The *Drosophila trithorax* gene encodes a chromosomal protein and directly regulates the region-specific homeotic gene *fork head*

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The activity of the *Drosophila* gene *trithorax* is required to maintain the proper spatial pattern of expression of multiple homeotic genes of the Bithorax and Antennapedia complexes. *trithorax* encodes two large protein isoforms of >400 kD. We have detected its products at 16 discrete sites on larval salivary gland polytene chromosomes, 12 of which colocalize with binding sites of several *Polycomb* group proteins. The intensity of *trithorax* protein binding is strongly decreased in larvae carrying mutations in another *trithorax* group gene *ash-1*, and in the *Polycomb* group gene *pco/E(z)*. A strong *trithorax* binding site was found at the cytological location of the *fork head* gene, a region-specific homeotic gene not located within a homeotic complex. Further analysis showed that *trithorax* protein binds at ectopic sites carrying *fork head* sequences in transformed lines. *Trithorax* binding occurs within an 8.4-kb regulatory region that directs *fork head* expression in several embryonic tissues including salivary glands. Consistently, expression of endogenous *fork head* RNA is greatly reduced in *trithorax* mutant embryos and in larval tissues. These results show that *trithorax* maintains expression of target genes by interaction with their regulatory regions and that this interaction depends on the presence of at least some of the other *trithorax* and *Polycomb* group proteins.

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The control of body segment identity during development in many organisms is achieved in large part by the activities of homeotic genes. In *Drosophila*, the product of each homeotic gene is distributed in a unique pattern in the embryo and determines the segmental identity of the cells in which it is expressed (for review, see McGinnis and Krumlauf 1992). Thus, the establishment and maintenance of correct patterns of expression for these genes is critical for the determination of the future fate of embryonic cells. The combined activities of the segmentation genes initiate the expression of the selector homeotic genes of the Antennapedia complex (ANT-C) and the Bithorax complex (BX-C) (Scott and Carroll 1987; Ingham 1988; Kaufman et al. 1990). Although many segmentation genes are only transiently expressed, their products fading away by the completion of gastrulation, the homeotic genes maintain their initially established pattern of expression through later development, both among and within segments. Therefore, additional

mechanisms and factors are required to preserve the homeotic gene expression patterns initiated at blastoderm. Two groups of genes, the *Polycomb*-group (*Pc-G*) (for review, see Paro 1990, 1993) and the *trithorax*-group (*trx-G*) (for review, see Kennison 1993), play a major role in the maintenance of, respectively, the repressed and the active state of homeotic gene expression during development. It has been proposed that the products of different *Pc-G* genes assemble in a multimeric complex only at target genes that are not actively transcribed, somehow locking them in an inactive state. This would imprint a determined state of the chromatin that could be inherited by the cellular progeny (Paro 1990). The four *Pc-G* proteins analyzed so far colocalized at a large number of sites on polytene chromosomes, suggesting that they often function together (Zink and Paro 1989; DeCamillis et al. 1992; Martin and Adler 1993; Rastelli et al. 1993). Moreover, it was shown that *Polycomb* (*Pc*) and polyhomeotic (*ph*) products are constituents of a large multimeric protein complex (Franke et al. 1992).

Contrasting with *Pc-G* repression is activation by *trx-G* genes. Genetic studies have identified several genes constituting the *trx-G* that are involved in the maintenance of the normal levels of homeotic gene ex-

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pression (Kennison and Tamkun 1988; Shearn 1989). Mutations in members of the *trx*-G produce additive effects with *trithorax* (*trx*) and with each other (Shearn 1989). Mutations in the genes of the *trx*-G, as well as *trx* itself, have the property of being suppressors of the *Pc* mutant phenotype (Kennison and Tamkun 1988). *trx* itself is required continuously throughout development from the early stages of embryogenesis until the late larval stages, as demonstrated by temperature-shift experiments and by clonal analysis (Ingham 1981, 1985). Loss-of-function mutations in *trx* often cause homeotic transformations similar to loss-of-function mutations in genes of the ANT-C and the BX-C. Transformations of the first and third thoracic segments toward the second thoracic segment, and transformations in the abdomen to a more anterior pattern, are seen in homozygotes, weak alleles, or hemizygous adults (Lewis 1968; Ingham and Whittle 1980; Ingham 1981). Embryos homozygous for lethal *trx* alleles display cuticular phenotypes, suggesting that *trx* is required for the normal level of activity of many homeotic genes (Duncan and Lewis 1982; Ingham 1983; Breen and Harte 1991). Immunostaining experiments showed that in *trx*⁻ mutant embryos expression of all BX-C genes and several ANT-C genes is reduced and is affected in a tissue-, parasegment-, and promoter-specific fashion (Mazo et al. 1990; Breen and Harte 1992). Genetic experiments suggest that *trx* interacts (directly or indirectly) with specific and discrete cis-regulatory regions of *Ubx* where *anterobithorax* and *postbithorax* mutations map (Castelli-Gair and Garcia-Bellido 1990).

The *trx* gene encodes five 11- to 14-kb RNA isoforms produced by differential splicing (Mozer and Dawid 1989; Breen and Harte 1991; Sedkov et al. 1994). Two 14-kb transcripts encode a protein of 3727 amino acids that has several Cys-rich regions with similarities to zinc finger-like DNA-binding domains (Mazo et al. 1990). Bacterial *trx*-LacZ fusion proteins containing Cys-rich regions bind zinc in vitro, consistent with the proposal that the *trx* product might be a nucleic acid-binding protein (Mazo et al. 1990). During cellularization, two of the *trx* RNAs are expressed in an unexpected posteriorly restricted pattern (Sedkov et al. 1994). These results suggest that the existence of this posterior expression domain is associated with a distinct *trx* function required for the proper expression of BX-C genes in the mesoderm and ectoderm as early as embryonic stages 10–11. Expression of the anteriorly expressed homeotic genes *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*) is unaffected in *trx* mutants at this stage. Instead, normal levels of ANT-C expression appear to require *trx* expression near the end of embryogenesis, a function possibly provided by products of the late embryonic *trx* transcripts (Sedkov et al. 1994).

