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Functional Reconstruction of *trans* Regulation of the *Ultrabithorax* Promoter by the Products of Two Antagonistic Genes, *trithorax* and *Polycomb*

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Maintenance of the "on-off" state of *Drosophila* homeotic genes in Antennapedia and bithorax complexes requires activities of the trithorax and Polycomb groups of genes. To identify *cis*-acting sequences for functional reconstruction of regulation by both *trithorax* and *Polycomb*, we examined the expression patterns of several *Ubx-lacZ* transgenes that carry upstream fragments corresponding to a region of approximately 50 kb. A 14.5-kb fragment from the *postbithorax/bithoraxoid* region of *Ultrabithorax* exhibited proper regulation by both *trithorax* and *Polycomb* in the embryonic central nervous system. Using a *Drosophila* haploid cell line for transient expression, we found that *trithorax* or *Polycomb* can function independently through this upstream fragment to activate or repress the *Ultrabithorax* promoter, respectively. Studies of deletion mutants of *trithorax* and *Polycomb* demonstrated that *trithorax*-dependent activation requires the central zinc-binding domain, while *Polycomb*-dependent repression requires the intact chromodomain. In addition, *trithorax*-dependent activity can be abrogated by increasing the amount of *Polycomb*, suggesting a competitive interaction between the products of *trithorax* and *Polycomb*. Deletion analysis of this fragment demonstrated that a 440-bp fragment contains response elements for both *trithorax* and *Polycomb*. Furthermore, we showed that the integrity of the proximal promoter region is essential for *trithorax*-dependent activation, implicating a long-range interaction for promoter activation.

The specification of body segments of *Drosophila melanogaster* requires the activities of at least eight homeotic genes located in the Antennapedia and bithorax complexes. Mutations that cause inactivation or misexpression of these genes alter segmental identities (39, 46, 52). Homeotic genes contain the highly conserved homeodomain and act as transcriptional regulators to modulate the activities of downstream genes (26, 31, 45).

Expression of homeotic genes follows complex and dynamic patterns during embryonic development (39, 65). A considerable number of genetic and molecular studies have been carried out with *Ultrabithorax* (*Ubx*), which controls the development of parasegments 5 and 6. It has been shown that two large *cis*-acting regulatory domains present in the ~50-kb *postbithorax/bithoraxoid* (*pbx/bxd*) upstream region and in the ~60-kb *anterobithorax/bithorax* (*abx/bx*) intron region are involved in segment-specific control of *Ubx* expression (3, 4, 23, 64). Analyses of *Ubx* transgenes have revealed that although small DNA fragments located within these regions can confer *Ubx*-like expression patterns on the linked *lacZ* reporter gene (56, 66, 74), larger DNA fragments are necessary for reconstruction of a more complete pattern (36, 74), indicating the presence of a complex array of regulatory elements.

At early stages of embryonic development, the expression domain of *Ubx* is initially established by several transiently expressed segmentation genes (30, 34, 35, 83) which encode proteins with either zinc finger domains or homeodomains. These proteins have been shown to bind specific DNA sequences located within the regulatory regions of *Ubx* (57, 66, 72, 87). The maintenance of the "on-off" state of *Ubx* during subsequent development is necessary for proper specification of cell fates. Genetic analyses indicate that many genes are involved in this step (38, 41). On the basis of their mutant phenotypes, these maintenance genes are divided into activator and repressor groups and are named after their prototypic members as the trithorax group (trx-G) and the Polycomb group (Pc-G), respectively. Recent studies have revealed that *trx* and several members of the Pc-G, including *Pc*, *polyhomeotic* (*ph*), *Posterior sex combs* (*Psc*), and *Polycomblike* (*Pcl*), encode polytene chromosome-associated proteins (16, 19, 43, 48, 49, 67, 88). Furthermore, *brahma*, a member of the trx-G, is implicated in promoter activation by its close similarity to a yeast transcription activator, SNF2/SWI2 (77). Thus, many maintenance genes are apparently involved in direct regulation of the *Ubx* promoter.

