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The *Drosophila trithorax* protein binds to specific chromosomal sites and is co-localized with *Polycomb* at many sites

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***trithorax* is required to stably maintain homeotic gene expression patterns established during embryogenesis by the action of the transiently expressed products of the segmentation genes. The large *trithorax* proteins contain a number of highly conserved novel motifs, some of which have been hypothesized to interact directly with specific DNA sequences in their target genes. Using antibodies directed against *trithorax* proteins, we show that they are bound to 63 specific sites on the polytene chromosomes of the larval salivary gland. *trithorax* binding is detected at the sites of its known targets, the Bithorax and Antennapedia complexes, despite the transcriptionally repressed state of these loci in the salivary gland. A temperature-sensitive *trithorax* mutation greatly reduces the number of binding sites. Simultaneous localization of *trithorax* and *Polycomb* indicates that many of their chromosomal binding sites coincide. We localized one *trithorax* binding site within a portion of the large 5′ regulatory region of the *Ubx* gene, to an interval which also contains binding sites for *Polycomb* group proteins. These results suggest that *trithorax* exerts its effects by binding directly or indirectly to specific DNA sequences in its target genes. Co-localization with *Polycomb* also suggests that interactions between these activators and repressors of the homeotic genes may be a significant feature of their mode of action.**

Key words: chromosome binding/homeotic genes/*Polycomb*/*trithorax*/*Ubx*

Introduction

The spatially restricted expression patterns of the homeotic genes in the Bithorax and Antennapedia complexes (BX-C and ANT-C) confer unique segmental identities on cells throughout the embryo. Beginning at the blastoderm stage, the concerted activities of transcriptional regulators encoded by the segmentation genes establish the restricted spatial domains and levels of expression of each of the homeotic genes. These transiently expressed segmentation gene products disappear during germ band extension, but the spatial patterns and levels of homeotic gene expression which they initiated are maintained throughout the rest of development. Indeed long-term stability of the patterns and levels of the homeotic gene expression is essential to their function in implementing stable determination of cell fates and differentiation of the normal body plan (Morata and García-Bellido, 1976; Struhl, 1982; Merrill *et al.*, 1987; Diederich *et al.*, 1989).

Additional negative and positive regulatory factors have been shown to play key roles in the maintenance of homeotic gene expression patterns throughout the rest of development. During germ band extension, the activities of the *Polycomb* group (Pc-G) proteins become required to maintain the spatially restricted patterns of homeotic gene expression (Lewis, 1978; Denell and Frederick, 1983; Jürgens, 1985; Struhl and Akam, 1985; Gould *et al.*, 1990; Simon *et al.*, 1992; McKeon *et al.*, 1994). The Pc-G proteins appear to be directly involved in repression since they are present in multiprotein complexes (Franke *et al.*, 1992) which are localized at specific chromosomal sites, including those of the homeotic genes (Zink and Paro, 1989; DeCamillis *et al.*, 1992; Martin and Adler, 1993; Rastelli *et al.*, 1993; Lonie *et al.*, 1994).

Long-term maintenance of homeotic gene expression also requires positive regulatory factors encoded by another group of genes, the *trithorax* group (trx-G) (Kennison and Tamkun, 1988; Shearn, 1989), *trithorax* (*trx*) itself being one of the best characterized trx-G members. *trx* mutant embryos and adults exhibit homeotic transformations similar to those seen in mutants of the ANT-C and BX-C genes *Scr*, *Antp*, *Ubx*, *abd-A* and *Abd-B* (Ingham and Whittle, 1980; Ingham, 1981, 1985). Genetic studies suggested that *trx* is required for expression of these ANT-C and BX-C genes. We found that *trx* embryos do indeed exhibit reduced levels of *Dfd*, *Scr*, *Antp*, *Ubx*, *abd-A* and *Abd-B*, which correlate with the specific segment identity transformations observed in their embryonic cuticles (Breen and Harte, 1993). Furthermore, each homeotic gene exhibits a complex pattern of parasegment-specific, tissue-specific and promoter-specific requirements for *trx*, indicating that maintenance of stable long-term expression of the homeotic genes is considerably more complex than previously suspected. This complexity suggests that the requirement for *trx* may not reflect a property of the homeotic genes themselves, but rather a property of specific factors and/or *cis* regulatory elements which control the expression of individual homeotic genes in specific subsets of their expression domains. We hypothesize that *trx* may be required to assist a subset of transcriptional activators which drive expression of individual homeotic genes in particular cells, perhaps by maintaining open chromatin domains which facilitate their binding to DNA.

Mutations in *trx* and other trx-G genes suppress the homeotic transformations caused by Pc-G mutations (Ingham, 1983; Kennison and Tamkun, 1988; Jones and Gelbart, 1993). The dominant homeotic derepression phenotypes of *Pc* mutants are also enhanced in flies

carrying an extra trx^+ gene (three copies) and suppressed in flies carrying only a single copy of trx^+ (Capdevila and García-Bellido, 1981; Kennison and Russell, 1987). These genetic interactions suggest that *trx* competes with or antagonizes the activities of the Pc-G proteins, and that stable determination of segment identity may depend critically upon the balance of these competing positive and negative regulatory factors acting on the homeotic genes.

The *trx* gene encodes multiple alternatively spliced mRNAs which encode two large protein isoforms (predicted: 365 and 405 kDa). The larger isoform contains an additional 368 N-terminal residues not present in the smaller isoform, all but six of which are encoded by alternatively spliced exon II (Mazo *et al.*, 1990; Breen and Harte, 1991; Sedkov *et al.*, 1994; Stassen *et al.*, 1995). The sequences of the *trx* proteins provide few clues to their mechanism of action. They contain several novel sequence motifs highly conserved in the human and mouse *ALL-1* proteins, which are implicated in the etiology of certain leukemias (Gu *et al.*, 1992; Tkachuk *et al.*, 1992; Ma *et al.*, 1993). One of these regions, extending over 700 residues, is rich in cysteines, some of which comprise novel zinc finger-like motifs (Mazo *et al.*, 1990; Stassen *et al.*, 1995). The other highly conserved region, comprising the 150 C-terminal residues, is also conserved in *E(z)*, a Pc-G protein (Jones and Gelbart, 1993) and *Su(var)3-9*, a modulator of position effect variegation (PEV) (Tschiersch *et al.*, 1994). This raises the possibility that the mechanism of action of *trx* may share some common features with these other chromosomal proteins.

To begin to investigate the mechanism of action of *trx*, we have used antibodies raised against *trx* to demonstrate that it is localized to specific sites on the polytene chromosomes, including those of its known targets in the BX-C and ANT-C.