Interestingly, there is a mammalian homolog of *trx*. Chromosomal translocations associated with leukemias in infants often have breakpoints in one of the introns of the *ALL-1/HRX* gene (Gu et al. 1992; Tkachuk et al. 1992). The human and mouse *ALL-1/HRX* genes encode proteins of similar size that share two regions of homol-

ogy with *trx* (Gu et al. 1992; Tkachuk et al. 1992; Ma et al. 1993). There is a homologous region in the central Cys-rich part of both proteins. The structure of this domain appears to be unique to the *trx* and *ALL-1/HRX* genes. The most striking homology (61% identity, 82% similarity over 215 amino acids), however, is found in the carboxy-terminal part of these proteins. *Enhancer of zeste* [*E(z)*], a gene from the *Pc*-G, encodes a protein of 747 amino acids with 53 amino acids at the carboxyl terminus that are 57% identical (70% similar) to the carboxy-terminal part of *trx*. This further emphasizes the importance of the carboxy-terminal domain of *trx* and suggests the existence of a new family of related proteins. Interestingly, mutations in *E(z)* have a strong maternal effect that is strongly suppressed by *trx*^{B11} (Jones and Gelbart 1993).

In the experiments described here we demonstrate that *trx* is a chromosomal protein. We detect *trx* protein binding at 16 discrete sites on polytene chromosomes, and show that its binding depends on the presence of other *trx*-G and *Pc*-G proteins. We also show that *trx* interacts in vivo with regulatory elements of the region-specific homeotic gene *fork head* and is required for its proper expression in multiple embryonic and larval tissues.

Results

Immunodetection of *trx* proteins in embryonic extracts

The *trx* locus encodes five alternatively spliced embryonic and larval transcripts (Sedkov et al. 1994). They encode two putative protein isoforms, *trxI* of 368 kD and *TrxII* of 404 kD, which differ in their amino-terminal sequence (Mazo et al. 1990; Sedkov et al. 1994). The predicted *trx* proteins (Mazo et al. 1990) contain two regions, a Cys-rich, zinc finger-like domain in the middle of the proteins, and the carboxy-terminal region, which are very similar to regions of a human homolog (Gu et al. 1992; Tkachuk et al. 1992). Another functionally important domain is located downstream of the Cys-rich region (E3 in Fig. 1), as we have shown recently that deletion of this domain in *trx*^{E3} mutants affects embryonic expression of the ANT-C genes *Antp* and *Scr*, and the *fork head* (*fkh*) gene (Sedkov et al. 1994). Based on these structural and functional data, we raised polyclonal antisera against these regions of *trx* (Fig. 1A). The N1 antibody was raised against the amino-terminal domain, which should be present only in the large protein isoform. Figure 1B shows that in an embryonic extract, the N1 antibody detects one large protein band that is most probably the larger *trx* protein isoform *trxII* (Fig. 1A). Antibodies L2, N4, and L9 were raised against protein domains that are present in both protein isoforms (Fig. 1A). These antibodies detect *trxII* as well as a faster migrating band that is likely to be the smaller *trx* protein isoform *trxI* (Fig. 1A,B). Both protein bands are absent in extracts prepared from embryos homozygous for *trx*^{B11} (Fig. 1B), a mutant allele containing a frameshift deletion in the

Figure 1. Detection of *trx* products in embryonic extracts. (A) A scheme for *trx* protein processing. N1, L2, N4, and L9 indicate protein domains against which the corresponding antibodies were raised. L2 and L9 protein domains are highly homologous between *trx* and ALL-1/HRX (Gu et al. 1992; Tkachuk et al. 1992). E3 (solid box) indicates the protein domain of 280 amino acids deleted in *trx*^{E3} (Mazo et al. 1990); an arrow shows that specific cleavage of both protein isoforms occurs within a region deleted in *trx*^{E3}. *trxI* and *trxII* are the full-size *trx* protein isoforms of 368 and 404 kD predicted from sequencing of *trx* cDNAs (Mazo et al. 1990) and from the structure of the alternatively spliced *trx* mRNAs (Sedkov et al. 1994). (B) A set of filters of embryonic extracts stained with anti-*trx* antibodies N1 (1:200), L2 (1:300), N4 (1:300), and L9 (1:370). (E3) Nuclear extracts from embryos collected from heterozygous *trx*^{E3} flies; (wt, left panels) total extracts from wild-type embryos; (B11) total extracts prepared from homozygous *trx*^{B11}

embryos selected as described in Materials and Methods; (wt, right panels) total extracts from the heterozygous *trx*^{B11} embryos selected in the same experiment. Equal amounts of protein were loaded in each pair of adjacent lanes (except for the right wt lane in L9), as determined by Ponceau S staining of filters after electroblotting. Additional weak bands of unknown origin are occasionally seen with the N1 and L2 antibodies. A weak band at 275 kD in the B11 lane stained with N1 + N4 antibodies is caused by slight contamination with heterozygous embryos that fail to stain or are stained lightly with *lacZ* at late stages. To visualize large protein isoforms with the L9 antibody, ~10 times more extract was loaded (right wt lane). Overloading of the gel results in some additional bands, which disappear when the concentration of extract is lowered (left wt lane). L9 was raised against the carboxy-terminal domain of *trx*, which is highly conserved in flies and other animals. It is possible that additional bands may be attributable to other proteins of this family. Lane L2 + N4 shows the difference in abundance of *trx* protein isoforms in 0- to 7- and 8- to 20-hr embryos. The molecular masses for the 275-, 240-, and 200-kD proteins were estimated using 310- and 295-kD *trx*-*LacZ* fusion proteins (A. Mazo, unpubl.), and 212- and 170-kD protein markers. The molecular mass determination for the *trxI* and *trxII* protein isoforms is estimated, as no protein markers of appropriate size are available for the SDS gels and calibration curves are not linear for slow migrating bands.

amino-terminal portion of the *Trx*-coding sequence (Mazo et al. 1990).

Both full-size *Trx* proteins were very difficult to detect, even though we used several protease inhibitors to minimize protein degradation. These bands are barely visible in extracts prepared from embryonic nuclei (except for *trx*^{E3}, see below). We believe that this is attributable to instability of the protein, for the following reasons. We consistently detect two much more abundant, faster migrating bands that are also absent in extracts from *trx*^{B11} embryos. As with the large bands, the N1 antibody detects only the slowly migrating band of 275 kD, whereas the L2 and N4 antibodies detect an additional 240-kD band (Fig. 1B). These proteins are most likely the stable products of proteolytic cleavage and contain the amino-terminal portions of the two full-size protein isoforms *trxI* and *TrxII*. To detect the carboxy-terminal portion of the cleavage product we used antibody L9 raised against the carboxy-terminal portion of *trx* protein (Fig. 1B). As with the other antibodies, L9 detects the *trxI* and *trxII* isoforms. In addition, this antibody detects a single band of 200 kD (Fig. 1B), which is likely to be a stable cleavage product of the carboxy-terminal portion of both protein isoforms. Based on the

