG mutations (ETP) group of proteins that have dual roles in activating and repressing transcription (Gildea et al., 2000).

Data suggesting a role for GAF in the regulation of transcriptional repression come from studies using reporter minigenes containing isolated cis-regulatory elements, PREs and TREs, from the bithorax complex genes (Busturia et al., 2001; Horard et al., 2000; Mishra et al., 2001). For example, the MCP138 silencer of the Abdominal-B gene contains two GAF and four PHO-binding sites. It is able to repress the PBX enhancer expression throughout larval development. This repression is abolished in Trl mutant background and when either PHO- or GAF-binding sites are mutated. Still, neither PHO- nor GAGA-binding sites alone are sufficient for repression indicating the need of both (and/or their interacting proteins) for the maintenance of repression (Busturia et al., 2001).

Additional data supporting the repressor role of GAF are the phenotypes resulting from genetic interactions between Trl and PcG mutant alleles. For example, the extra sex comb phenotype of Polycomb mutations is enhanced in Trl heterozygous mutant background (Busturia et al., 2001; Strutt and Paro, 1997). Moreover, it has been shown that GAF binds to both active and inactive PREs (Strutt et al., 1997), interacts with the nucleosome remodelling factor (NURF) complex (Tsukiyama et al., 1994), with TRX (Poux et al., 2002), with SIN3 corepressor complex, through SAP18 (Espinás et al., 2000) and with PC (Horard et al., 2000).

We have further investigated the role of Trl in the regulation of homeotic genes to better understand its function. We have studied whether these proposed activating and repressing roles were influenced by the developmental stage to help identify candidates for the interacting factors that may control these two functions.
Materials and methods

Fly strains and genetic interactions

The mutations used are described elsewhere (Flybase, 1999). The Trithorax-like mutations were \(Trl^{R85}\), \(Trl^{1Sc}\) and \(D(3L)\)e-M21. The homeotic gene mutations include \(Ubx^{130}\) (included in the TM2 balancer chromosome), \(Ubx^{TM1}\), \(Abd-B^{TM1}\), \(Cbx^{T2;3}\)apXa, \(Mcp^{1}\), \(Mcp^{1}\) and \(Antp^{rev1}\) on \(TM3\), \(Sb\). When examining the effect of \(Trl\) mutations, crosses carrying the mutation in the female or in the male were performed, and no major differences were observed. Special care was taken to avoid overcrowding conditions. A minimum of 100 flies were studied for each different mutant combination. To measure the wing area of the \(Cbx^{+}\)/ and \(Cbx^{T}\)/\(Trl^{R85}\) flies, the wings were dissected, mounted and photographed. The areas were calculated as a number of pixels/wing and a mean value was obtained in both cases. We have studied roughly 100 wings of each genotype. To study the enhancement effect of \(Trl^{R85}\) on \(Ubx^{130}\) haplo-insufficient phenotype, males \(Trl^{R85}\)/\(TM3\)FP were crossed to \(TM2\)/\(TM6\)C females. Additionally, a \(Trl^{R85}\)/\(MKRS\): \(pho\) \(\times\) \(ct^{P}\) stock was built and crossed to \(TM2\)/\(TM6\)C to study the effect of \(pho\) and \(Trl\) on the enhancement of the \(Ubx^{130}\) phenotype. We looked at the double heterozygous combinations between \(Trl^{R85}\) and \(D(2)vgD\), \(Axs^{223}\), \(Pcm^{1/3}\) and \(Pcm^{1/3}\)/\(+\) show an average of 2.5 extra sex comb hairs in either the second or third leg. In comparison, an average on 3.2 hairs were found in the second and third leg of the double heterozygotes flies. We also looked at the interactions with the following \(trxG\) mutations; \(osa^{2}\), \(kiss^{2}\), \(lola^{R02512}\), \(trx^{E2}\) and \(brm^{E2}\) \(trx^{E2}\), \(brm^{Antp^{+}}\), \(brm^{Antp^{j}}\), \(mor^{r}\) and \(mor^{j}\). We examined the number of the sex comb hairs, the wings held out phenotype, the pigmentation of the abdominal segments and antennal and/or arista transformations. We did not find any significant genetic interaction between these mutant alleles and \(Trl^{R85}\).