Genetic studies have shown that the Pc-G is responsible for locking homeotic genes in the "off" state to delineate the anterior boundary of their expression domains (20, 46, 82). Detailed analyses of genetic interactions between various *Ubx* mutations and *Pc* mutations suggest that *Pc* exerts its effects primarily through regulatory sequences in the *pbx* and the *abx* regions (14), which are located ~30 kb upstream and ~40 kb downstream of the *Ubx* start site, respectively. Pc proteins contain a chromodomain motif which is highly conserved in proteins involved in position-effect variegation (61, 80). Protein products of *Pc* and *ph* are colocalized at approximately 100 sites on polytene chromosomes and have been shown to be

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constituents of multimeric protein complexes (25). Recently, a chromatin cross-linking study has demonstrated that Pc protein can be detected in extended regions of inactive *Ubx* and *abdominal-A* (*abd-A*) genes in cultured SL-2 cells (58). These results support the view that a transcriptionally inactive chromatin structure is organized by Pc-G protein complexes for the maintenance of the "off" state (59, 60).

How are homeotic genes differentially activated in the presence of ubiquitously distributed Pc proteins (25, 61)? Genetic studies indicate that *trx* may play a crucial role. It has been observed that embryos and adults of trx^{-} Pc^{-} double mutants show fewer abnormalities than do those bearing the Pc^{-} mutation alone (13, 69, 71). Many *trx* alleles were uncovered in a systematic screen to isolate suppressors of *Pc* (41). Similar to *Pc*, *trx* exhibits a pleiotropic effect on various homeotic genes (10, 32, 33, 70). *trx* mutations also appear to interact with the same set of regulatory mutations of *Ubx* as *Pc* mutations do (14). Molecular studies have shown that *trx* encodes a large polypeptide of 3,759 amino acids with several clusters of unusual cysteine-rich zinc-binding motifs in the central region (51). Recently, a human *trx* homolog, *ALL-1/HRX/Htrx1*, implicated in ontogeny of acute juvenile leukemias has been identified, and sequence comparison has revealed substantial similarity in the central zinc-binding domain and a striking sequence identity (~60%) in the C-terminal 210 amino acids (28, 62, 79). The subdomain of this C-terminal region has also been found in several other proteins (37, 54, 80). However, the functional significance of these conserved domains and the molecular mechanisms by which trx protein interacts with the *Ubx* promoter and counteracts the effect of Pc-G remain poorly understood.

To understand the molecular mechanisms by which *trx* and *Pc* control *Ubx* expression, we have established a convenient system for reconstruction of regulatory interactions by these genes. Here, we show that proper regulation by both *trx* and *Pc* can be established by transient assays with a *Drosophila* haploid cell line. We have also used this system to map the regulatory sequences to a 440-bp region. Furthermore, we show that the proximal promoter is essential for *trx*-dependent activation.

MATERIALS AND METHODS

***Drosophila* transformation and strains.** Germ line transformation of *Ubx-lacZ* constructs was carried out as described elsewhere (74). The transformant lines used in this study were designated A to E, according to the nomenclature shown in Fig. 2A. All the transformant lines contain inserts on the second chromosome and are either balanced over *CyO* (Fig. 2A, lines A, B, C, and D) or maintained as homozygotes (line E). The expression pattern of *lacZ* in the central nervous system (CNS) was examined in more than one line for each construct, and only results for lines with the most representative patterns are shown. The B line described here was previously named bxd14.5 (74). Mutant alleles used for this study were *Df(3R) red-P93*, *l(3)tr Sb/In (3L) P*$^{+}$ *In (3R) P18*, *Me Ubx e*^{4} for trx^{-} and *Pc*^{4} *Ki p*^{p} *e*^{s}*/TM3* for Pc^{-}.