apparent sizes of the cleavage products, we predicted that a cleavage site might occur close to a protein sequence deleted in *trx*^{E3}. To test this, we analyzed nuclear extracts prepared from heterozygous *trx*^{E3} embryos. Figure 1B shows that these extracts are notably enriched in the large protein isoforms. In *trx*^{E3} extracts, the *trxI* and *trxII* isoforms migrate faster because of the 280-amino acid deletion in the *trx*^{E3} mutant (wild-type proteins in lanes E3 are not seen because of their low abundance in extracts from nuclei). The large E3 isoforms appear to be stable, as there are no bands that would correspond to their cleavage products, that is, bands migrating faster than 275 and 240 kD. Therefore, it is likely that the proteolytic cleavage of *trx* proteins occurs within a region deleted in *trx*^{E3}. These results show that *trx* produces two large, unstable protein isoforms, *trxI* and *TrxII*. These proteins may then undergo a specific proteolytic cleavage, probably within the same sequence, giving rise to three stable *trx* protein isoforms. Figure 1B also shows that in early embryos the large protein isoform and the corresponding degradation product are less abundant than in late embryos. On the contrary, the smaller protein isoform is more abundant in early embryos. This is consistent with RNA splicing results, which showed

that in early embryos the large protein isoform is encoded only by the minor early zygotic *trx* RNA E1 (Sedkov et al. 1994).

Antibody staining of tissues

The L2 antibody detects *trx* protein in embryonic nuclei, and the distribution is similar to that of late zygotic *trx* RNA (Fig. 2A–C). The staining patterns with N4 and L9 antibodies are virtually identical. In addition to Western blot analysis, the specificity of the L2 antibody was confirmed using *trx* mutant embryos. The progeny of *trx*^{B11} embryos balanced with a *TM3, fushi tarazu (ftz)-lacZ* balancer chromosome were double-stained with *trx* and β -galactosidase antibodies. At late embryonic stages when maternal protein is presumably decreased, homozygous *trx* mutant embryos, those not staining for β -galactosidase showed little or no staining with *trx* antibody (data not shown). Because *trx* protein translated from maternally derived *trx* RNA is quite abundant at

early stages, we were not able to detect a spatially restricted pattern characteristic of early zygotic *trx* transcripts (Sedkov et al. 1994). The N1 antibody stains late embryos similarly, but it does not stain embryos efficiently before germ band retraction (data not shown). These observations correlate with the alternative splicing patterns that we have described previously (Sedkov et al. 1994). *trx* produces one maternal and one major early zygotic RNA, neither of which contain an exon encoding the amino-terminal part of the protein to which N1 antibody was generated. Therefore, N1 recognizes mainly the late embryonic *trx* protein isoform, which contains the amino-terminal region.

trx antibody also stains a variety of tissues in third-instar larvae. The strongest staining is seen in imaginal discs, salivary glands, and gut tissues (Fig. 2I–K). Staining of the brain was weak, while staining was almost absent in the ventral ganglia (data not shown). In the eye–antennal disc, *trx* is expressed in most of the nuclei,

Figure 2. Developmental expression of the *trx* protein. (A) A syncytial blastoderm embryo, showing nuclear localization of *trx* protein. (B,C) A stage-14 embryo *trx* protein and RNA are present at a higher level in the embryonic CNS. (D–K) A dissected third-instar larva shows staining in the nuclei of imaginal discs (D–G), the salivary glands (I), and gut tissues (J,K). (D–G) Localization of the *trx* protein and RNA (H) in eye–antennal (D), wing (E), dorsal metathoracic (halter) (F), and mesothoracic leg (G,H) discs. In all but C and H immunostaining was with L2 antibody. (C,H) In situ hybridization using digoxigenin-labeled RNA probe (Sedkov et al. 1994). (gc) Gastric ceca; (mg) midgut; (Mt) Malpighian tubules; (pv) proventriculus.

whereas in wing, haltere, and mesothoracic leg discs it is expressed predominantly in the posterior regions. These patterns are all similar to distribution of *trx* RNA (Fig. 2C–H).

trx protein is associated with 16 loci on polytene chromosomes

To determine whether *trx* protein is associated with specific chromosomal sites, we used antibodies N4 and L2 to stain salivary gland polytene chromosomes. Strong, highly reproducible labeling was observed when antisera against either of these two *trx* protein domains were

used (Fig. 3A). A similar pattern was seen with unpurified antiserum (data not shown). No staining was detected when preimmune sera from the same animals were used. The specificity of the staining pattern was confirmed by the absence of distinct stained bands on polytene chromosomes prepared from homozygous *trx*^{E12} mutant larvae (selected as described in Materials and methods).

We observed 16 strong polytene chromosome-binding sites using antibodies generated against *trx* protein domains L2 and N4. A list of these *trx*-binding sites is presented in Table 1. Occasionally we observed additional weak bands. Because the appearance of these weak

Figure 3. Effect of *ash-1* and *pco/E(z)* mutations on *trx* binding to polytene chromosomes. Salivary gland chromosomes were prepared from wild-type and mutant larvae raised at 29°C and stained with L2 antibody. (A) Immunostaining of squashed salivary gland chromosomes of wild-type larvae. The large arrowhead indicates the band at 98D1, the cytological location of *fkh*. (B) Several very weak *trx*-binding bands (small arrowheads) occasionally remain on chromosomes prepared from homozygous *ash-1*^{Vb234}. Many of the weak *trx* bands seen on wild-type chromosomes are seen on chromosomes from larvae homozygous for *PCO25* (C) and *E(z)*^{S2} (data not shown). (D) In salivary gland nuclei of wild-type larvae *trx* protein shows a punctated pattern. Although *trx* protein is localized at high levels in the salivary gland nuclei of *ash-1*^{Vb234} (E) and *PCO25* mutants (F), staining is more diffuse. (G) Northern blot showing that levels of the 14-kb *trx* RNA are similar in wild-type (wt) larvae, *ash-1*^{Vb234} (*ash-1*), and *PCO25* (*pco*) larvae raised at 29°C. (*rp49*) Hybridization of the same filter with an *rp49* probe.