Staining of embryos and imaginal discs was performed using standard protocols. The \(P[PBX-Ubxxp-lacZ]\), the \(P[PBX-MCP138-Ubxpp-lacZ]\), line [T9] on the second chromosome and the \(P[PBX-MCP138\ GAGA\ amat\ Ubxpp-lacZ]\), line [T4] reporter constructs have been previously described (Busturia et al., 2001). Stocks carrying the \(P[PBX-MCP138-Ubxpp-lacZ]\) and \(Trl^{R85}\) were built to study \(lacZ\) expression. To analyse the embryonic expression of \(UBX\) in the \(Cbx^{1}/\Trl^{R85}\) embryos, males \(Cbx^{+}\)/\(+\) were crossed to females \(Trl^{R85}/TM6\)BGFP. Similarly, males \(Mcp^{1}/\Trl^{R85}\) were crossed to \(Mcp^{1}/\Trl^{R85}\) females to analyse the expression of \(ABD-B\) in \(Mcp^{1}/\Trl^{R85}\) embryos. When studying the effect of \(Trl\) mutations, the mutant embryos were intentionally not marked to better compare the levels of expression. Moreover, the comparison was done in embryos stained in the same tube. The following antibodies were used: rabbit anti-\(\beta\)-\(gal\) (Vector Labs), mouse anti-\(Ubx\) FP.3.38 (White and Wilcox, 1984), rabbit anti-\(abd-A\) (Macias et al., 1990), mouse anti-\(Abd-B\) (Celniker et al., 1990), rat anti-\(Scr\) (Riley et al., 1987), mouse anti-\(Antp\) (Condie et al., 1991), rabbit anti-sc (Skeath et al., 1992), guinea pig anti-senseless (Nolo et al., 2000), mouse anti-en (Patel et al., 1989).

FLP-induced germ line \(Trl^{R85}\) clones

A recombinant chromosome bearing \(P[FRT(w^{hs})2A]\) and the \(Trl^{R85}\) was constructed. Females \(yw\) \(P[hs70-FLP122]\); \(CyO\); \(TM6\)B/TM2 were crossed to males \(yw\); \(P[w^{+},ovo^{D1}]\) \(P[FRT(w^{hs})2A]/TM3\)B. The resulting males \(P[hs70-FLP\ 122]\); \(Sp\); \(P[w^{+},ovo^{D1}]\) \(P[FRT(w^{hs})2A]/TM6\)B were then crossed to females \(yw\); \(Trl^{R85}\) \(P[FRT(w^{hs})2A]/TM6\)B. The progeny of this cross were heat-shocked at 4, 5 and 6 days AEL for 2 h at 37°C. Single mosaic females \(yw\) \(P[hs70-FLP122]/yw\); \(P[w^{+},ovo^{D1}]\) \(P[FRT(w^{hs})2A]/Trl^{R85}\) \(FRT24\) from the above cross were crossed to males \(D(3L)\)e-M21/TM6BGFP and the fertility and the phenotype of the progeny were studied. The verification of recombination in the females was done by the observation, in the mosaic females, of variegates eyes and of the phenotype observed when inducing somatic clones (see results). For the germ line clones, we stained the ovaries of the mosaic females with DAPI using standard protocols.

FLP-induced \(Trl^{R85}\) somatic clones

A recombinant chromosome containing multiple wing hair (\(mwh\), \(Trl^{R85}\) and \(P[FRT(w^{hs})2A]\) was constructed. Females \(yw\) \(P[hs70-FLP122]\); \(mwh\ \ Trl^{R85}\) \(P[FRT(w^{hs})2A]/TM6\)B were crossed to males \(yw\); \(hs\)-\(nGFP\); \(CD71^{v}\), \(P[FRT(w^{hs})2A]/TM6\)BGFP or to males \(yw\); \(Dp(1;3)xc^{4}\), \(Mii^{55}\); \(P[ubi-GFP]\), \(P[FRT(w^{hs})2A]/TM6\)B when the clones were induced in a \(Minute\) mutant background. The progeny were heat-shocked at 37°C for 15 min at 0–24, 24–48, 48–72, 72–96, 96–120 h AEL. Clones in the imaginal discs were scored for the absence of \(GFP\) expression and, in the adult cuticle for the \(mwh\) and yellow markers. Adults bearing clones were selected in the dissecting microscope and mounted for a detailed analysis.

Results

Trithorax-like germ line mutant clones

Mutant germ line clones generated using the null \(Trl^{R85}\) mutant allele (Farkas et al., 1994) were used to observe the phenotype and the expression of homeotic proteins in embryos lacking the maternal and zygotic GAF contribution. The dominant female sterile (DFS) Flip-recombinase system was used (Chou et al., 1993) to generate germ line \(Trl^{R85}\) clones (Liaw, et al., 1995). Mosaic females were crossed to males carrying a deficiency of the \(Trl\) gene over a
GFP marked balancer chromosome. Most of the mosaic females (807 studied) were sterile. Only 23 of them laid few eggs. The phenotype of the few progeny obtained was quite variable, ranging form unfertilised eggs in most cases to embryos developing cuticle with variable defects. The phenotypic variability has also been previously reported for genes involved in oogenesis (Suzanne et al., 2001).