Results

Immunodetection of trx proteins

Polyclonal antibodies were raised against a *trpE−trx* fusion protein containing a region common to both the predicted smaller and larger *trx* protein isoforms (Breen and Harte, 1991). To avoid possible cross-reactivity with other proteins, the region of *trx* selected for immunogen does not include any part of its highly conserved regions and has no significant homology to any other proteins in current databases. The immune serum was depleted of anti-*trpE* antibodies by pre-incubating it with a total protein extract from bacteria expressing only the *trpE* portion of the fusion protein at high levels. Figure 1A shows that this antiserum detects a protein of the expected molecular weight in extracts of bacteria expressing the *trx* fusion protein. Figure lB shows that this antiserum also recognizes two very high molecular weight proteins present in nuclear extracts of *Drosophila* embryos. They migrate more slowly than the highest molecular weight marker used (thyroglobulin large subunit, 330 kDa) and so are in the size range predicted for the *trx* proteins. Figure 1C shows that isoform-specific antibodies raised independently against the N-terminal 172 residues present only in the larger isoform also detect a protein of similar size. This antiserum also detects a smaller species with an apparent mol. wt of ~300 kDa, smaller than either of

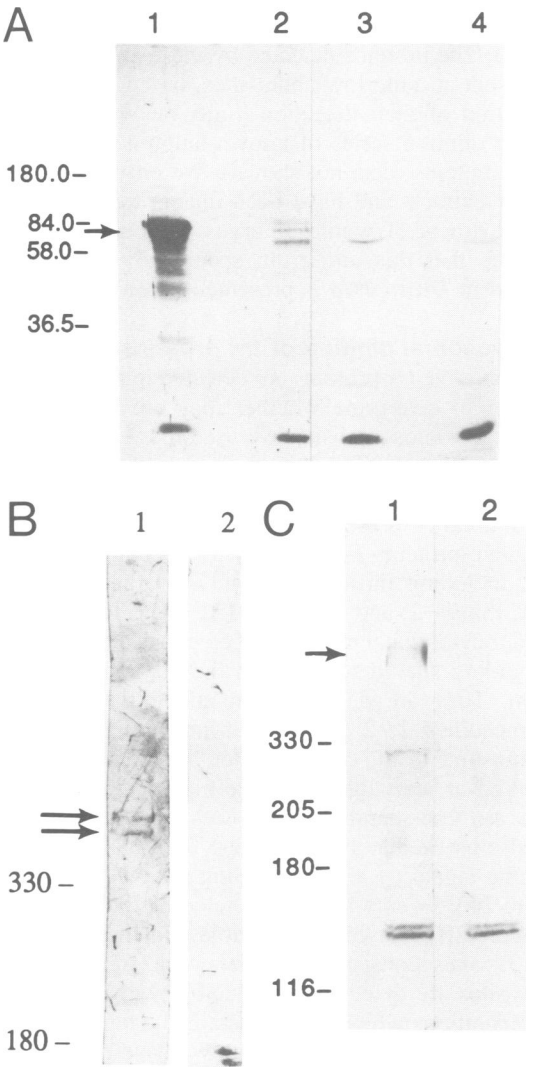

Fig. 1. Characterization of anti-*trx* antibodies by Western analysis. Polyclonal rabbit antibodies were raised against a *trpE−trx* fusion protein containing *trx* residues 2355–2739, which are common to the predicted *trx* isoforms. (**A**) Detection of *trx* fusion proteins in bacterial protein extracts. Lanes 1 and 3: extracts containing *trpE−trx* fusion protein (68 kDa, predicted); lanes 2 and 4: extracts containing the partial *trpE* protein (36 kDa) expressed from pATH vector alone. Lanes 1 and 2 were incubated with immune serum, lanes 3 and 4 with pre-immune serum. Proteins of the expected molecular weights are detected only on the blots incubated with immune serum. The absence of a *trpE* band in lane 2 (shown to be present in high abundance on Coomassie staining) indicates that the fusion protein band in lane 1 is recognized exclusively by antibodies directed against the *trx* part of the fusion protein. Additional bands in lanes incubated with pre-immune serum (lanes 3 and 4) are due to the presence of some non-specific anti-bacterial antibodies present in that serum. (**B**) Detection of *trx* proteins in *Drosophila* embryo nuclear extracts with the same antiserum. Lane 1 was incubated with immune serum, lane 2 with pre-immune serum. Two large proteins are detectable in the size range of the two predicted *trx* proteins (365 and 405 kDa), i.e. above the 330 kDa thyroglobulin marker. (**C**) Detection of *trx* proteins in *Drosophila* embryo nuclear extracts using isoform-specific antiserum raised against N-terminal 172 *trx* residues [a separate gel run from (B)]. Lane 1 was incubated with immune serum, lane 2 with pre-immune serum. This antiserum also detects a protein of similar size, above the 330 kDa marker. The doublet present in both pre-immune and immune lanes below the 180 kDa marker is due to antibodies present in the pre-immune serum that cross-react with other fly proteins. It also serves to indicate that approximately equal amounts of total protein are present in each lane.

the predicted *trx* isoforms. This may be a breakdown product. The proteins detected by these antisera appear to be present at quite low abundance, based on independent calibration of their detection limits by Western analysis, using a dilution series of known amounts of purified *trx* fusion proteins (data not shown). We only detect them in nuclear extracts and have been unable to detect them in crude extracts of whole embryos or adult flies. Further evidence that this antiserum specifically recognizes *trx* proteins in *Drosophila* is presented below.

Chromosomal binding of trx proteins

Since putative *trx* proteins are detected in nuclear extracts, in order to determine whether they are associated with specific chromosomal sites we used these antisera to stain polytene chromosome spreads prepared from late third instar larval salivary glands. Figure 2 and Table I show that antibodies present in the common region antiserum recognize proteins associated with specific chromosomal sites, including those of known *trx* target genes in the BX-C (89El−2) and ANT-C (84Al−B2). Table I summarizes our cytological mapping of 63 consistently observable *trx* binding sites detected with the common region antiserum. They are detectable in euchromatic regions on chromosomes 1, 2 and 3, but not on the tiny fourth chromosome or the chromocenter. The staining intensities observed at individual sites are highly reproducible, but there are considerable differences in the intensities of signals observed at different sites. Ten sites always exhibit a strong signal (+++), including the ANT-C (shown in Figure 2C). Twenty-one sites, including the BX-C (shown in Figure 2B), show a somewhat less intense signal (++), and 31 sites consistently show weak signals (+). The weak sites are detected reproducibly with this antiserum in preparations which are well stained overall. The region of the *en* locus (48A3–4), which we have recently shown to be regulated by *trx* (Breen and Harte, 1995), consistently exhibits a weak to moderate signal. The variation in signal intensities among sites may reflect the number of *trx* binding sites at a given locus and/or variations in the *trx* binding affinity for specific sites. We do not observe many additional weak sites which are not seen consistently, but this does not rule out the possibility that there are additional undetected sites.