Table 1. Comparison of *trx* and *Pc-G* and *zeste* protein-binding sites on salivary gland chromosomes

trx site	Pc-G and zeste sites	
2D	2D	<i>Pc ph Psc</i>
4C1-4	4C	<i>Pc ph Psc* Su z</i>
7A		
7B	7B	<i>Pc ph Psc Su z</i>
22F	22F/23A	<i>Psc* Su z</i>
29EF	29E	<i>Pc ph Psc* Su</i>
49E/F1,2	49EF	<i>Pc ph Psc Su z*</i>
56BC	56C	<i>Pc ph Psc Su z*</i>
57EF		
69EF		
70A	70A/B	<i>Pc ph Psc* z</i>
72A		
84A4,5	84A/B	<i>Pc ph Psc Su z*</i>
85E1,2	85E1	<i>Pc</i>
98D1,2	[98C/D]	<i>Pc ph</i>
99B1,3	99AD	<i>Pc ph Psc* Su z*</i>

No *trx* protein is detected either at the centromere or on the fourth chromosome. The data for *Psc*, *Su(z)2* (*Su*), and *zeste* (*z*), and the data for the coincidence of *Pc/ph* and *Psc* or *Su(z)2* sites, were derived from Rastelli et al. (1993). The asterisk at some *zeste*-binding sites indicates that stained bands do not colocalize precisely with the *Psc* and *Su(z)2* bands at these cytological locations (Rastelli et al. 1993). The data for *Pc* and *ph* were derived from a revised list of sites for *Pc* protein by Paro and Zink (1992) and from DeCamillis et al. (1992). Brackets at 98C/D indicate that data for *Pc* and *ph* differ between Paro and Zink (1992) and DeCamillis et al. (1992).

bands was poorly reproducible, probably depending on the quality of the chromosome preparations, we do not include mapping of these bands here. One of the *trx*-binding sites (84A4,5) is at the location of the ANT-C, which is a well established genetic target of *trx* (Ingham 1985; Breen and Harte 1993). However, we do not detect *trx* protein at the BX-C (89E), although this complex is also a genetic target of *trx*.

The protein products of *Pc* and *ph*, members of the *Pc-G*, are completely colocalized at ~80 binding sites on polytene chromosomes (DeCamillis et al. 1992). The *Psc* and *Su(z)2* proteins, products of two other members of the *Pc-G*, share a substantial number of binding sites with each other and with *Pc* and *ph* (Martin and Adler 1993; Rastelli et al. 1993). Twelve *trx*-labeled bands coincide with binding sites of *Pc-G* proteins and the *zeste* protein (Table 1). We found that seven *trx* sites coincide with sites of all four *Pc-G* proteins. Given the relatively small number of *trx* binding sites, it is unlikely that this overlap is coincidental. However, *trx* and *Pc-G*-binding sites coincide only within the limits of resolution of cytological examination, that is, their binding sites may be separated by 100–200 kb, as estimated by others (DeCamillis et al. 1992; Rastelli et al. 1993).

Mutations in *ash-1* and *pco/E(z)* affect *trx* protein binding to chromosomes

An important genetic feature of the *trx-G* genes is their

interaction with each other and with *Pc-G* genes (Capdevila and Garcia-Bellido 1981; Kennison and Tamkun 1988; Shearn 1989). To investigate this further, we tested whether binding of the *trx* protein to polytene chromosomes is altered in animals carrying mutations in *ash-1* and *fs(1)h*, two members of the *trx-G*, and in *pco/E(z)*, a *Pc-G* gene. Because complete loss of many of these genes' function is lethal at early stages, we used temperature-sensitive loss-of-function alleles. Temperature-sensitive alleles of *ash-1*, *fs(1)h* and *pco/E(z)* have been described that can survive until late stages after the removal of zygotic function (Gans et al. 1975; Shearn et al. 1978; Jones and Gelbart 1990; Phillips and Shearn 1990).

We collected embryos homozygous for the temperature-sensitive alleles *ash-1*^{Vb234}, *fs(1)h*^{1V24}, and two alleles of *pco/E(z)*, *PCO25* and *E(z)*^{S2}, at 18°C for 48 hr and then transferred them to 29°C until the third-instar larval stage. To control for the efficiency of *trx* binding to polytene chromosomes under these conditions, we immunostained on the same slides polytene chromosomes of wild-type larvae raised at 29°C. We did not detect any abnormalities in *trx* binding to chromosomes of wild-type larvae raised at high temperature (Fig. 3A). However, in three mutants tested, *trx* binding was significantly altered. The strongest effect was observed in *ash-1* mutants: *trx* binding was almost completely abolished in the majority of nuclei. Only in a small number of nuclei could we still see a few very weak bands at their usual locations (Fig. 3B). In *PCO25* mutants raised at 29°C *trx* binding was also very weak, but it was still observable at most of the usual sites in many nuclei (Fig. 3C); in *E(z)*^{S2} mutants the effect on *trx* binding was similar (data not shown). We did not detect any consistent changes in the intensity of *trx* binding to polytene chromosomes in *fs(1)h* mutant larvae (data not shown).

ash-1 and *pco/E(z)* mutations do not cause any significant decrease in *trx* RNA levels (Fig. 3G), suggesting that these gene products are not required for regulation of *trx* expression. The level of *trx* protein also remains the same, and it is still seen in all salivary gland nuclei (Fig. 3E,F). However, there is a noticeable change in the subcellular distribution of *trx* protein. In wild-type nuclei it accumulates primarily in spots (Fig. 3D). A similar punctuated pattern has been described for *Pc* protein (Messmer et al. 1992). In nuclei of *ash-1* and *pco/E(z)* mutants, *trx* staining appears to be more diffuse (Fig. 3E,F), apparently reflecting its dissociation from the chromatin. These results show that the stability of *trx* binding depends on products of some genes from both the *trx-G* and *Pc-G*. The fact that *trx* binding was not completely lost in these mutants might be explained by the incomplete inactivation of their proteins at the non-permissive temperature, as was suggested for *E(z)* by Jones and Gelbart (1990).

trx protein binds to the *fkh* regulatory region

A list of potential targets of *trx* protein based on polytene chromosome staining is speculative, as each polytene band may contain multiple genes. We have conducted a

Figure 4. *trx* protein chromosomal binding to *fkh* regulatory sequences is localized to an 8.4-kb DNA fragment. The *fkh* and *distal to fork head* (*dfk*) transcription units are shown at the top; (□) the *fkh* open reading frame. (Left) The fusion genes that were used in this work; (right) the expression patterns of these fusion genes (adapted from Weigel et al. 1990). Stippled bars indicate that *trx* protein binds to polytene chromosomes at the location of the insert in the corresponding line. Open bars indicate absence of *trx* binding to the inserts. (fg) Foregut; (amg) anterior midgut; (hg) hindgut; (Mt) Malpighian tubule; (pmg) posterior midgut; (sg) salivary glands; (cns) central nervous system.

detailed analysis of *trx* protein binding at 98D1. One of the genes in this region is *fkh*, a region-specific homeotic gene that has a general role in development of the embryonic gut and salivary glands (Jürgens and Weigel 1988; Weigel et al. 1989; Weigel and Jäckle 1990). We used *fkh* P-element-transformed lines to ask whether *trx* protein is localized to *fkh* DNA.