Immunohistochemical staining and embryo dissection. Embryos 12 to 16 h old were fixed and devitellinized as described elsewhere (1). For immunochemical staining, the rehydrated embryos were first blocked with 5% serum in PBT (1× phosphate-buffered saline [PBS], 0.15% Triton) and then incubated with either a mouse monoclonal antibody against UBX (FP.3.38 [84]) or a rabbit polyclonal antibody against β-galactosidase (Cappel). After extensive washing, the embryos were incubated first with appropriate biotinylated secondary antibody and then with avidin-biotin complexes (Vector). The chromogenic reaction was carried out with 0.5 mg of diaminobenzidine per ml and 0.03% H_2O_2. For double-staining, β-galactosidase antibody was used first and the diaminobenzidine reaction was developed in the presence of 0.08% $NiCl_2$. After extensive washing and blocking, UBX staining was carried out. trx^{-} embryos were identified by their *Ubx* expression pattern in the CNS. To isolate the nerve cord, stage 15 embryos (12) were dissected in 50% glycerol–1× PBS solution with tungsten needles under a dissecting microscope. The isolated nerve cords were mounted in Fluoromount-G (Fisher) between cover glasses and examined with a Zeiss Axioplan microscope equipped with differential interference contrast optics.

Plasmids. The basal *Ubx-lacZ* vector Ubx_0 was previously described as pMBO1241 (74). It contains *Ubx* sequences from positions −1763 to +1136 of the *Ubx* promoter and approximately 15-kb sequences corresponding to *lacZ*, *rosy*, the pUC-based vector. In addition to these common sequences, Ubx constructs A to E contain the following upstream sequences (see Fig. 2A for the coordinate): A, 9.4-kb *Sal*I fragment from positions −27.5 to −18.1; B, 14.5-kb *Sal*I fragment from positions −18.1 to −3.7; C, 5.7-kb *Bam*HI fragment from positions −5 to +0.7; D, 11.5-kb *Hin*dIII fragment from positions 0 to +11.5; E, 10.5-kb *Sal*I fragment from positions +11.5 to +22. Ubx-CAT contains *Ubx* promoter sequences from positions −3149 to +353 and was obtained from M. Krasnow (42). Adh-CAT and Hsp70-CAT were obtained from C. Thummel (78).

For expression of *trx* and *Pc*, Act5C-promoter-containing vectors were used. For cloning of trx-1, the *trx* sequence from nucleotides 1 to 12,244 (based on the *trx* sequence in GenBank) was first cloned into the *Eco*RI-*Xba*I sites of pBluescript (Stratagen) and then recloned into the *Apa*I-*Not*I sites of pAct5CppA-SK, which was modified from pAct5CppA (29) by an insertion of the entire polylinker of pBluescript at *Kpn*I-*Sac*I sites. trx-2 was constructed by deleting the *Bgl*II fragments from nucleotide 7034 to the polylinker. trx-3 was constructed by deleting from the *Nhe*I site at nucleotide 4256 to the *Not*I site in the polylinker. For the construction of trx-4, the ~7-kb *Apa*I-*Bgl*II fragment from the 5′ part of the cDNA was replaced by a 2,448-bp *Apa*I-*Nco*I fragment. Both the *Nco*I and *Bgl*II sites were repaired by Klenow enzyme to restore the reading frame. Pc-1 was constructed by inserting the 1.1-kb *Ase*I fragment of a *Pc* cDNA clone (61) into the *Eco*RV site of pAct5CppA. To construct Pc-2, an 83-bp *Bam*HI-*Eco*47III DNA fragment corresponding to the coding sequences immediately before the chromodomain was generated by PCR amplification (5′ primer, ATGTGGATCCTAAAAATGACTGGTCGAGGC; 3′ primer, AGTCAGCGCTCGTACACTAGATCGACTGG) and used to replace the *Bam*HI-*Eco*47III fragment of Pc-1. Consequently, Pc-2 has a deletion of 27 codons from the 26th to the 54th codon with the addition of two codons (E and R). The Pc-3 clone was generated by deleting the internal 236-bp *Bst*XI fragment. After repair by T4 polymerase, the reading frame was terminated 1 codon after the junction, resulting in a truncated Pc with 254 codons. The Pc-4 clone was generated by replacing the internal *Bcl*I fragment corresponding to codons 175 to 354 with a *Bam*HI-containing linker (GATCACGGATCCGT) to restore the reading frame. Consequently, four additional codons (H, G, S, and V) were created in the junction. The 354th codon for the Val residue was restored in this mutant.