The unfertilised eggs exhibited a defect in micrycle formation (Fig. 1B), most likely accounting for their unfertilised state. Some of the few embryos that developed defective cuticle were Trl maternal− zygotic+, indicating that the phenotype could not be paternally rescued. Fig. 1D, shows the phenotype of a Trl maternal− zygotic+ embryo. The anterior part of the embryo is completely defective and the denticle belts are not properly formed. Unfortunately, the phenotypes of the small amount of embryos obtained do not allow us to conclude about the state of transcription of the homeotic genes in these conditions. These results indicate that Trl is involved in oogenesis (Liaw et al., 1995), a conclusion also already suggested by previous studies using Trl1/c, a weaker mutant allele (Bhat et al., 1996).

Expression of homeotic proteins in Trithorax-like homozygous mutant embryos

We have studied the expression of homeotic proteins in homozygous TrlR85 embryos. The expression of sex comb reduced (SCR), Antennapedia (ANTP), Ultrabithorax (UBX), abdominal-A (ABD-A) and Abdominal-B (ABD-B) proteins were all examined. Also, we have analysed the expression of the engrailed (EN) protein. We observe that in TrlR85 homozygous embryos, there is a clear reduction in the expression of UBX and EN proteins (Fig. 2). Although difficult to quantify, a reduction of at least 50% compared with the wild-type expression is likely. No effect was seen in the expression of SCR, ANTP, ABD-A and ABD-B (data not shown). Therefore, the effect of absence of Trl on the regulation of some homeotic genes can be observed as early as embryonic development, independently of the proposed strong GAF maternal effect (Bhat et al., 1996; Farkas et al., 1994).

We next looked at the expression of UBX in the imaginal discs of two different Trl mutant alleles: Trl1/c and TrlR85, a hypomorphic and null alleles, respectively. Homozygous Trl1/c larvae survive, their imaginal discs are normal and show wild-type levels of UBX expression. Homozygous TrlR85 larvae do not reach third instar. In contrast, some of the TrlR85/Trl1/c larvae develop till third instar and, remarkably, their imaginal discs are very small and show normal levels of UBX expression (Fig. 2).

Requirement of Trithorax-like throughout larval development

Somatic clones lacking the function of Trl were induced at different times during development in both wild-type and in Minute mutant backgrounds. Clones induced in a wild-type background were small and showed much weaker phenotypes than those observed in a Minute mutant background, which confers a growth advantage (Morata and Ripoll, 1975). Therefore, we will describe the phenotype of the clones induced in a Minute mutant background marked with yellow and multiple wing hair as cuticular markers.

Clones induced at 0–24 h after egg laying (AEL) did not develop. Clones induced at 24–48, 48–72, 72–96 or 96–120 h AEL exhibit very similar phenotypes and appear throughout the entire body. As expected, clones induced earlier are larger and affect a greater part of the tissues. We analysed clones in the whole adult cuticle, paying special attention to those phenotypes indicative of either inactivation or derepression of homeotic genes. In general, most

Fig. 1. TrlR85 germ line clones. (A) Wild-type unfertilised embryo. (B) TrlR85 germ line clone unfertilised (maternal− zygotic−) egg. Note the defective micrycle (arrow head) compared to (A). (C) Wild-type cuticle larva. (D) maternal− zygotic− embryo. The anterior part of the embryo and the denticle belts are abnormal.
clones (except in the abdomen) display lack of bristles and none of the clones present any obvious homeotic transformation. In the head, the antennae, including the arista, are defective (Fig. 3I). Clones in the eyes, the proboscis and palpi sometimes reduce their sizes. In the thorax, the notum is often split and there is a clear disappearance of both macrochaetae and microchaetae (Figs. 3D, F, G). In some cases, very few small yellow bristles appear in the territory of the clone, marked with mwh, suggesting that the clone bristles might not have had enough time to grow (Figs. 3F, G). Previous studies have shown that overexpression of homeotic genes in the notum often results in a split thorax phenotype and are usually accompanied by round dark vesicular structures (Casares et al., 1996). The phenotype of the TrlR85 clones does not have this feature.

In the wing margins, clones running along the dorso-ventral border usually delete the triple row of bristles (Fig. 3Q). In the wing blade, they often develop blisters, reduced their sizes (Fig. 3T) and, interestingly, they induce a wing “held-out” phenotype (Fig. 3B). This phenotype has been associated with the inactivation of the Antp P2 promoter (Vazquez et al., 1999). In the legs, most of the clones showed