Extensive studies employing germ-line transformation have identified several *cis*-regulatory regions of *fkh* (Weigel et al. 1990; Fig. 4). A *fkh* P-element construct, *P[NS-fkh]* (Fig. 4), contains the transcribed region as well as some 5'- and 3'-flanking sequences and can partially rescue the mutant phenotype of a complete loss-of-function allele upon germ line transformation (Weigel et al. 1989). This construct contains DNA sequences that direct *fkh* protein expression in most of its normal domains, including the anterior and posterior midgut, Malpighian tubules, hindgut, and salivary glands. Another construct, *NX1.lacZ*, contains the entire 8.4 kb of 5'-nontranscribed sequences present in the *P[NS-fkh]* construct (Fig. 4). The expression of β -galactosidase in *NX1.lacZ* embryos is very similar to the pattern of endogenous *fkh* protein in *P[NS-fkh]* embryos (Weigel et al. 1990). By immunostaining of polytene chromosomes with *trx* antibody we find that *trx* binds at the insertion sites of the construct *P[NS-fkh]* in two transformed lines (DW-19, in Fig. 5, and DW-20, data not shown). We further localized *trx* binding to the 8.4-kb DNA fragment of construct *NX1.lacZ* (line DW4-25, data not shown). Our preliminary results suggest that *trx* binds to the distal portion of this DNA fragment (data not shown). In contrast, we did not detect *trx* binding at the insertion site of the *HZP-Sp.lacZ* construct (line DW5-12). This construct contains part of the 3'-nontranscribed sequences present in the *P[NS-fkh]* construct (Fig. 4) and directs strong β -galactosidase expression in the anterior and posterior midgut primordia in the extended germ band embryo.

trx is required for normal levels of *fkh* expression in embryonic and larval tissues

To address the question of the functional significance of

trx binding to *fkh* regulatory regions we compared expression of the reporter gene in the line DW4-25 (construct *NX1.lacZ*) in wild type and *trx*^{B11} homozygous mutant embryos by whole-mount in situ hybridization. No defects were detected in *trx* mutant embryos in the expression of *lacZ* in its two terminal domains at the blastoderm stage (data not shown). However, the level of *lacZ* expression is substantially reduced in its domains of expression in stage 14 and later embryos (the reduction is very similar to that of endogenous *fkh* in Fig. 6b, and therefore is not shown here). These results suggest that the 8.4-kb 5' regulatory region of *fkh* contained in the construct *NX1.lacZ* is a direct target of *trx* protein.

This 8.4-kb *fkh* genomic DNA fragment directs expression of β -galactosidase in a subset of the endogenous *fkh* pattern but does not drive a *fkh*-related pattern in the foregut and central nervous system (CNS) (Weigel et al. 1990; Fig. 4). Therefore, we wished to know whether *trx* activity is required for *fkh* expression in these embryonic tissues. Consequently, we analyzed the expression of en-

Figure 5. Immunohistochemical localization of *trx* protein to *fkh* regulatory sequences. (Top) *trx* binding site at 29EF on a wild-type chromosome; (middle) *trx* binding in the transformed line DW4-19 (construct *P[NS-fkh]*; Weigel et al. 1990), in which 15.3 kb of 5' nontranscribed *fkh* DNA is inserted, resulting in a new site of *trx* binding at 31A; (bottom) in situ hybridization of pCaSpeR- β -gal DNA to polytene chromosomes of the same transformed line. The site of insertion of the *fkh* transformant corresponds to the new *trx*-binding site at 31A.

Figure 6. *trx* is required to maintain transcription of *fkh* in embryos and larvae. (a,b) Expression of *fkh* in *trx*^{B11} homozygous embryos (b), as well as in *trx*^{E12} and *trx*^{B16} (data not shown), is severely reduced at early stage 14 in all expressing tissues compared with wild-type embryos (a); anterior is to the left. (c–j) In wild-type larvae *fkh* RNA is expressed at high levels in hematopoietic cells (c), salivary glands (e), at the midgut–hindgut boundary (arrowhead, see g and h) and in gastric ceca (i). In all of these tissues we observed strong reductions in the level of *fkh* transcript in *trx*^{E12} as well as in *trx*^{B16} (data not shown) homozygous animals (d,f,h,j). *fkh* RNA is also expressed in imaginal discs in wild-type larvae. Data for *fkh* expression in imaginal discs in *trx* mutants is unavailable, as their development is arrested in homozygous *trx*[−] larvae (B. Kuzin et al., unpubl.); similar effects were described by Shearn (1989) for *ash-1* and *ash-2*, two other genes from the *trx*-G. Expression of *rp49* [*Minute(3)99D*] RNA in gastric ceca (k) and salivary glands (l) of *trx*^{E12} mutants is not affected. Scale bar, 100 μ M. (b) Brain; (cns) central nervous system; (dhv) dorsal heart vessel; (gc) gastric ceca; (hg) hindgut; (lg) lymph glands; (mg) midgut; (Mt) Malpighian tubules; (pv) proventriculus; (sg) salivary glands.

ogenous *fkh* RNA in *trx* mutant embryos. As seen in Figure 6b, expression of *fkh* RNA in late *trx*[−] embryos is decreased in all tissues including foregut and CNS. Weigel and co-workers found that 10 kb of the 3'-nontranscribed DNA sequences direct *fkh* expression in the CNS, and they have constructed P-element-transformed lines carrying this DNA fragment (Weigel et al. 1990; Fig. 4). However, when we immunostained polytene chromosomes from larvae transformed with a corresponding construct (*HZSpBglacZ*), we did not observe the appearance of a new binding site at the location of the insert in three transformed lines tested. Similarly, we did not detect any substantial changes in the expression of the reporter gene in *trx* mutants (data not shown). This shows that although the expression of endogenous *fkh* RNA is reduced in *trx* mutant embryos in all tissues where it is expressed, *trx* effect on *fkh* expression in salivary glands and its chromosomal binding is limited to a subset of *fkh* regulatory sequences.

Similar to its effect in the embryo, lack of *trx* function causes a drastic decrease of *fkh* expression in larvae in all expressing tissues examined, including the lymph glands, the salivary glands, the hindgut–midgut boundary, and the gastric ceca (Fig. 6). Consistent with a direct regulatory interaction between *trx* and *fkh*, we have found that *trx* is expressed in all tissues where *fkh* is expressed (Fig. 2). To test the specificity of the *trx* effect on *fkh* expression we examined whether lack of *trx* function would affect expression of genes at loci where *trx* protein does not bind. Therefore, we used a probe specific to the ribosomal protein *rp49*, which has been assigned to the *Minute(3)99D* locus (Kongsuwan et al. 1985). No *trx* protein binding to 99B was detected in our experiments (Table 1). In situ hybridization showed that *rp49* RNA is expressed at the same level in the larval salivary glands and gastric ceca of both wild type (data not shown) and *trx*^{E12} animals (Fig. 6k,l). These results confirm that *trx* functions as a specific, positive regula-

tor required to maintain normal levels of *fkh* expression late in embryogenesis and in larvae.