Mutants with deletions in the B fragment were constructed as follows. *Sal*I-*Kpn*I fragments corresponding to the right half or the left half of the B fragment were deleted from Ubx-B to construct Ubx-B1 or Ubx-B2, respectively. Ubx-B120 and other smaller constructs were made in $Ubx_0(r\Delta)$, which was derived from Ubx_0 by deleting the *Sma*I-*Bgl*II fragment corresponding to the entire *rosy* gene. The restriction fragments used for cloning were *Bam*HI-*Bgl*II for B120, *Eco*RI-*Bgl*II for B140, *Afl*II-*Bgl*II for B150, *Pst*I-*Bgl*II for B160, *Eco*RI-*Afl*II for B142, and *Afl*II-*Pst*I for B151 (details will be provided upon request). For the construction of the B120-Uβ series, the *Sac*I-*Hpa*I fragment spanning the *Ubx* promoter of B120 was replaced by corresponding fragments from Uβ, UβΔ, Uβ-GAGA, Uβ-NTF, and Uβ-zeste clones (44). All the plasmids used for transfection experiments were double-banded through a CsCl gradient (68).

Cell culture and transfection. *Drosophila* haploid cell line 1182-4 (18) was maintained in Schneider medium (Life Technologies) or in a 1:1 mixture of M3 and D22 media (M3-D22 medium) (17) at 25°C with constant humidity. Both media contain 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Life Technologies), 50 U of penicillin per ml, and 50 μg of streptomycin (Life Technologies) per ml. Cells were grown in T flasks (Falcon) and diluted threefold with fresh medium every 5 to 7 days. Cell doubling time was about 2 to 3 days in both media. Cells tend to adhere to the flask more tightly in Schneider medium than in M3-D22 medium and therefore need mild trypsinization under the former condition. For this reason, the M3-D22 medium was used for later experiments. Nevertheless, no significant differences were observed for transfection experiments.

For transfection, 2 ml of cells was seeded at a density of 2×10^{6} cells per ml for each well in 6-well plates (Falcon). After 20 to 24 h, each well was transfected with a total of 5 μg of DNA by the calcium phosphate technique (22, 42). Cells were incubated for 72 to 76 h before harvest. Because of the large size difference for constructs used in this study, the molar ratio of the effector to the reporter, instead of the weight, was maintained for assays to be compared. In general, the entire DNA mixture was composed of effector and reporter at a 5:1 ratio unless otherwise indicated. For the internal control, Ubx-CAT or ADH-CAT was kept at the molar ratio used for the *Ubx-lacZ* construct, while 50 ng of Act-CAT was used in each transfection for its high level of activity. When pAct5CppA was added as the control for the effector and when different *Ubx-lacZ* constructs were studied, the weight difference was compensated for by the addition of pBluescript.

RNA analysis. Total RNA was isolated from cultured cells by directly lysing the cells on the plates with 4 M guanidinium thiocyanate and then performing CsCl gradient centrifugation through a 5.7 M CsCl cushion (68). Poly(A)$^{+}$ RNA was then purified through Dynabeads Oligo $(dT)_{25}$ (Dynal). cDNA was synthesized with SuperScript II RNase H^{-} reverse transcriptase according to the instructions of the vendor (Life Technologies). For PCR, primer sequences that flank the introns of each gene were chosen. The 5′ and 3′ primers of *ph* were TGGATCGTCGTGCATTGAAGTTTA (nucleotides 496 to 519 of the cDNA sequence) and GGCTGAATGAATGCTGCAAGGTGA (nucleotides 746 to 726), respectively. The 5′ and 3′ primers of *trx* were CACATCACCCAGTGCCATTC

CGA (from positions 3019 to 3041 of the cDNA sequence) and GGCTGAATG AATGCTGCAAGGTGA (from positions 3336 to 3313), respectively. The 5′ and 3′ primers of *Pc* were GACAATGCGACCGACGATCCAGT (from positions 884 to 906 of genomic sequences) and GTGGCTTGATGCGTGTCCACA TC (from positions 1508 to 1486), respectively. The amplification reaction was carried out as follows: the first cycle was 94°C for 3 min, 67°C for 1 min, and 70°C for 1.5 min; the subsequent 29 cycles were 94°C for 1 min, 67°C for 1 min, and 70°C for 1.5 min; and a 5-min extension was done at 70°C. The PCR products were then fractionated on a 4% agarose gel.