To confirm that these sites represent specific binding to *trx* proteins, we analyzed chromosome binding sites in nuclei from larvae homozygous and hemizygous for trx^{1}, a temperature-sensitive allele (Ingham and Whittle, 1980). The trx^{1} DNA lesion, a 9 kb insertion, possibly of a novel mobile element, lies upstream of the coding sequence (Breen and Harte, 1991) and so is likely affecting expression of *trx* rather that the protein itself. Figure 3 illustrates that the polytene chromosomes from a homozygous trx^{1} larva raised at the restrictive temperature show a greatly reduced number of detectable *trx* binding sites with the common region antiserum; some nuclei show no detectable binding sites. Even fewer residual binding sites are observed on average in $trx^{1}/Df(3R)red^{P52}$ hemizygotes raised at 29°C. In contrast, the full inventory of binding sites is seen on chromosomes from wild-type larvae cultured continuously at 29°C, or from trx^{1} homozygotes raised continuously at 22°C, a permissive temperature, or from animals with only one copy of trx^{+} $[+/Df(3R)red^{P52}]$

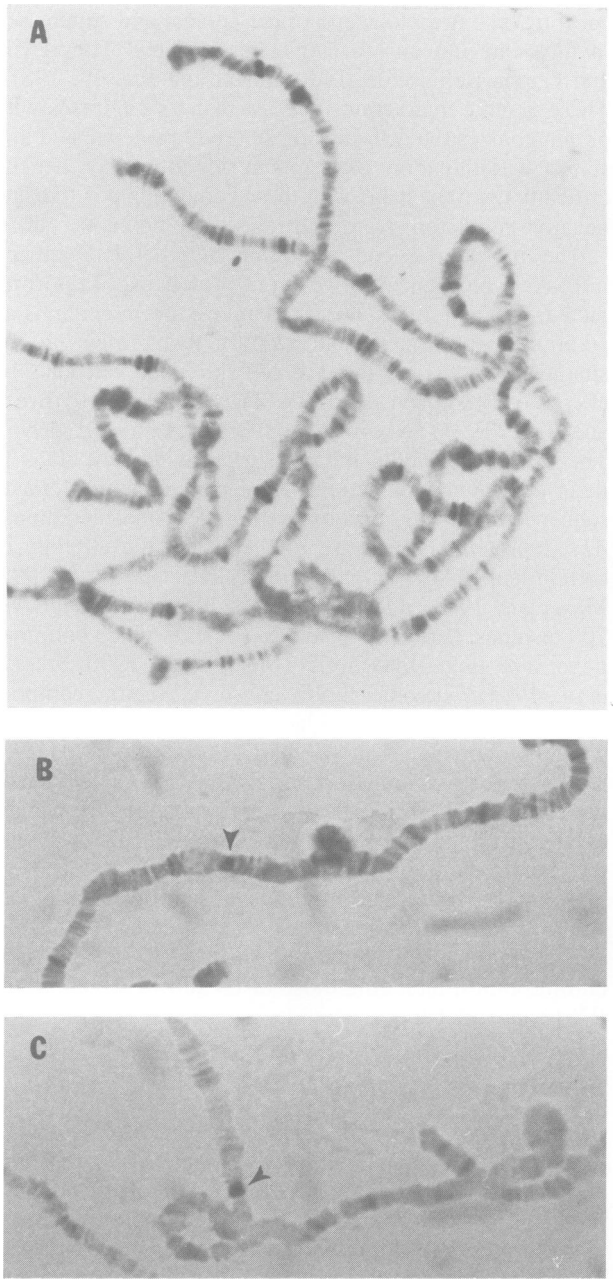

Fig. 2. (**A**) Immunolocalization of the *trx* protein on salivary gland polytene chromosomes of wild-type third instar larvae. (**B**) Binding to the BX-C region at 89E1–2. (**C**) Binding to the ANT-C region at 84A1–2 and 84B1–2.

(data not shown). Figure 4 graphically summarizes our quantitative analysis of these data, gathered from the examination of a large random sample of nuclei from identically treated trx^{1} and wild-type larvae. The mean number of residual binding sites detectable in trx^{1} nuclei is five compared with 50 sites in wild-type nuclei in this sample. The latter number (50) does not reflect a departure from the 63 sites listed in Table I. It is simply the average number of scorable sites in any single nucleus, due to inability to score sites obscured by other chromosome arms and, to a lesser extent, inability to detect some of the weakest sites due to normal variability in their signal intensities. No correction for these factors was made since they are expected to affect the mutant sample

Table I. *trx* chromosome binding sites

trx	Signal		Pc-G	Sites for	*Pc*	*ph*	*Psc*	*Su(z)2*	*z*
Chromosome 1									
2F	++	*							
4C	++	*	‡	4C	*Pc*	*ph*	*Psc*	*Su*	*z*
6D	++	*							
7A	+++	*							
7B	+++	*	‡	7B	*Pc*	*ph*	*Psc*	*Su*	*z*
8C	+								
11C	++								
11F	++								
16D	++		‡	16D	*Pc*	*ph*	*Psc*	*Su*	*z*
17D/E	++		?	17E	*Pc*	*ph*			
Chromosome 2									
22A	+++	*	‡‡	22A	*Pc*	*ph*	*Psc*	*Su*	
22B	+			22B	*Pc*	*ph*	*Psc*	*Su*	*z*
25A	+	*							
26A	++	*	‡	26A			*Psc*	*Su*	*z*
26E	+								
29F	+++	*	?	29E	*Pc*	*ph*	*Psc*	*Su*	
30C	+		‡	30C	*Pc*	*ph*			
33B	++		‡	33B	*Pc*	*ph*			
34B	++								
35B	++		?	35A/B	*Pc*	*ph*	*Psc*	*Su*	
36E	+								
37B	+		‡	37B	*Pc*	*ph*			
38B	+								
39A/B	+								
43E	+								
45B/C	++		?	45C	*Pc*	*ph*	*Psc*		z
46C	+		‡	46C	*Pc*	*ph*	*Psc*		z
46D	+								
48A	+		‡	48A	*Pc*	*ph*	*Psc*		z
49F	+++	*	?	49E/F	*Pc*	*ph*	*Psc*	*Su*	*z*
50D	+								
52C	+								
53C/D	++		?	53C					z
56C	+++	*	‡	56C	*Pc*	*ph*	*Psc*	*Su*	*z*
56E/F	+		‡	56E/F					z
58E	+								

proportionately and therefore have little effect on the magnitude of the difference observed between the two samples. In the sample of trx^1 nuclei, the probability of a site remaining is directly proportional to its signal intensity on wild-type chromosomes; the residual sites most frequently observed were those normally exhibiting the strongest signals in wild-type nuclei. This suggests that the trx^1 mutation reduces the concentration of active *trx* protein and that stronger signals may reflect a higher affinity of these sites for *trx*. The BX-C and ANT-C binding sites, which show moderate and fairly strong signals, respectively, are undetectable in >85% of the trx^1 nuclei in which these sites were scorable.

