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-
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 homology 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 trxB12 (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, trxl of 368 kD and Trxl 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 trxE3 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 antibodies 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 trxl (Fig. 1A,B). Both protein bands are absent in extracts prepared from embryos homozygous for trxB12 (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 trxE3 (Mazo et al. 1990); an arrow shows that specific cleavage of both protein isoforms occurs within a region deleted in trxE3. trxl and trxll 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^{+/-} 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 trxl and trxll 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.

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that in early embryos the large protein isoform is encoded only by the minor early zygotic trx RNA El [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^{111} embryos balanced with a 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,
whereas in wing, haltere, and mesothoracic leg discs it is expressed predominantly in the posterior regions. These patterns are all similar to distribution of \textit{trx} RNA [Fig. 2C–H].

\textit{trx} protein is associated with 16 loci on polytene chromosomes

To determine whether \textit{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 \textit{trx} protein domains were used [Fig. 3A]. A similar pattern was seen with unpurified antisera [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 \textit{trx}^{E12} mutant larvae [selected as described in Materials and methods].

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

\begin{figure}
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\caption{Effect of \textit{ash-1} and \textit{pco/E(z)} mutations on \textit{trx} binding to polytene chromosomes. Salivary gland chromosomes were prepared from wild-type and mutant larvae raised at 29\(^\circ\)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 \textit{fkh}. (B) Several very weak \textit{trx}-binding bands [small arrowheads] occasionally remain on chromosomes prepared from homozygous \textit{ash-1}^{b16234}. Many of the weak \textit{trx} bands seen on wild-type chromosomes are seen on chromosomes from larvae homozygous for \textit{PCO25} [C] and \textit{E(z)}^{172} [data not shown]. (D) In salivary gland nuclei of wild-type larvae \textit{trx} protein shows a punctated pattern. Although \textit{trx} protein is localized at high levels in the salivary gland nuclei of \textit{ash-1}^{Vb336} [E] and \textit{PCO25} mutants [F], staining is more diffuse. (G) Northern blot showing that levels of the 14-kb \textit{trx} RNA are similar in wild-type [\textit{wt}] larvae, \textit{ash-1}^{Vb336} [\textit{ash-1}], and \textit{PCO25} [\textit{pco}] larvae raised at 29\(^\circ\)C. [ip49] Hybridization of the same filter with an \textit{rp49} probe.}
\end{figure}
bands was poorly reproducible, probably depending on the quality of the chromosome preparations, we do not include mapping of these bands here. One of thetrx-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 ofPc andph, 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 Psc-G proteins and the zeste protein (Table 1). We found that seven trx sites coincide with sites of all four Psc-G proteins. Given the relatively small number of trx binding sites, it is unlikely that this overlap is coincidental. However, trx and Psc-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).

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

| trx site | Pc-G and zeste sites |
|----------|----------------------|
| 2D | 2D | Pc ph Psc |
| 4C1-4 | 4C | Pc ph Psc* Su z |
| 7A | 7A | Pc ph Psc Su z |
| 22F | 22F/23A | Psc* Su z |
| 29EF | 29E | Pc ph Psc* Su |
| 49EF/1,2 | 49EF | Pc ph Psc Su z* |
| 56BC | 56C | Pc ph Psc Su z* |
| 57EF | 70A/B | Pc ph Psc* Su z |
| 69EF | 70A/B | Pc ph Psc* Su z |

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 ofPc/ph and Psc orSu(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 andph 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).

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 andfs(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 ofash-1,fs(1)h, andpco/E(z)have been described that can survive until late stages after the removal ofzygotic 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, fs(1)h, and pco/E(z), 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 a few mutants tested, trx binding was significantly altered. The strongest effect was observed inash-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 oftrx binding to polytene chromosomes infs(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 oftrx 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 oftrx protein. In wild-type nuclei it accumulates primarily in spots [Fig. 3D). A similar punctated pattern has been described for Psc 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 oftrx 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 nonpermissive 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 oftrx 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; (ens) central nervous system.

A 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 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-

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\textsuperscript{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.

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.

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

To address the question of the functional significance of
trx directly regulates fkh

dogenous 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 intrx 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" 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 trxl and trxlII with antibodies raised against four trx protein domains (Fig. 1) and have shown that they are expressed at different stages of embry 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* gene 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 chromosom al 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 trxl binding sites and that these sites occur at the cytological location of only those genes that are regulated by trxl 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 homeoteics. 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 *Suz(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*, *Suz(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 fortrx 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 “bromo-domain,” 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 HZSpBglacZ] 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 mutants, 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; Sedikov 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[tz-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^B10/TM3.Sb.e.Ser was a gift of J. Kennison. trx^B10 is a P-element insertion in the intronic region [Mozer and Dawid 1989]. y;trx^22/TM3,y^-^-^-^,Sb.e.Ser was provided by A. Shearn. Complementation of trx^22^-^-^-^ deficiency is described by Breen and Harte [1991]. y;PC025, red/TM3,y^-^-^-^,Sb.e.Ser were provided by A. Shearn. E(z)^[2]y^-^-^-^, e^-^-^-^E(z)^[2]y^-^-^-^ was provided by R. Jones. fs(1)h^-^-^-^, V^-^-^-^ was provided by S. Haynes. Homozygous trx^B11^-^-^-^ animals die before or upon hatching; trx^B11^-^-^-^ survive until third-instar larvae, whereas trx^B10^-^-^-^ die as second-instar larvae. Transformed fkh lines P[w^-^-^-^:N5-fkh] [DW4-19,20], P[w^-^-^-^:N1.lacZ118] [DW4-25], P[y^-^-^-^;HZSp5.lacZ] [DW5-12], and P[y^-^-^-^;HZSp5bg.lacZ] [DW5-7,8,9] (in the case of fs(1)h^-^-^-^, V^-^-^-^) was 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 500 g 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/g of embryos, for 30 min at 4°C. Extract samples were separated on a 5% discontinuous SDS–polyacrylamide gel, electroblotted 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 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 NiNTA resin (Qiagen). Immunization of rabbits and affinity purification of antisera for L2 and L9 was 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 500 g 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/g of embryos, for 30 min at 4°C. Extract samples were separated on a 5% discontinuous SDS–polyacrylamide gel, electroblotted 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 EcoRI–HindIII 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 fhk, trx and rp49 RNA probes. Staging of embryos was according to Campos-Ortega and Hartenstein [1985]. Homozygous trx^-^-^-^ larvae developed much slower than wild-type larvae and were selected on this basis from the progeny of either y;trx^22/TM3,y^-^-^-^, Sb.e.Ser or y;trx^B11/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^B11^-^-^-^), and by their underdeveloped imaginal discs (in the case of trx^B11^-^-^-^). trx^B11^-^-^-^ is used to show that the observed changes in fkh expression in larval tissues are not attributable to removal by the trx^22^-^-^-^ 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 RNA was extracted from third-instar larvae by the guani­dinium 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 [Magnis, MSII]. 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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