Fig. 2. Expression of EN and UBX in TrlR85 homozygous embryos. (A) Wild-type embryonic UBX expression—details shown in (C). (B) UBX expression in TrlR85—details shown in (D). (E) Wild-type EN expression—details shown in (G). (F) EN expression in TrlR85—details shown in (H). Note the difference of expression levels along the A/P axis. (I) Wild-type wing imaginal disc stained with UBX. Expression is seen in the cells of the peripodial membrane. (J) TrlR85/TrlR85 wing disc. Note the differences in size compare to the (I). (K) Expression of UBX in a wild-type haltere disc. (L) Expression of UBX in TrlR85/TrlR85/TrlR85 haltere disc. Expression is normal, but the size of the haltere disc is dramatically reduced (compare K and L).
lack of bristles and we do not see any clone showing transformation of one leg into another (Fig. 3M). In the first leg of males, mutant clones showed a reduction of the number of sex comb hairs which, invariably, is accompanied by a lack of surrounding bristles in the clones (Fig. 3K). The reduction of the number of sex combs hairs could indicate that the Scr gene is being inactivated. Particular attention was focused on clones in the haltere because it seems to be the most sensitive structure to be transformed in Trl mutations (Farkas et al., 1994). Many clones are found in the haltere, but none show any transformation towards wing. In the abdomen, clones appear in all the segments in both males and females. In no case, the mutant clones show transformation towards more anterior or posterior segments. The clones in the genitalia and analia follow the same pattern of missing bristles affecting the vaginal denticles in females and the claspers in males (Fig. 3O).

None of the clones show a transformation similar to those produced in either trithorax (Ingham, 1985) or Polycomb mutant clones (Busturia and Morata, 1988), as could have been expected if Trl functioned as an activator/repressor throughout development. It could be argued that a putative reduction of only 50% in the expression of homeotic proteins (see above) in TrlR85 homozygous cells is not enough to produce morphological visible transformations. However, haplo-insufficient phenotypes due to the lack of one dose of either Ubx or Abd-B proteins are observed in the adult flies, suggesting that a reduction of 50% produces an observable mutant phenotype.

These results suggest that the Trl function is not required for homeotic gene expression throughout larval development. However, TrlR85 clones clearly exhibit defects in both the development of macro- and microchaetae in the notum, and the bristles in the head, wings and legs (Fig. 3), suggesting that Trl might be required for other functions during larval development.

**Homeotic gene expression in the TrlR85 somatic clones**

We investigated whether homeotic gene expression was affected in the TrlR85 clones in the imaginal discs. Some of the phenotypes observed in the mutant clones in the adult might have been indicative of inactivation of Scr and Antp homeotic genes. However, when we look at the expression of ANTP and SCR in the clones in the wing and leg discs, we do not see changes in its expression compared to wild-type. Moreover, the split thorax and the deletion of the wing triple row phenotypes are sometimes observed when homeotic genes are over-expressed (Busturia and Morata, 1988).

Analysis of the UBX expression in the imaginal discs containing TrlR85 clones shows either over-expression in the wing disc or under-expression in the haltere discs (Fig. 4F) at anytime of clone induction. Although this is in agreement with Brown et al.’s (2003) findings, they did not look at the UBX expression in TrlR85 clones throughout larval development. Likewise, ABD-A and ABD-B expression is not observed in the wing and haltere discs in the TrlR85 clones, neither EN expression was affected in the imaginal discs.

Because lack of bristles was a prominent phenotype (Fig. 3)—particularly in the notum—we investigated several proneural genes (Campuzano and Modollel, 1992) for expression changes in mutant clones. Analysis of the expression of the scute protein shows it to be normal. In sensory organ development, the proneural genes activate senseless expression which, in turn, activates and maintains the expression of proneural genes (Nolo et al., 2000). An analysis of senseless protein expression shows it to be absent in wing discs TrlR85 clones induced at late (120 h AEL) developmental stages (Fig. 4C).

These results support our conclusion that Trl is required during embryogenesis for the activation of the homeotic genes but it is dispensable during imaginal disc development for its regulation. Moreover, they also support the conclusion that other genes might require the function of Trl at later stages of development.

**Trithorax-like embryonic regulation of MCP-mediated silencing**

Studies using minigene constructs containing PREs have suggested that Trl plays a role in repression of the homeotic genes. However, none of these studies distinguish when, during development, the repressive function takes place. The MCP138 silencer of the Abd-B gene (Busturia and Bienz, 1993; Muller et al., 1999) contains four PHO- and two GAF-binding sites. We have previously shown that the MCP138 silencer element is able to maintain the repression of the PBX enhancer (Muller and Bienz, 1991) (in the P[PBXMCP138] construct) anterior to...
ps6 (posterior compartment of the haltere disc) throughout larval development (Busturia and Bienz, 1993; Busturia et al., 2001). However, this repression is abolished when either the flies containing the $P\{PBXMCP138\}$ construct are in a $Trl^{R85}$ background, or when the GAF-binding sites are mutated ($P\{PBXMCP138\} \text{GAGA mut}$ construct). In both cases, the imaginal discs show a lacZ expression in ps4 (anterior compartment of the wing disc) and ps5 (posterior compartment of the wing disc and anterior compartment of the haltere disc) (Busturia et al., 2001). These results did not indicate at what developmental stage the derepression occurs.