Discussion

trx proteins undergo specific proteolytic cleavage

The *trx* locus produces five unusually large embryonic and larval transcripts of 11–14 kb (Mozer and Dawid 1989; Breen and Harte 1991; Sedkov et al. 1994), which encode two predicted protein isoforms of ~400 kD (Mazo et al. 1990). Accordingly, we have detected both protein isoforms *trxI* and *trxII* with antibodies raised against four *trx* protein domains (Fig. 1) and have shown that they are expressed at different stages of embryo development. However, in the embryo, both full-size *trx* protein isoforms are highly unstable. They appear to be cleaved within the region of amino acids 2131–2411, as this would be consistent with observed cleavage products, and since deletion of this region in the *trx*^{E3} mutation leads to a significant stabilization of both protein isoforms (Fig. 1B). As a result of this specific proteolytic cleavage three stable isoforms of 275, 240 and 200 kD appear (Fig. 1). The first two proteins represent the amino-terminal halves of the full-size proteins; the 275 kD isoform contains an additional 362 amino acids at the amino terminus (Mazo et al. 1990; Sedkov et al. 1994), while the 200 kD protein represents the carboxy-terminal portion of both *trx* protein isoforms I and II (Fig. 1). The predicted molecular weights for these three isoforms are ~240, 200, and 160 kD, that is, 30–40 kD smaller than those estimated from their mobility on SDS gels. This may indicate that *trx* proteins undergo additional post-translational modifications. We are currently investigating this possibility, as well as the question of the functional significance of the apparent *trx* protein processing.

trx encodes a chromosomal protein

trx protein binds specifically to 16 sites on the salivary gland polytene chromosomes, that is, to a much smaller number of sites than any of the *Pc-G* proteins examined so far. This may be attributable to the fact that its mode of action differs from that of *Pc-G* genes. *trx* is a transcriptional activator that is required for normal levels of expression of the *BX-C*, the *ANT-C* (Mazo et al. 1990; Breen and Harte 1993), and *fkh* (this work) in many tissues. Because we do not detect binding at the *BX-C*, it is likely that we are detecting a minimum number of *trx*-binding sites and that these sites occur at the cytological location of only those genes that are regulated by *trx* in the salivary gland cells. This assumption is also supported by the fact that *trx* chromosomal binding in salivary glands is limited to the *fkh* regulatory regions that are active in these cells. We observed *trx* protein binding at the cytological localization of *ANT-C*. Expression of the *Antp* and *Scr* RNAs (and proteins) and the Deformed protein have been shown to be reduced in *trx* mutant embryos (Breen and Harte 1993; Sedkov et al. 1994). The

only homeotic gene of the *ANT-C* and *BX-C* essential for the development of embryonic salivary glands is *Scr*, and it is the only homeotic gene expressed in embryos in parasegment 2, which gives rise to the salivary gland placodes (Panzer et al. 1992). However, neither of the *ANT-C* genes is expressed at high levels in embryonic salivary glands. We have no information concerning expression of the *ANT-C* genes in third-instar salivary gland cells. Therefore, we do not know at present which, if any, of the genes of this complex are activated by *trx* in larval salivary glands.

The majority of *trx*-labeled bands coincide with binding sites of *Pc-G* proteins, indicating that the *Pc-G* and *trx-G* may have other common targets besides the homeotics. Because of the limit of resolution of cytological localization, this may not be the case for all shared binding sites. For example, a *Pc*-binding site was found at 98C/D (Paro and Zink 1992) close to the cytological location of *fkh* (98D1), although it has been shown that *fkh* expression is not affected in *Pc* mutants (Jürgens and Weigel 1988). Therefore, it is quite possible that *Pc* binding is not associated with the *fkh* gene and that *Pc* and *trx* regulate different genes at this cytological location. However, it is possible that *trx* has several binding sites at this location and that some of them coincide with *Pc*-binding sites. Several *trx*-binding sites coincide with the chromosomal locations of the *Pc-G* genes, suggesting that members of these groups may control each other, forming a regulatory network. We detect *trx* protein at the cytological locations of *ph* (2D2-3), *Pcs* and *Su(2)* (49EF), and *Sex combs on midleg* (85EF) (Lindsley and Zimm 1992). Genetic data on the interaction of *trx* with *Pc*-group genes is limited to *Pc* and *extra sex combs* (Ingham 1983; Kennison and Tamkun 1988). Locations of other *trx*-binding sites overlap with those of *cut* (7B1-2), *decapentaplegic* (22F1,2) (Lindsley and Zimm 1992) and *HNF-4(D)* (29E) (Zhong et al. 1993). No data has been reported on the interaction of *trx* with any of these genes.

The question remains whether *trx* protein binds directly to DNA or whether it is a non-DNA binding chromosomal protein. The *trx* protein contains several Cys-rich, zinc finger-like regions that bind zinc in vitro (Mazo et al. 1990). Only one of these protein domains (amino acids 762–865) has similarity with a known DNA-binding motif. It consists of two zinc finger-like structures resembling a hormone receptor DNA-binding domain. Some of the *Pc-G* products may bind DNA directly, whereas others may not. *Pc* protein has no recognizable DNA-binding domains (Paro and Hogness 1991) and does not bind DNA in vitro (cited by Zink et al. 1991), whereas *ph*, *Su(z)2* and *Psc* contain potential zinc finger-like motifs and do bind DNA in vitro, although not in a sequence-specific manner (cited in DeCamillis et al. 1992; Rastelli et al. 1993). Therefore, one can speculate that of the proteins that form a multimeric complex, only some interact directly with DNA while others interact only with other members of the complex or with other chromosomal proteins. The site specificity of such a complex might be provided by *Pc-G* genes that have

not yet been analyzed. Alternatively, site specificity might be a property of the complex as a whole and might not be DNA specific in nature. Recently, Orlando and Paro (1993) showed that in *Drosophila* tissue culture cells Pc protein quantitatively covers large regulatory regions of repressed BX-C genes. They argue that this observation, taken together with the large number of Pc-binding sites on polytene chromosomes, makes it difficult to imagine that Pc-G complexes are targeted over such large regions by specific DNA sequences.

trx binding to chromosomes depends on ash-1 and pco/E(z)