A similar pattern of sites is also seen with antibodies raised independently against the 172 N-terminal residues of the larger *trx* isoform. However, detection of the weaker sites is much more variable with this antiserum. Similar preparations incubated with the pre-immune serum show no signal (data not shown). Table I indicates some of the sites that are consistently observable with this antibody, although exhaustive analysis of all detectable sites was not done. In particular, all the strong sites seen with the other antibody are also detected with the isoform-specific antibody. These strong sites include the ones most likely to persist in those trx^1 control nuclei which do show residual sites with the other antiserum. The detection of these sites by two different antisera raised against different parts of the *trx* protein indicates that these sites, as well as those which are consistently abolished in trx^1, are due to specific recognition of *trx* proteins by antibodies present in both antisera.

trx is co-localized with Pc at many chromosomal sites

Even at the relatively low level of resolution of this type of cytological analysis, it is evident that up to 32 of our *trx* binding site assignments appear to coincide with previously reported binding sites for one or more of the Pc-G and *zeste (z)* proteins (Zink and Paro, 1989; DeCamillis *et al.*, 1992; Martin and Adler, 1993; Rastelli *et al.*, 1993). To investigate this further, we used dual fluorescence confocal microscopy to simultaneously localize the *trx* and *Pc* proteins on polytene chromosomes. Figure 5 shows that, at this level of resolution, the *trx* and *Pc* signals coincide precisely at many sites, producing a novel yellow signal resulting from superimposition of the red and green signals from the different fluorochromes used to visualize the *trx* and *Pc* proteins, respectively. We did not map individual sites, but there are at least 30 sites at which binding of both proteins coincides. This may be

Table I. *Continued*

trx	Signal[a]		Pc-G[b]	Sites for	*Pc*	*ph*	*Psc*	*Su(z)2*	*z*
Chromosome 3									
61C	+	*	‡	61C	*Pc*	*ph*		*Su*	
61F	+	*	‡	61F	*Pc*	*ph*	*Psc*	*Su*	
63E	+								
64D	++								
65E	+		‡	65E				*Su*	
66B	+								
66F	+		?	66E/F	*Pc*	*ph*	*Psc*		
69C/D	++		?	69C	*Pc*	*ph*	*Psc*	*Su*	*z*
70A	++		?	70A/B	*Pc*	*ph*	*Psc*		z
70D	++		?	70D/E	*Pc*	*ph*	*Psc*	*Su*	*z*
84A	+++	*	‡	84A/B	*Pc*	*ph*	*Psc*	*Su*	*z*
84B	+++	*	‡	84A/B	*Pc*	*ph*	*Psc*	*Su*	*z*
84D	+++	*	‡	84D	*Pc*	*ph*	*Psc*	*Su*	*z*
85D	++	*	‡	85D				*Su*	*z*
86C	+		‡	86C	*Pc*	*ph*	*Psc*	*Su*	
88C	++								
88D	++								
89B	+	*	‡	89B	*Pc*	*ph*	*Psc*		
89E	++	*	‡	89E	*Pc*	*ph*	*Psc*	*Su*	*z*
91E	+								
92B	+								
93D	++								
94F	+								
98D	+++	*	?	98C/D	*Pc*	*ph*			
99B	+++	*	?	99A/D	*Pc*	*ph*	*Psc*	*Su*	*z*
100A	+	*	‡	100A	*Pc*	*ph*	*Psc*		
100F	+	*	‡	100F	*Pc*	*ph*	*Psc*		

Binding sites are reported to the lettered division, although in many cases they have been mapped to higher resolution. Sites are nominally classified as strong (+++), moderate (++) or weak (+) based on their consistent appearance in at least 50 nuclei. Some sites also seen consistently with the isoform-specific antibodies are indicated by an asterisk, although that analysis was not exhaustive. Sites of likely *trx* co-localization with previously reported Pc-G binding sites are indicated in the 'Pc-G' column. A ‡ indicates that the reported sites are identical; a question mark indicates that the sites reported overlap, but differ in the resolution with which they were determined. This is followed by a list of the corresponding Pc-G sites [taken from a recent revised compilation of Pc-G sites (Rastelli *et al.*, 1993)] and the specific proteins which have been shown to bind at that site.

an underestimate, since the fixation time was optimal for *Pc*, but not *trx*. Nevertheless, this number corresponds closely to the number predicted from independent mapping of *Pc* and *trx* binding sites (Table I).

trx binds within the 5′ regulatory region of the Ubx gene

To map *trx* binding sites at higher resolution, we focused on the well-characterized *Ubx* gene, which we had previously shown requires *trx* for stable long-term maintenance of its expression in many cells throughout its expression domain (Breen and Harte, 1993). Irvine *et al.* (1991) previously showed that a *Ubx–lacZ* reporter containing the 35.4 kb flanking the *Ubx* transcription start exhibited long-term maintenance, while a similar construct containing only the 22.2 kb flanking the start site did not, suggesting that a *trx* response element resides within the distal 13 kb of the 35 kb construct. Simon *et al.* (1993) identified only one region of *Ubx* that conferred long-term maintenance of *Ubx–lacZ* expression within appropriate parasegmental boundaries: a 14.5 kb fragment containing the region between −13.5 and −28 kb upstream of the *Ubx* promoter. Figure 6 shows that a previously described transformant containing this 14.5 kb segment (Simon *et al.*, 1990) creates a new *trx* binding site at the insertion site of this construct (Simon *et al.*, 1993). Transformants with similar constructs containing other regions of the *Ubx* 5′ regulatory region do not create new *trx* binding sites (V.Chinwalla and P.J.Harte, unpublished). This indicates that *trx* binds directly or indirectly to sequences within this region of the *Ubx* gene. Several Pc-G proteins, including *ph* (DeCamillis *et al.*, 1992), *Psc* (Chan *et al.*, 1994) and *Pc* (A.Chiang and W.Bender, personal communication; V.Chinwalla and P.J.Harte, unpublished), have been shown to bind within this same interval on polytene chromosomes, further suggesting that co-localization of *trx* and *Pc* binding sites may reflect close proximity on a molecular scale.