In embryos carrying a $P\{PBX\}$ construct (Muller and Bienz, 1991), the expression is basically the same as $P\{PBXMCP138\}$, although its expression does not last as long as in $P\{PBXMCP138\}$ embryos, where lacZ expression is silenced anterior to ps6 till the end of embryogenesis both in the epidermis and very weakly in the central nervous system (Fig. 5). This indicates a role of MCP138 in the maintenance of PBX expression. Since we have found that $Trl$ is required only early in development to regulate the expression of the homeotic genes, we have further investigated whether absence of the GAF and/or the GAF-binding sites is required for the MCP138 silencing in embryogenesis. To do that, we have looked at the lacZ expression of $Trl^{R85}$ homozygous embryos carrying the $P\{PBXMCP138\}$ construct, and at the wild-type embryonic lacZ expression of the $P\{PBXMCP138\}\text{GAGA mut}$ transgenic line. We have found that in both cases, the expression of lacZ is derepressed anterior to ps6 (Fig. 5) in a variable way both in the epidermis and the CNS. These results indicate that both GAF and GAF-binding sites are required in the early embryo for the silencing activity of the MCP138 element and suggest that the effect previously seen in the imaginal discs (Busturia et al., 2001) is likely a consequence of the effect in the embryo.

***Trithorax-like genetic interactions***

It has been shown that $Trl^{R85}/Ubx^{130}$ flies show enhancement of the $Ubx^{130}$ haplo-insufficient phenotype (Farkas et al., 1994). Also, $Trl$ mutations increase the penetrance of the phenotype of trxG mutant alleles (Gildea et al., 2000). These results indicate that GAF belongs to the trxG activating proteins. However $Trl$ mutations also enhance the PcG mutant phenotypes (Busturia et al., 2001; Strutt et al., 1997), suggesting therefore, that GAF could also have a repressor role in the maintenance of homeotic gene expression. To define better the function and the time of action of $Trl$ in the regulation of homeotic gene expression, we have performed a detailed analysis of the $Trl$ genetic interactions resulting phenotypes.

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**Fig. 4. Imaginal discs phenotypes.** (A) Merged image of a wing imaginal disc showing the GFP expression (green) and senseless protein expression (red). (B) Lack of GFP expression (green) indicates the territory of the clones. (C) Senseless expression (red) is lost in the $Trl^{R85}$ clone induced at 96 – 120 h of development. (D) Merged image of a haltere imaginal disc showing the GFP expression (green) and the Ubx expression (red) indicating the territory of the clones. (E) GFP lack of expression indicates the presence of $Trl^{R85}$ clones. (F) Expression of UBX (red) is normal all along the haltere.
A striking observation is the low frequency of enhancement of the Ubx haplo-insufficient phenotype in TrlR85/Ubx130 flies. The penetrance reported for this phenotype ranges from 0.27% (Hur et al., 2002), 4% (our experimental conditions) and 6% (Schwendemann and Lehmann, 2002). We have further studied whether this penetrance could be also affected by the lack of PHO protein. Interestingly, the TrlR85/Ubx130; pho1/+ flies show an enhancement of the Ubx130 phenotype in 18% of the flies, indicating a novel activator role for PHO.

We investigated the ability of Trl to modulate, by suppressing or enhancing, the gain of function phenotypes associated with the over-expression of homeotic proteins. We focused on the Antp\textsuperscript{rev1}-, Cbx1- and Mcp1- dominant homeotic mutations. Antp\textsuperscript{rev1}/+ flies show Antp protein ectopically in the antenna causing a transformation towards legs (Zeng et al., 1993). We see that 37% of Antp\textsuperscript{rev1}/TrlR85 flies show the antenna to leg phenotype, compared to the 25% observed in Antp\textsuperscript{rev1}+/+. Therefore, there is a small but significant enhancement of the phenotype in TrlR85 background. Homozygous and heterozygous Mcp1- adults show a transformation of the abdominal A4 segment towards the A5 as indicated by the strong dark pigmentation in A4 in the adult males (Fig. 6B) (Lewis, 1978). This is due to the ectopic expression of Abd-B protein in the A4 segment, where it is normally repressed (Celniker et al., 1990). In Mcp1+/ flies, the pigmentation of the A4 segment is observed in 100% of the flies with constant and high expressivity (the whole A4 segment is fully pigmented). However, in 96% of the Mcp1+/TrlR85 males, patches of depigmentation are observed in the A4 segment (Fig. 5C). This indicates that absence of Trl causes reduction of the Mcp1- phenotype. Cbx1- flies show a transformation of the posterior wing towards the posterior haltere (Casanova et al., 1985) due to ectopic expression of UBX in the wing (Cabrera et al., 1985). One hundred percent of the Cbx1/+ flies show a wing to haltere transformation with variable expressivity (Figs. 6D, E). Also, Cbx1/TrlR85 flies show a 100% penetrance of the wing to haltere transformation, but with much higher expressivity (Figs. 6F, G) when compared to Cbx1/+ flies. We have calculated the wing areas in both the Cbx1/+ and Cbx1/TrlR85 flies and found that Cbx1/TrlR85 flies show a 40% reduction of wing size compared to wing size of Cbx1/+ flies. This indicates that lack of GAF enhances the Cbx1 transformation. In summary, TrlR85 modulates the gain of function phenotype in mutations of the three genes studied. The modulation ranges from weak enhancement (Antp\textsuperscript{rev1}) to strong enhancement (Cbx1), and to strong suppression (Mcp1). We have studied whether the effect of these interactions could already be reflected in the expression of the homeotic proteins in the embryo. ABD-B is expressed from ps10 to ps14 in the wild-type embryo. Mcp1/Mcp1- embryos show an ectopic expression of ABD-B in ps9. We have failed to detect a reduction of ABD-B expression in ps9 in TrlR85/Mcp1- embryos, most probably because the effect is very subtle. We have found that UBX expression is quite normal in Cbx1/+ embryos (Fig. 6H). However, in Cbx1/TrlR85 embryos, the expression of UBX is derepressed anterior to ps5 (Fig. 6I), indicating that the enhancement of the Cbx1 phenotype in Cbx1/TrlR85 flies can be observed as early as embryogenesis.