We do not yet know whether *trx* acts alone or as part of a multimeric complex. If such a complex does exist, other likely components would be the products of other *trx*-G genes. Mutations in other members of the *trx*-G were identified by their interaction with *trx* and by their suppression of the phenotype of *Pc* mutations. For some of them, including *ash-1*, *ash-2*, and *fs(1)h*, the genetic interaction with *trx* was characterized quite extensively (Digan et al. 1986; Shearn 1989). Shearn (1989) suggests that the proteins in question function stoichiometrically as subunits of a multimeric complex, rather than catalytically in a linear pathway. In agreement with this, we have found that although *trx* is expressed at a high level and is still localized to nuclei in larvae carrying mutations in *ash1*, it appears to have dissociated from the chromosomes. This implies that *ash1* encodes a cofactor that is required for *trx* binding to chromosomes *in vivo*. In contrast, *trx* protein binding was not visibly affected in *fs(1)h* mutants. Relatively little is known about how the *fs(1)h* product functions, but some clues may come from its structural homology to another member of the *trx*-G, *brahma* (*brm*). Both genes encode the "bromodomain," a protein domain that is also found in several other eukaryotic proteins, some of which are involved in transcriptional activation of their target genes (Haynes et al. 1992). *brm* is required during larval development for the activation of multiple homeotic genes, including *Scr*, *Antp*, *Ubx*, and *Abd-B*; genetic evidence suggests that *trx* and *brm* interact in the activation of *Ubx* (Tamkun et al. 1992). *brm* encodes a protein that is closely related to the yeast activator SWI2/SNF2, and both proteins have extended homology with ATP-dependent DNA, DNA-RNA, and RNA helicases (Laurent et al. 1990). In yeast, SWI2/SNF2 does not bind DNA directly but is required in concert with several other proteins to activate transcription of a subset of regulated genes. There is evidence that SWI2/SNF2 exists in a complex with other gene products (Cairns et al. 1994). It was speculated that the *brm* protein complex opens up the chromatin structure and enables *trx* to activate transcription of target genes (Tamkun et al. 1992; Travers 1992). If *fs(1)h* is an essential component of the *brm* protein complex, our data would indicate that this mechanism is not involved in facilitating *trx* binding to chromosomes.

trx-G genes maintain the activated state of target gene expression and might counteract the repressive effects of

Pc-G genes. At this time there is no evidence that any of the products of the groups interact directly. The fact that *trx* binding is decreased in *pco/E(z)* mutants is, therefore, the first indication that these gene products might be involved in similar biochemical processes. Interestingly, two other *Pc*-G proteins, *Psc* and *Su(z)2*, dissociate from their sites on polytene chromosomes in the same *E(z)* mutant (Rastelli et al. 1993), although binding of the *zeste* protein is not affected. Absence of the *E(z)* product causes general decondensation of chromosomes, suggesting that *E(z)* plays a major role in maintaining the integrity of chromosomes (Rastelli et al. 1993). It is also intriguing that the *trx* and *pco/E(z)* proteins contain a highly homologous domain at their carboxyl termini (Jones and Gelbart 1993), which is also conserved in a human homolog of *trx*.

fkh is a direct target of the trx protein

As shown by Weigel and co-workers, 25-kb of *fkh* DNA contain regulatory elements sufficient to direct *fkh* expression in all but one of its domains of expression in the embryo (Weigel et al. 1990). Their analysis of different classes of mutants led to the conclusion that regulatory elements required for the initiation and maintenance of *fkh* expression in the embryonic foregut are located 5' to the genomic fragment contained in construct *NS-fkh* (Fig. 4). Because transformant lines carrying upstream sequences are not available we could not investigate *trx* binding to this *fkh* upstream region. With this exception, we have analyzed *trx* protein binding to salivary gland polytene chromosomes of transformed lines carrying all 25 kb of *fkh* DNA. Interestingly, we detected *trx* binding only to DNA sequences that contain, among others, the regulatory elements directing *fkh* expression in the embryonic salivary glands. Although *trx* is required for normal levels of *fkh* expression in the CNS, we did not detect *trx*-binding sites in the corresponding regulatory element. It is possible that this result reflects the tissue specificity of *trx* binding to chromosomes. Specifically, although *fkh* is under *trx* control in both the salivary glands and CNS, in each particular tissue *trx* is bound to the regulatory element that is active in that tissue. However, the DNA sequences of constructs that do not bind *trx* in salivary glands (*HZPSp.lacZ* and *HZSpBg.lacZ*) do not contain any of the *fkh* 5' nontranscribed sequences. Therefore, at present, we cannot rule out the possibility that *trx* binds to the *fkh* promoter or to some non-tissue-specific regulatory element that is responsible for the overall level of *fkh* expression and that is located in the 5' nontranscribed region.

The expression level of endogenous *fkh* RNA in all embryonic and larval tissues is reduced in *trx* mutants. *fkh* RNA does not vanish entirely in *trx*⁻ late embryos, and in this respect the effect of *trx* mutation is analogous to its effect on other homeotic genes (Mazo et al. 1990; Breen and Harte 1993; Sedkov et al. 1994). The observation that *trx* mutation does not completely abolish *fkh* expression, and that the *trx* effect is seen only in late embryos, may account for the fact that although both *trx*

and *fkh* mutants display cuticle defects in similar anterior and posterior regions, the defects caused by *trx* in these regions are much less severe (Ingham 1983; Sato and Denell 1987; Weigel et al. 1989). *fkh* is expressed in lymph glands (Fig. 6c), the hematopoietic organs where larval blood cells originate (see Gateff 1978 and references within). Interestingly, in light of the apparent involvement of a *trx* homolog in hematopoiesis in humans (Gu et al. 1992; Tkachuk et al. 1992), *trx*⁻ larvae showed a dramatic decrease of *fkh* expression in the lymph glands (Fig. 6d). An interesting possibility is that the same gene hierarchy may be involved in similar developmental processes in *Drosophila* and mammals.

This work shows that a gene product essential for maintaining homeotic gene expression, *trx*, binds specifically to its target genes in vivo, suggesting that it is a direct transcriptional regulator of these targets. *trx* binding in vivo depends on the presence of some, but not all, *trx*-G and *Pc*-G gene products. In addition, we have found a previously unknown target of *trx*, the region-specific homeotic gene *fkh*, and have presented evidence that *trx* maintains expression of this gene in embryos and larvae by interaction with its regulatory region.