Discussion

trx chromosome binding

The binding of *trx* to 63 specific sites on the polytene chromosomes strongly suggests that it is either a sequence-specific DNA-binding protein or that it is physically associated with such a protein(s). Its binding to a known biological target in the BX-C, and the regions of the ANT-C and *en* loci also strongly suggests that the regulatory effects of *trx* are mediated by direct interactions with its target genes. Kuzin *et al.* (1994) recently reported that other anti-*trx* antibodies recognize 16 sites on the polytene chromosomes, but they failed to detect binding at the BX-C at 89E. Their sites correspond to the stronger sites reported here, with some minor differences in site assignments. While the presence of a constriction at 89E does make the appearance of the binding site at the BX-C somewhat dependent on how well spread the chromosomes are, we suspect that their failure to detect

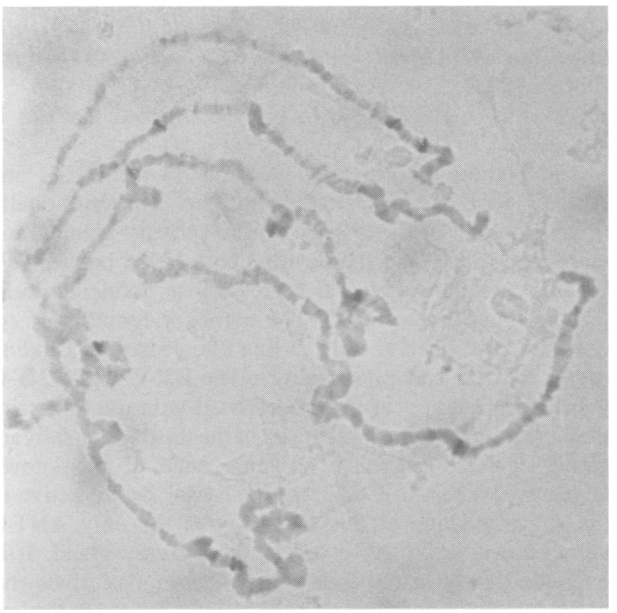

Fig. 3. *trx*^{1} has a greatly reduced number of binding sites. A representative homozygous *trx*^{1} nucleus, from an animal raised at 29°C, showing the residual pattern of binding sites. Only a few sites, corresponding to the strongest signals in wild-type chromosomes, remain detectable and residual sites tend to have weaker signals. Some *trx*^{1} nuclei show no detectable binding sites.

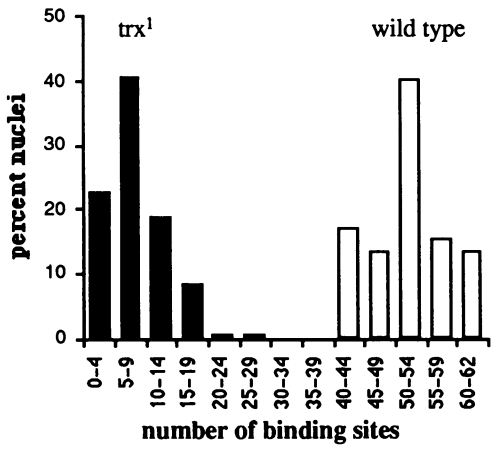

Fig. 4. Distributions of the number of chromosome binding sites per nucleus in large matched samples of *trx*^{1} mutant (black) and wild-type (white) nuclei. The distributions are non-overlapping. *trx*^{1} nuclei have a greatly reduced number of sites per nucleus (mean = 5; mode = 5; median = 5.5) compared with wild-type (mean = 50.1; mode = 50; median = 50.6) and residual sites tend to have weaker signals. The pattern of binding site loss in *trx*^{1} nuclei is not random. Weak sites are always lost and some nuclei contain no detectable sites. When residual sites are evident, those with strongest signals in wild type have the highest probability of being retained: 49F, 99B, 98D, 22A, 29F, 7B. Of the *trx*^{1} nuclei in which ANT-C (84A/B) and BX-C (89E) binding sites were scorable, 86% had no detectable signal at the ANT-C and 87% had none at the BX-C.

the BX-C binding may reflect either differences in the preparation of chromosomes or lower sensitivity of their antibodies on this material.

While there may be additional *trx* binding sites that we have not detected, their relatively small number indicates that *trx* is not a global transcriptional activator or co-activator, but has a more specialized function. This conclusion is also consistent with the limited phenotypic spectrum

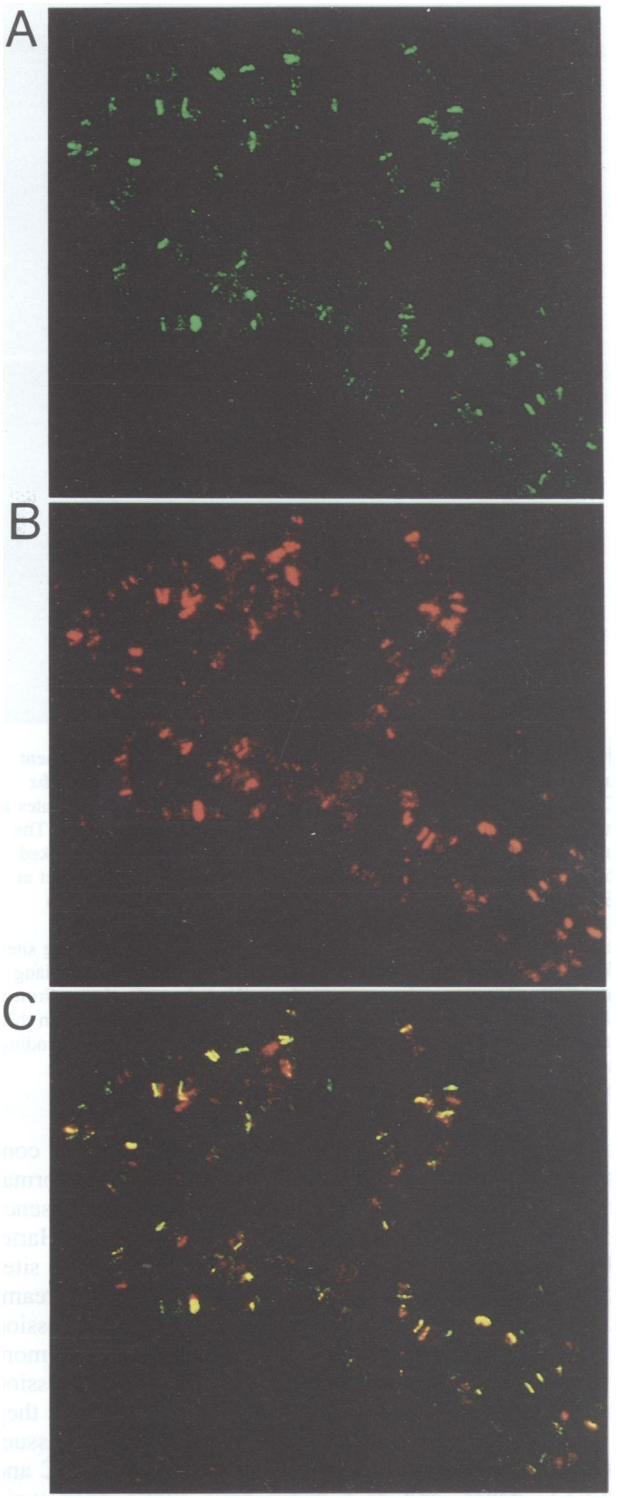

Fig. 5. Co-localization of *trx* and *Pc* chromosome binding sites by confocal microscopy. (**A**) Wild-type polytene chromosomes stained with anti-*Pc* antibodies and visualized using FITC-conjugated anti-rabbit secondary antibodies (green fluorescence). (**B**) The same polytene chromosome preparation used in (A) stained with anti-*trx* antibodies and visualized using rhodamine-conjugated anti-rabbit secondary antibodies (red fluorescence). (**C**) Merged red (*trx*) and green (*Pc*) fluorescence images in (A) and (B) (to yield yellow). The yellow bands identify sites at which *Pc* and *trx* proteins are co-localized. Green and red bands identify sites at which only one of the proteins is bound. The fixation time was 15 s, optimal for *Pc*, instead of 20 s, the optimum for *trx*. Under these conditions not all *trx* sites may be detectable.