Taking together, these results may suggest the Trl functions have both activator and repressor functions. However, we favour the model where Trl has only one function but its

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**Fig. 5.** TrlR85 regulation of MCP-mediated silencing. In all the cases, embryonic β-galactosidase expression is shown. (A) P[PBX-MCP138-GAGAmut-Ubxpp-lacZ]. (B) P[PBX-MCP138GAGAmut-Ubxpp-lacZ]. (C) P[PBX-MCP138-Ubxpp-lacZ]. (D) P[PBX-MCP138-Ubxpp-lacZ] in TrlR85 homozygous. Arrow heads indicate PS6.
mutations produce dual activator and repressor effects (see Discussion).

Discussion

Temporal requirement of Trithorax-like for the regulation of homeotic genes

We have shown that the expression of UBX and EN in Trl homozygous embryos is significantly reduced (Fig. 2). These results indicate that, independently of the strong maternal effect of the GAF (Bhat et al., 1996; Farkas et al., 1994), it is possible to observe that Trl regulates the expression of Ubx and en genes as early as embryonic development.

It is not clear why only a reduction of UBX and EN expression is observed, and not the other homeotic proteins studied. However, several possibilities exist to explain these results. The most direct is that Scr, Antp, abd-A and Abd-B genes are simply not regulated by GAF. Another possibility is that the change in protein level in the null mutant is below the detection sensitivity limit of the assay. However, similar promoter-specific reductions in homeotic gene expression have been reported when studying the effect of trx (Breen and Harte, 1993) and PcG mutations (Soto et al., 1995). As Trl has been proposed to have a very broad and general role in gene regulation (van Stennsel et al., 2003), we favour the explanation that the differences observed are caused by the different specificity/sensitivity of the homeotic genes to transcriptional changes. This different sensitivity is also
reflected in the phenotypes observed when studying interactions of TrlR85 and bithorax genes null mutant alleles. For example, TrlR85/Ubx130 shows an enhancement of the Ubx130 haplo-insufficient phenotype in adult flies, but neither TrlR85/Ubx130 nor TrlR85/AbdBM1 flies show an enhancement of the haplo-insufficient phenotype of Ubx and Abd-B mutations, respectively (data not shown). Interestingly, Ubx130 (a breakpoint on the TM2 balancer chromosome) and Ubx1 show different sensitivity, although both are null mutant alleles. This implies that pairing of the chromosomes might also influence the sensitivity to transcriptional changes (Duncan, 2002; Goldsborough and Kornberg, 1996) in TrlR85 embryos. Moreover, ether-induced phenocopies of the Ubx gene are the most often homeotic transformations found and, curiously, the frequency is higher in a Ubx130 than in a Ubx1 mutant background (Capdevilla and Garcia-Bellido, 1974). Finally, mutations in the RNA polymerase II gene (Rpl1215, coded by the Ubx-like gene in Drosophila) exclusively show Ubx mutant phenotypes (Greenleaf et al., 1980). These observations, although not conclusive, support the idea that Ubx may be more sensitive to changes in transcription than other homeotic genes, which is consistent with our findings that only a reduction of UBX expression is observed in TrlR85 embryos.