Materials and methods

Drosophila strains

The fly strains used are as follows: *trx*^{B11}, *red*, *e*/TM3, *Sb*, *P*[*ftz-lacZ*]. *trx*^{B11} is a frameshift deletion that terminates the presumptive *trx* open reading frame after 17.5% of its length (Mazo et al. 1990). *trx*^{B16}/TM3, *Sb*, *e*, *Ser* was a gift of J. Kennison. *trx*^{B16} is a P-element insertion in the *trx* intronic region (Mozer and Dawid 1989). *y*; *trx*^{E12}/TM3, *y*⁺, *Sb*, *e*, *Ser* was provided by A. Shearn. Complementation of *trx*^{E12} deficiency is described by Breen and Harte (1991). *y*; *PCO25*, *red*/TM3, *y*⁺, *Sb*, *e*, *Ser* (= *E*[*z*]³²) and *ash-1*^{Vb234}, *red*/TM3, *Sb*, *e*, *Ser* were provided by A. Shearn. *E*(*z*)^{S2}, *e* (= *E*[*z*]⁶¹) was provided by R. Jones. *fs(1)h*¹, *V*²⁴ was provided by S. Haynes. Homozygous *trx*^{B11} animals die before or upon hatching; *trx*^{E12} survive until third-instar larvae, whereas *trx*^{B16} die as second-instar larvae. Transformed *fkh* lines *P*[*w*⁺; *NS-fkh*] (DW4-19,20), *P*[*w*⁺; *NX.I.lacZ118*] (DW4-25), *P*[*ry*⁺; *HZPSp.lacZ*] (DW5-12), and *P*[*ry*⁺; *HZSpBg.lacZ*] (DW5-7,8,9), originally described by Weigel et al. (1990), were generously provided by H. Jäckle.

Antibody production and purification

Expression of parts of a *trx* cDNA (L2, nucleotides 3426–6058; L9, nucleotides 9990–12115) as LacZ fusion proteins was described previously (Mazo et al. 1990). Two parts of the *trx* cDNA (N1, nucleotides 866–1894; N4, nucleotides 6395–8107) were expressed in the Qiagen expression system and purified on Ni-NTA resin (Qiagen). Immunization of rabbits and affinity purification of antiserum for L2 and L9 was as described (Glover 1987); N1 and N4 antisera were purified on affinity columns with the corresponding bacterially expressed proteins.

Western blot analysis

Total embryonic protein extracts were prepared as described (Rastelli et al. 1993) by homogenizing embryos directly in the SDS sample buffer [3% SDS, 100 mM Tris (pH 6.8), 10% glycerol, 1 mM PMSF, 1 mg/ml of leupeptin]. The homogenates

were sonicated to solubilize the chromatin and boiled for 5 min, and the cell debris was removed by centrifugation at 14,000 rpm for 15 min. To prepare embryonic extracts deficient for *trx* protein, we used flies heterozygous for *trx*^{B11}, balanced with a TM3 chromosome carrying a reporter gene expressing the *lacZ* gene under the control of the *ftz* regulatory region. After aging for ~15 hr, the embryos were dechorionated, treated with heptane for 10 min, and stained with X-gal for 1 hr. All but the homozygous mutant embryos developed a blue color (Rastelli et al. 1993). Embryos that remained white were collected under the microscope and used to prepare mutant extracts. The rest of the embryos were used to prepare control extracts (Fig. 1B). To prepare nuclear extracts, nuclei were isolated from embryos as described by Franke et al. (1992), with some modifications. Sucrose (0.35 M) was added to the homogenization buffer, and the homogenate was precleared by centrifugation at 500g for 1 min. Finally, purified nuclei were extracted in 20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 2 mg/ml of leupeptin, 2 mg/ml of pepstatin, 2.5 mg/ml of aprotinin, at ~0.2 ml/gram of embryos, for 30 min at 4°C. Extract samples were separated on a 5% discontinuous SDS-polyacrylamide gel, electrophoretically transferred onto Immobilon-P membranes (Millipore), and detected after incubation with primary antibodies diluted to 1:200–1:1000 and then with secondary antibodies conjugated to peroxidase (Vector). The signals were enhanced with the silver amplification system for peroxidase (Amersham).

Antibody staining of tissues

The staining of whole-mount embryos essentially followed the procedure of MacDonald and Struhl (1986) using an ABC kit and DAB substrate (Vectastain). Larvae were dissected in PBS and stained according to Pattatucci and Kaufman (1991). Immunostaining of polytene chromosomes from salivary glands was according to Zink and Paro (1989) with minor modifications; reproducible results were obtained when the chromosome squashing and immunostaining were performed in the same day. Purified *trx* antisera N1, N4 and L2 were used at 1:50–1:100 dilution.

In situ hybridization to whole-mount embryos and larval tissues

For in situ hybridization, embryos and larval tissues were fixed and processed as described previously (Tautz and Pfeifle 1989; Kramer and Zipursky 1992), except that single-stranded RNA probes were used. A region of *fkh* cDNA (nucleotides 1879–2564; Weigel et al. 1989), a region of *trx* cDNA (nucleotides 3541–6172; Sedkov et al. 1994), and a *Eco*RI–*Hind*III fragment of genomic DNA containing the *rp49* coding region (Kongsuwan et al. 1985) were amplified by polymerase chain reaction (PCR) and subcloned into Bluescript. These clones were used to prepare digoxigenin-labeled antisense *fkh*, *trx* and *rp49* RNA probes. Staging of embryos was according to Campos-Ortega and Hartenstein (1985). Homozygous *trx*⁻ larvae develop much slower than wild-type larvae and were selected on this basis from the progeny of either *y*; *trx*^{E12}/TM3, *y*⁺, *Sb*, *e*, *Ser* or *trx*^{B16}/TM3, *Sb*, *e*, *Ser* flies. Wild-type and mutant larval tissues were processed in the same tube; their identity was verified by the brownish staining of their mouth hooks due to their being homozygous *y*⁻ (in the case of *trx*^{E12}), and by their underdeveloped imaginal discs (in the case of *trx*^{B16}). *trx*^{B16} is used to show that the observed changes in *fkh* expression in larval tissues are not attributable to removal by the *trx*^{E12} deficiency of genes other than *trx* (Breen and Harte 1991). In situ hybridization to poly-

tene chromosomes was carried out with biotinylated pCaSpeR- β -gal DNA as a probe. Embryos and larval tissues were photographed under Nomarski optics, and chromosomes were photographed under bright-field illumination using an Olympus Vanox microscope.

Northern blot analysis

Total RNA was extracted from third-instar larvae by the guanidium thiocyanate/phenol procedure and was fractionated by electrophoresis through 0.8% formaldehyde agarose at 4 V/cm for 14–18 hr and transferred by capillary blotting to a nylon membrane (Magna, MSI). RNA isolation and hybridization were performed as described in Mozer and Dawid (1989). The same clones as for in situ hybridization were used to prepare *trx* and *rp49* DNA probes.

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