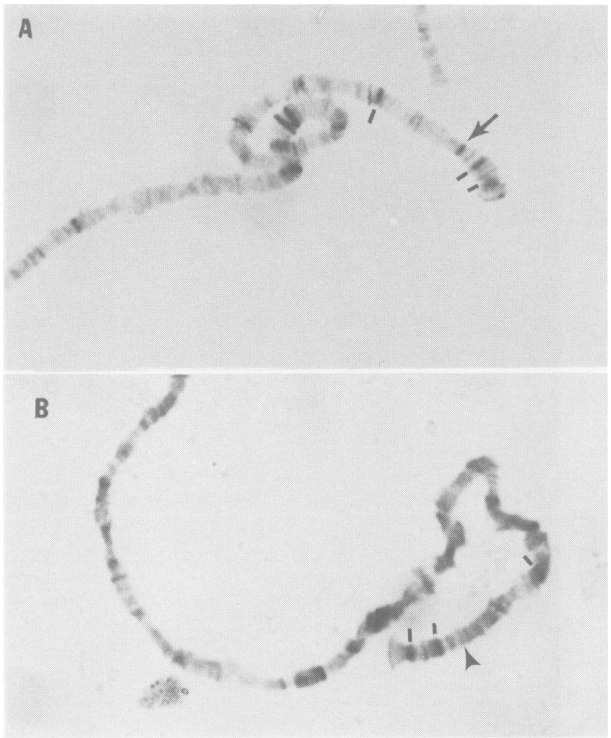

Fig. 6. *trx* binds within the *Ubx* 5′ regulatory region. (**A**) P element transformant line 85–39, which contains a 14.5 kb segment of *Ubx* DNA (from −13 to −27 kb 5′ of the transcription start site) creates a new *trx* chromosome binding site on the tip of chromosome 3L. The new site, which is not present in wild-type chromosomes, is marked by an arrow and corresponds to the insertion site of this construct at 62A, previously determined by chromosome *in situ* hybridization (Simon *et al.*, 1993; A.Chiang and W.Bender, personal communication). This same interval of *Ubx* also contains binding sites for the Pc-G proteins *ph* (DeCamillis *et al.*, 1992) and *Pc* (A.Chiang and W.Bender, personal communication). We independently confirmed the presence of a new binding site for *Pc* at this same 62A site in this transformant (V.Chinwalla and P.J.Harte, unpublished). (**B**) *trx* binding sites on the tip of a wild-type 3L chromosome. The absence of a binding site at 62A is indicated by an arrowhead.

of complete loss-of-function *trx* alleles, whose most conspicuous features are restricted to homeotic transformations which can be accounted for by reduction or absence of expression of specific homeotic genes (Breen and Harte, 1993). It is possible that some of the *trx* binding sites outside the BX-C and ANT-C correspond to 'downstream' targets of the homeotic genes, whose reduced expression in *trx* mutants might be obscured as subsets of the more inclusive phenotypes associated with reduced expression of BX-C and ANT-C genes. It is also possible that they include target genes which exhibit quite restricted tissue-specific requirements for *trx*, as do *en* and the BX-C and ANT-C genes, and as a result have subtle phenotypic manifestations which have so far gone undetected in *trx* mutants. It is also possible that some of the *trx* binding sites on the polytene chromosomes do not have any functional significance. In any case, the functional significance of particular *trx* binding sites can only be evaluated when the identity of the transcription units associated with these sites is established and independent molecular and genetic evidence demonstrates that their expression is dependent on *trx*. Some of the candidate target genes, based on the close correspondence of their cytogenetic location with *trx* binding sites, include *cut* (7B3), *eve* (46C3–11), *Psc*, *Su(z)2* (49E2−F1), *dev* (70D1–2), *roe* (84D2), *sic* (85D8–12), *neu* (86C1–8), *mor* (89B1–4), *fkh* (98D2–3), *tll* (100A5−B2) and *Su(var)3-12* (100F3–5). Kuzin *et al.* (1994) have recently confirmed that *fkh* is a target.

The binding of the Pc-G proteins to the BX-C and ANT-C loci is consistent with the transcriptionally repressed state of these genes in the salivary gland (Zink and Paro, 1989), and the positive correlation between *Pc* binding and transcriptional repression in Schneider cells (Orlando and Paro, 1993). The activator function of *trx* does not lend itself readily to this interpretation. Binding of *trx* to *Ubx* and presumably other BX-C and ANT-C genes does not appear to be correlated with the transcriptional status of these target genes in the third instar salivary gland, at least qualitatively, since none of the known targets of *trx* in the BX-C and ANT-C or *en* are active in the salivary gland at this time. *Scr* is the only ANT-C gene known to be expressed in the salivary gland. It is expressed early in the embryonic placode that gives rise to the salivary gland (Panzer *et al.*, 1992), but not in the late larval salivary gland (M.Scott, personal communication). This suggests that *trx* binding is not sufficient to activate or maintain expression of its target genes, either because other essential factors are missing or because Pc-G repressors are also present at these sites. It also raises the possibility that binding of *trx* to its targets may be essentially 'constitutive', maintenance of repressed versus active transcriptional states being regulated by whether Pc-G proteins bind. It is also possible that binding to the ANT-C could reflect the prior history of *Scr* activity in the salivary gland placode.

Co-localization of trx and Pc-G proteins

At this cytological level of resolution, the *trx* and *Pc* proteins are co-localized at a minimum of 30 chromosomal sites. While cytological co-localization cannot be readily translated to a molecular scale, the number of sites at which *trx* and *Pc* binding coincides is much greater than would be expected by chance, suggesting that it may have functional significance and that it might reflect their close proximity on a molecular scale, particularly in light of the antagonistic genetic interactions between *trx* and Pc-G genes. Given the positive correlation between Pc-G binding and the transcriptional status of its target genes (Orlando and Paro, 1993), it is possible that the remaining *trx* binding sites correspond to genes which are active in the salivary gland, but which also contain Pc-G binding sites which would be occupied in tissues where these genes are repressed.

We have demonstrated that *trx* binds within a 14.5 kb segment of the large 5′ regulatory region of *Ubx*, which also contains binding sites for Pc-G proteins. The failure of constructs containing the promoter-proximal 22 kb of DNA flanking the *Ubx* transcription start to exhibit long-term maintenance suggests that functional *trx* response elements may be confined to the distal 6 kb of this interval. This 6 kb was recently shown to contain a functional *trx* response element as well as the sole autonomously functional Pc-G response element in the *Ubx* 5′ regulatory region, which has been mapped to a 1.6 kb interval (Chan *et al.*, 1994). This suggests that *trx* and Pc-G binding sites and response elements may be more intimately associated.