Homozygous TrlR85 clones induced at different stages of larval development do not show any kind of phenotype indicating homeotic transformations (Fig. 3). Moreover, we do not see any changes of expression of the ANTP, SCR, UBX, ABD-A, ABD-B and EN (Fig. 4F) in the TrlR85 clones in the imaginal discs, consistent with the absence of homeotic phenotypes observed in the adult cuticle. Although reduction in the number of the sex comb hairs was observed in the clones, we believe that this is an effect of the invariably lack of bristles accompanying the clones. The fact that reduction of sex comb hairs has been observed in Trl13c homozygous flies or in spt16/+; Trl13c/+ double mutant combinations (Jansen et al., 2000, Shimojima et al., 2003) most probably indicates that the activity of the Scr gene is affected, but this may happen very early in development. Therefore, we could conclude that Trl is not required for the regulation of the homeotic genes during larval development. This apparent dispensability of GAF during larval development could be due, at least in part and only for the homeotic gene regulation, to the persistence of sufficient stable wild-type GAF from TrlR85/+ heterozygous cells giving rise to clones. It is, however, worth emphasising that the lack of homeotic phenotypes cannot be explained by the nature of the mutant allele because TrlR85 is a null allele. Nor can it be explained by perdurance of the maternal Trl protein because we see an effect in homeotic gene expression in embryogenesis and, furthermore, because some other non-homeotic phenotypes observed are not rescued by the proposed long perdurance of the maternal component.

We consistently observed that the clones exhibit lack of bristles phenotypes (Fig. 3). These phenotypes are often associated with mutations in genes involved in control of cell cycle, mitosis and/or chromosome segregation (Ueda et al., 1992). Trl is known to be involved in the regulation of these processes (Bhat et al., 1996; Trunova et al., 2001). Therefore, the lack of bristles phenotypes may be more related to a Trl function in cell cycle and division than to the regulation of the homeotic genes. Mutations in mitotic genes often result in very small or absent imaginal discs, but the homozygous mutant larvae still survive because of the dispensability of mitotic genes for larval growth (Ripoll et al., 1992). Therefore, the larval survival of some Trl mutant combinations could be due to its function in mitosis rather than to the proposed long perdurance of GAF (Bhat et al., 1996). The reduced size of the TrlR85/Trl13c imaginal discs reported in this work (Figs. 4G–H) supports this conclusion.

Temporal requirement of Trithorax-like for MCP-mediated silencing

We have shown that MCP-mediated silencing during embryogenesis requires the function of GAF. We previously reported this requirement in TrlR85/+ larvae carrying the P[PBXMCP138] construct (Busturia et al., 2001). These results indicate that GAF protein is required in the early embryo for the silencing activity of the MCP138 element, and that the effect seen in the imaginal discs is likely a consequence of the effect in the embryo. We were not able to study the Trl temporal requirements for the MCP-mediated silencing throughout larval development (i.e., to study the expression of lacZ in TrlR85 homozygous clones) because these constructs contain FRT elements flanking the MCP138 fragment, and the recombination will take place between the FRTs both in cis and in trans, thus making it impossible to distinguish the effects of both recombination events. However, recent results by others (Brown et al., 2003) have indicated that homozygous TrlR85 clones induced at some time in larval development and in a Minute mutant background do not affect the repression conveyed by the MCP800 element, an 800-bp DNA fragment that includes the MCP138 sequence (Busturia et al., 2001). Therefore, considering both results—the expression in the embryo and the lack of requirement of Trl for the silencing of MCP800 reported by Brown et al.—it can be concluded that MCP silencing requires GAF early in embryogenesis, and that its function seems dispensable throughout larval development.

Function of Trithorax-like in the regulation of homeotic gene expression

Repression is the default state of many eukaryotic genes. This state has to be modified for the gene to be further dynamically activated or repressed. Our results show that Trl has an important role very early in development, but it is later dispensable for the regulation of homeotic genes. This is distinct from the role it plays in cell cycle and mitosis.
during larval development (Bhat et al., 1996; Trunova et al., 2001). Based on our results, we propose a model in which Trl is one of the earliest acting genes regulating the homeotic genes expression. In this model, the role of Trl is to establish very early in development, via chromatin remodelling, a transcriptional ground state of many genes, including the homeotic genes. This chromatin ground state is a pre-requisite for further signalling resulting in negative and/or positive transcriptional gene regulation. Our model implies that GAF function may be up-stream to other specific regulatory events. Finally, GAF interaction with its target genes can result in either activation or repression, depending not on an intrinsic property of GAF and its regulatory proteins partners, but on the characteristic of the particular regulated gene.

In our model, the repressed default state of the Ubx gene is modified by GAF and, most probably, many other factors. As a result, the Ubx gene is ready to be activated or repressed in different parts of the body by the action of the corresponding regulators (e.g., the segmentation genes), but independently of GAF. Later in development, maintenance of Ubx expression is also regulated by the action of trcG and PcG genes, most likely independently of GAF. Supporting an early and only role of GAF in the regulation of the homeotic genes is the observation that the formation of hypersensitive nuclease sites (nucleosome-free regions) by GAF, is transient, that is, takes place only early in development (cited as unpublished results in Bhat et al., 1996).