Chloramphenicol acetyltransferase (CAT) and β-galactosidase assays. Transfected cells were mildly trypsinized (when grown in Schneider medium) or resuspended (in M3-D22 medium) by repetitive pipetting. Cells were then collected by centrifugation at 5,000 × *g*, 4°C for 5 min. The cell pellet was washed three times with 1× PBS. Finally, cells were resuspended in 100 μl of 0.25 M Tris-HCl (pH 7.8) and lysed by freezing at −70°C and thawing at 37°C three times. Cell debris were removed by a 15-min centrifugation at 4°C. Protein concentration of the cell extracts was determined by the method of Bradford with gamma globulin as the standard (Bio-Rad). Cell extracts were stored at −70°C.

For a standard CAT assay, 50 μg of protein extract was assayed with 0.1 μCi of [^{14}C]chloramphenicol for 60 min (27). A PhosphorImager (Molecular Dynamics) was used for quantitation of acetylated chloramphenicol following thin-layer chromatography. β-Galactosidase activity was assayed as described elsewhere (68). The reaction mixtures were incubated at 37°C until a significant level of color intensity developed. For reactions which gave more than 60% conversion in CAT assays or more than 0.8 optical density unit in β-galactosidase assays, experiments were repeated with less extract or a shorter incubation time. The activities of CAT and β-galactosidase were independently calculated by the standard formula for each enzyme (68). When CAT activities were monitored as the internal control, the β-galactosidase activity was first normalized with the CAT activity for each individual sample and then averaged for each duplicate. Variation between duplicates was generally below 20%. The relative activities of different constructs were calculated by using one set of samples as the reference. The reference is specified in the figure legend for each experiment. Data presented in the figures are the averages of relative activities from at least three separate experiments.

RESULTS

Proper regulation by *trx* and *Pc* is mediated through a fragment from the *pbx*/*bxd* region. *Ubx* expression in ectoderm-derived embryonic tissues is restricted to the posterior region from parasegment (PS) 5 to PS 13 (2, 84, 85). Since the effects of both *trx*$^-$ and *Pc*$^-$ mutations on *Ubx* expression in the CNS have been well studied (2, 9, 10, 51, 86), we began to search for regulatory sequences that can drive the expression of the *lacZ* reporter gene in a posteriorly restricted pattern in the CNS. Germ line transformation was carried out for five *Ubx-lacZ* transgenes that altogether cover ~50 kb of sequence upstream of *Ubx* (Fig. 2A, lines A to E), and the expression patterns of these transgenes in the CNS were examined. As shown in Fig. 1A, the Ubx-B construct shows strong *lacZ* expression in posterior neuromeres corresponding to PS 6 to 13 but only residual expression in anterior neuromeres (panel b). In these posterior neuromeres, two symmetrical clusters of cells, one located in the center and the other near the periphery, have strong *lacZ* expression. Comparison of this pattern with the patterns of endogenous *Ubx* (Fig. 1B, panel c) or *abd-A* (data not shown) indicates that *lacZ* is expressed primarily in the posterior compartment. In contrast, other constructs show no expression (i.e., Ubx-A) or high levels of *lacZ* expression in anterior neuromeres where *Ubx* is not normally expressed (Ubx-C, -D, and -E). Since the B fragment corresponds to the overlapping sequences between the *pbx* and the strong *bxd* regions (Fig. 2A) in which mutations cause severe defects in *Ubx* expression in PS 6 (2, 36, 86) and since it is the only upstream fragment that drives *lacZ* expression in a manner similar to that of the endogenous *Ubx*, we conclude that this fragment contains important regulatory information for *Ubx* expression.