We are investigating this possibility further by higher resolution mapping of *trx* binding sites.

Simultaneous binding of *trx* and *Pc* to the same region of *Ubx* also suggests that binding of either protein does not preclude binding of the other, at least qualitatively. This is particularly interesting in light of the evidence that *Pc* is bound extensively throughout the entire *Ubx* and *abd-A* transcription units in Schneider cells, where they are stably repressed, perhaps forming some kind of heterochromatin-like repressive domain (Orlando and Paro, 1993). Whether this reflects the situation in polytene chromosomes is unknown.

The genetic interactions between *trx* and Pc-G, i.e. the suppression of *Pc* phenotypes by *trx* mutations and reduced *trx* gene dosage as well as the enhancement of *Pc* phenotypes by increased *trx* gene dosage (Capdevila and García-Bellido, 1981; Ingham, 1983), have been interpreted to indicate that the activities of *trx* and Pc-G proteins are antagonistic at some level, the activity of *trx* perhaps modulating or countering the repressive effects of Pc-G proteins. If so, their co-localization (and simultaneous binding) suggests that this does not involve competition for chromosome binding at specific genes, at least qualitatively, but perhaps involves competition or antagonism at some other level. Further delimitation of the DNA interval to which *trx* and *Pc* co-localize in this region of *Ubx* would make this a firmer conclusion and is in progress. The previous observation that *Pc* protein is absent from a transcriptionally active *Abd-B* gene, but bound extensively throughout the adjacent transcriptionally silent *Ubx* and *abd-A* genes in Schneider cells (Orlando and Paro, 1993), suggests that the maintenance of active versus repressed states, while requiring *trx*, is effectively regulated by whether the negative Pc-G factors do or do not bind. In contrast, binding of *trx* to a transcriptionally inactive *Ubx* gene in the salivary gland suggests that *trx* binding may be constitutive; while necessary for maintaining an active transcriptional domain, it is not sufficient. It will be interesting to see whether other trx-G proteins co-localize with *trx* and exhibit similar 'constitutive' binding to silenced target genes.

The role of trx in maintenance of homeotic gene expression

Genetic studies suggest that *trx* counters the repressive effects of Pc-G proteins. The suppression of both the *Pc* phenotypes and the ectopic homeotic gene expression in *trx*, Pc-G double homozygotes (Ingham, 1983; Jones and Gelbart, 1993; T.R.Breen and P.J.Harte, unpublished) indicates that *trx* has a transcriptional stimulating activity in the absence of Pc-G activity (T.R.Breen and P.J.Harte, unpublished) and does not stimulate transcription indirectly solely by acting as an 'antirepressor' of Pc-G-mediated repression. Such 'anti-Pc-G' activity might simply be another manifestation of a primary transcriptional stimulating activity of *trx*. This would not be inconsistent with the general view that transcriptional activators function by altering the outcome of competition between transcriptional initiation factors and structural components of chromatin (Pc-G proteins?) for binding to DNA (Workman *et al.*, 1991; Workman and Kingston, 1992). *trx* might do so directly or indirectly, by facilitating the binding of other activators.

Several pieces of evidence suggest that *trx* may act at the level of chromatin structure. *trx* shares several novel highly conserved motifs with other chromosomal proteins suspected to act by modulating chromatin structure, including *E(z)*, a Pc-G protein (Jones and Gelbart, 1993) and *Su(var)3–9*, a modulator of PEV (Tschiersch *et al.*, 1994), and *Pcl*, another Pc-G gene (Lonie *et al.*, 1994; Stassen *et al.*, 1995). In addition, two other trx-G genes have been characterized which appear likely to stimulate transcription through their effects on chromatin structure. *Trithorax-like* encodes the *Drosophila* GAGA factor (Farkas *et al.*, 1994), which is required to achieve normal levels of homeotic gene expression *in vivo* (Farkas *et al.*, 1994), stimulates transcription from the *Ubx* promoter *in vitro* (Biggin and Tjian, 1988) and binds to specific sites on the polytene chromosomes (Tsukiyama *et al.*, 1994). GAGA acts as an antirepressor of histone H1-mediated inhibition of RNA polymerase II transcription *in vitro* (Croston *et al.*, 1991), and GAGA binding promotes disruption of adjacent nucleosome structure (DNase I hypersensitive sites) around the *hsp70* promoter in an *in vitro* chromatin assembly system (Tsukiyama *et al.*, 1994). Deletion of GAGA binding sites in the *hsp26* gene greatly reduces its inducibility and abolishes constitutive hypersensitive sites (Lu *et al.*, 1993). In addition to their *trx*-like phenotypes, GAGA mutations also enhance PEV-mediated repression (Farkas *et al.*, 1994). As we observed for *trx* (Breen and Harte, 1993), GAGA mutations also affect expression of *Abd-B* differentially within its expression domain, suggesting that GAGA functions in maintaining active chromatin configurations at specific *cis* regulatory elements, perhaps facilitating the binding of specific other factors essential for transcriptional activation (Farkas *et al.*, 1994).

Another trx-G gene, *brahma*, encodes a homolog of the yeast transcriptional activator SWI2/SNF2 (Peterson and Herskowitz, 1992; Tamkun *et al.*, 1992) and closely related human proteins BRG1 and hbrm (Khavari *et al.*, 1993; Muchardt and Yaniv, 1993). All four are members of a novel family of putative ATP-dependent DNA helicases found in stable multiprotein complexes (Peterson *et al.*, 1994). Purified human SWI complexes stimulate transcription from reconstituted chromatin templates *in vitro* by facilitating binding of other activators (Kwon *et al.*, 1994), as well as the TATA binding subunit of the TFIID complex, which cannot otherwise bind to nucleosomal DNA (Imbalzano *et al.*, 1994). Their DNA helicase activity is presumed to weaken histone:DNA contacts, causing local changes in the stability or positioning of nucleosomes sufficient to enhance access and binding of other activators to DNA. SWI complexes have no demonstrable DNA binding activity and *brm* is apparently not stably associated with specific chromosomal sites *in vivo* (J.Tamkun, personal communication). The ATP dependence of GAGA-mediated nucleosome disruption (Tsukiyama *et al.*, 1994) raises the possibility that the activities of these two trx-G factors might be coupled.