Establishing a given chromatin status for the homeotic genes is probably not an exclusive role of GAF. Other proteins that have been shown to act synergistic with GAF may also participate in this function (Faucheux et al., 2003; Huang et al., 2002; Hur et al., 2002; Mahmoudi et al., 2003; Schwendemann and Lehmann, 2002). It is difficult to predict which of the proteins that genetically interact with GAF might be involved in this early function. Temporal requirement experiments should address this question.

However, there are some indications of what proteins might be involved in this co-regulation. The Pipsqueak protein binds to almost the same DNA sequences as GAF, shows a complete overlap with GAF in their binding sites in polythene chromosomes and has dual activator/repressor functions on the regulation of homeotic genes (Huang et al., 2002; Schwendemann and Lehmann, 2002). GAF dispensability for the regulation of homeotic genes could also be due to the ability of Pipsqueak (or other related protein) to compensate for loss of GAF. Zeste protein has also dualistic functions and binds DNA sequence specifically (Hur et al., 2002; Rastelli et al., 1993). Both proteins could be good candidates to collaborate with GAF in its early function. Interestingly, we have found that TrlR85/Ubx130; pho1/+ flies show a significant enhancement of the Ubx130 haplo-insufficient phenotype compare to TrlR85/Ubx130 flies. Moreover, the interaction of PHO with its DNA target sequence is facilitated by GAF (Mahmoudi et al., 2003). These results suggest that PHO may also cooperate with GAF for its early function. Finally, the establishment of hypersensitive nuclease sites and a synergistic increase in transcription in the Alcohol dehydrogenase (Adh) gene, require the presence of both binding sites for GAF and Adf-1 (a transcriptional regulator of Adh) (Pile and Cartwright, 2000). Like Adh expression, the regulation of the homeotic genes may require multiple regulators that act synergistically to achieve a required level of chromatin modification that can then be modified further to reach the activated or repressed transcriptional state.

Because of its ability to bind DNA in a sequence specific manner, it has been proposed that GAF might be involved in the recruitment of the PcG/trxG complexes to DNA throughout development (Horard et al., 2000; Poux et al., 2002). Our results show that GAF is required only in early development. During this time, there is likely to be an interaction, albeit transient, with PcG/trxG proteins. Interactions later in development may occur between those PcG/trxG proteins involved in cell cycle regulation.

**Trithorax-like mutations produce dual loss of activation and loss of repression effects**

The phenotypes observed when studying Trl genetic interactions suggested a dual activator/repressor function of the GAF. However, as described above, we think that the Trl function in the regulation of the homeotic genes is to establish a chromatin ground state very early in development. This chromatin ground state is susceptible to both activation and repression but does not initiate either activity. We propose that Trl does not have a dual activator/repressor role. Rather Trl mutations produce a dual loss of activation and repression effects. Why are opposite effects observed? Is this consistent with our model? We believe that the effect of Trl mutations depends on the balance of the activators and repressors proteins acting on a particular sequence and on the cross-regulatory interactions between proteins regulating a given gene. In TrlR85 homozygous embryos, the level of UBX expression is reduced but, curiously, its pattern of expression is quite normal. This can be interpreted as Trl being an activator of Ubx expression. However, we think that absence of GAF creates a global reduction of transcription that potentially affects equally to all the activators and repressors of the Ubx gene, including the Ubx gene itself. This produces a balanced reduction of negative and positive transcriptional regulation, resulting in a pattern of UBX expression that is still quite normal. Therefore, we propose that the reduced level of UBX expression is not due to Trl acting as an activator of Ubx. Rather, it is due to the global effect that Trl mutations have in the Ubx expression regulating genes.

When studying the Trl function on a given regulatory DNA sequence, for example, the PBXMC138 element or the sequences in the Chel and the Mep mutations, the effect of absence of Trl depends on the balance between activators
and repressors acting on that particular sequence. In a Trl mutant background, the global reduction of activation and repression affects the ability of the PBXMCP138 regulators to act. In this case, the effect of absence of GAF is primarily observed as loss of repression because negative regulation in this sequence is most likely dominant to positive regulation (Zhang et al., 1991). This model also explains the effect of TrlR85 on the enhancement of the ChxI and the partial suppression of the McpI phenotypes. In the case of the ChxI mutation, repression is dominant and therefore the effects seen in TrlR85/ChxI flies would be predominantly loss of repression. In the case of McpI, the TrlR85 effect is explained as global inactivation of the McpI regulators, which results in a still partial suppression of the phenotype because repression is dominant in the McpI sequence.

Finally, we have also shown that GAGA-binding sites are required during embryogenesis for the MCP138-mediated silencing, further indicating a repressor function of GAF. In the P[PBX MCP138GAGAmut] embryos, GAF function is normal, but mutation of the GAF-binding sites results in a phenotype equivalent to complete lack of function of GAF in the MCP138 sequence.

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