The activities of these reasonably well-characterized trx-G proteins suggest that *trx* may also act at the level of chromatin structure. The different tissue- and parasegment-specific requirements of individual homeotic genes for *trx* suggest that *trx* might also stimulate transcription indirectly by facilitating the binding of specific other factors which,

for example, direct *Abd-B* expression in a subset of cells within its expression domain (Breen and Harte, 1993). If so, it might provide a basis for an explanation for the proposed 'constitutive' binding of *trx* at sites which also bind Pc-G proteins. Binding of *trx* to a target gene may be of no consequence unless that gene is transcriptionally active/activated as a consequence of the binding of other activators, whose binding or activity may depend on *trx*. In those cells where the target gene is not active at the time Pc-G-mediated stable repression is normally implemented, Pc-G binding would establish a stable repression domain and override or prohibit any subsequent effects of the *trx* protein which remains bound. Such a model could imply that the source of the antagonistic gene dosage effects observed between *trx* and Pc-G may actually be the trx-dependent binding or transcriptional activating activities of other factors, high rates of transcription being ultimately responsible for the inhibition of Pc-G binding.

Materials and methods

Fly strains and mutants

The wild-type strain used in these studies was Oregon R. trx^1 is a spontaneously occurring temperature-sensitive mutation isolated and characterized by Ingham and Whittle (1980), and further characterized by Breen and Harte (1991). For experiments demonstrating reduced number of chromosome binding sites in trx^1 nuclei, homozygous trx^1 and hemizygous $trx^1/Df(3R)red^{P52}$ flies were raised continuously at a restrictive temperature of 29°C. Parents were cultured at 29°C for at least 4 days before collecting eggs destined for chromosome preparations. Identically treated wild-type flies were used as controls in this experiment. While even fewer residual binding sites are observed on average in $trx^1/Df(3R)red^{P52}$ hemizygotes raised at 29°C, trx^1 homozygotes from a homozygous stock were used to generate the large sample used for quantitative analysis in Figure 4, simply because it was more efficient to have all larvae be of the relevant genotype. trx^1 larvae raised continuously at the permissive temperature of 22°C were also examined and shown to have a wild-type pattern of binding sites (data not shown).

Antibody preparation

Two different polyclonal antibodies were used in this study. One was raised against *trx* residues 2355–2739, which are common to both *trx* isoforms. An 800 bp *Eco*RI–*Bam*HI fragment encoding these residues was subcloned into the pATH *trpE* fusion vector (Koerner *et al.*, 1991). The junction between the *trpE* and *trx* coding sequences of this construct was sequenced to verify that the fusion was in frame. Fusion protein was gel purified and used directly to immunize rabbits. To remove antibodies directed against the *trpE* portion of the fusion protein, antiserum was pre-incubated with total protein extract from induced bacteria containing the *trpE* vector with no insert. For chromosomal staining, serum from which *trpE* antibodies were removed was used directly or further purified by passing over an Affigel BlueTM (Pharmacia) column to remove bulk serum proteins (proteases, albumin). Pre-immune serum showed no background staining on polytene chromosome spreads (data not shown). Isoform-specific antibodies were raised against the N-terminal residues present only in the larger isoform. A 500 bp polymerase chain reaction (PCR) fragment encoding residues 1–172 was subcloned into the pQE-9 vector (Qiagen), which adds six histidine residues to the N-terminus of the protein. Fusion protein was purified by metal chelate chromatography on Ni^{2+}-nitrilotriacetate Sepharose (Qiagen) (Hochuli and Piesecki, 1992), further gel purified and used directly to immunize rabbits.

Western blot analysis

For Western analyses of bacterial proteins, extracts containing *trpE–trx* fusion proteins were separated by SDS–PAGE and electroblotted onto nitrocellulose. For Western analyses of fly proteins, crude nuclear extracts were prepared from *Drosophila* embryos as described previously (Kamakaka *et al.*, 1991). Approximately 200 μg of protein were loaded per lane and separated on a 6% SDS–PAGE gel cross-linked with diallyltartardiimide (DATD). Following electrophoresis, proteins were electrotransferred to nitrocellulose or nylon membranes. DATD was used in place of bis-acrylamide as a cross-linker to create gels of greater porosity which allow the large *trx* proteins to enter the gel and migrate with unrestricted mobility. The blots were processed using a 1:10 000 dilution of the primary antiserum for bacterial Westerns and a 1:200 dilution for fly Westerns. Biotinylated anti-rabbit secondary antibodies (Chemicon) were used at 1:4000 dilution and detected using the Vectastain Elite ABC-alkaline phosphatase system (Vector) as per the manufacturer's instructions.

Immunostaining of polytene chromosome spreads

Chromosome spreads were prepared from wandering third instar larvae and stained with polyclonal anti-*trx* primary antibodies and biotinylated secondaries as previously described (Zink and Paro, 1989; DeCamillis *et al.*, 1992). The optimal fixation time for detection of *trx* protein on chromosome spreads was determined to be 20 s. Primary antiserum was used at 1:50 dilution. Biotinylated anti-rabbit secondary antibodies (Chemicon) were used at 1:2000 dilution and detected using the Vectastain Elite ABC-horseradish peroxidase system (Vector). Photographs were taken with a Zeiss Axioplan photomicroscope using a 63× planapo lens and Kodak Ektachrome or TMax 100 film. For the analysis of chromosome binding sites in trx^1 mutants, the number and locations of binding sites per nucleus were scored in a sample of 295 trx^1 nuclei (from 21 animals) and 152 wild-type nuclei, prepared under identical conditions from animals reared continuously at 29°C. The same batches and dilutions of primary and secondary antisera were used for wild-type and mutant samples, which were processed in parallel on the same days.

trx binding to 5′ regulatory sequences of the Ubx gene

Binding of *trx* was localized within a 14.5 kb DNA segment of the *Ubx* 5′ regulatory region by immunostaining polytene chromosomes of a previously described transformant line 85-39, containing this region and inserted at 62A on chromosome arm 3L (Simon *et al.*, 1993), a location at which we have never seen *trx* binding in wild-type chromosomes.

Co-localization of Pc and trx proteins on polytene chromosomes

Double labeling of polytene chromosomes was carried out using affinity-purified anti-*Pc* (kindly provided by R.Paro) and anti-*trx* antibodies (both at 1:50 dilution) which were differentially detected by reacting with either rhodamine-labeled (for *trx*) or fluorescein-labeled (for *Pc*) secondaries. The optimal fixation times for *trx* and *Pc* differ and the difference, while slight, is significant. Since the *trx* binding site pattern was more reproducible in our hands, fixation was for 15 s, the optimal time for visualization of *Pc* sites. Under these conditions, not all *trx* sites may be detectable. Since both primary antibodies were raised in rabbits, chromosome immunostaining was carried out sequentially [anti-*Pc* primary and fluorescein isothiocyanate (FITC)-labeled secondary followed by anti-*trx* primary and rhodamine-labeled secondary, with standard wash cycles after each antibody binding step]. Controls for the specificity of the reaction of individual differentially labeled secondaries involved leaving out the second (anti-*trx*) primary to determine if any of the chromosomally bound first primary (anti-*Pc*) remained available for binding by the final secondary. In these controls, no signal from the final secondary was detected (data not shown), indicating that the sequential use of the two rabbit primaries and differentially labeled secondaries provided the required specificity for the simultaneous imaging of the two proteins.

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