To determine whether this 14.5-kb B fragment is relevant for *trx* and *Pc* regulation, we examined the effects of *trx*$^-$ and *Pc*$^-$ mutations on *lacZ* expression. The CNS of a *trx*$^-$ embryo double-stained with antibodies against UBX (in brown) and β-galactosidase (in black) is shown in Fig. 1B. The expression of *lacZ* is severely reduced in a subset of peripheral cell clusters in each PS (indicated by arrowheads in Fig. 1B, panel d) but much less so in other cells. Since *Ubx* expression is also severely affected in these cells in *trx*$^-$ embryos, we conclude that the B fragment must be under the regulation of *trx*. The expression pattern of *lacZ* in *Pc*$^-$ mutants is shown in Fig. 1C. Similar to that of the endogenous *Ubx*, *lacZ* expression extends anteriorly to supraesophageal ganglions in *Pc*$^-$ mutants (Fig. 1C, compare panels c and d). Therefore, the Ubx-B transgene is also under proper regulation of *Pc*. The effect of *Pc*$^-$ mutation on the same fragment was also observed at earlier stages (73). Our results are in good agreement with a recent study in which a 6.5-kb 2212H6.5 DNA fragment that overlaps extensively with the distal part of the B fragment has been shown to respond to *Pc*$^-$ and *trx*$^-$ mutations in embryos and imaginal discs, respectively (15).

***Ubx* activation by *trx* in cultured cells.** The presence of multiple zinc-binding motifs in *trx* led us to speculate that *trx* may be directly involved in promoter activation (51). This possibility is supported by the finding that trx proteins are associated with polytene chromosomes (16, 43). To examine this issue further, transient assays were used to determine the effect of *trx* on the *Ubx* promoter in cultured *Drosophila* cell lines.

The *Ubx-lacZ* constructs originally used for germ line transformation were used in transfection experiments. To minimize the variability of the assays, transfections were carried out with the same molar ratio for a *Ubx-lacZ* construct and a control plasmid, Ubx-CAT, that also contains a basal *Ubx* promoter (see Materials and Methods for details). The β-galactosidase activities from these *Ubx-lacZ* constructs were normalized against the CAT activity. As shown in Fig. 2B, when transfected into the haploid cell line 1182-4 (17), Ubx-A, -C, -D, and -E constructs all show activity similar to that of the construct containing only the basal promoter (Ubx_0) in the absence of *trx*. In contrast, Ubx-B reproducibly shows a much higher level of activity (about fourfold over that of Ubx_0). This activity presumably reflects the interaction of certain *cis*-acting regulatory sequences with endogenous factors. Since it is independent of the orientation of the fragment (data not shown), for convenience, it is referred to as the enhancer activity. Cotransfection of *trx* further stimulates *lacZ* expression from Ubx-B more than threefold over the enhancer activity. In general, this *trx*-dependent activity shows a reciprocal relationship with the enhancer activity: when the enhancer activity is low, the *trx*-dependent stimulation is higher and vice versa (Table 1). It seems that there might be certain limiting factors for how much the overall stimulation level can be reached in haploid cells. It is also interesting that when transfection was carried out in SL-2 cells, neither enhancer activity nor *trx*-dependent activity was detected (data not shown). This might be expected from the observation that endogenous *Ubx* and *abd-A* genes are completely repressed by the Pc-G in SL-2 cells (58). Cotransfection of *trx* also appears to slightly stimulate *lacZ* expression from Ubx-A, -C, and -D constructs over the basal activity level. However, because the magnitude of stimulation is low and the β-galactosidase activities of these constructs are usually below the sensitivity of detection (less than 0.05 optical density unit), the significance of these increases is not clear. To further determine whether the effect of *trx* is specific to the distal regulatory sequences of *Ubx*, we have also examined the responses of the *Ubx* proximal promoter and the promoters of alcohol dehydrogenase and actin 5C and found that none of these promoters is significantly affected by *trx* (data not shown). Thus, the detection of a *trx*-dependent activity in the

FIG. 1. Expression pattern of *Ubx-lacZ* transgenes in embryonic CNS. (A) Expression patterns of *lacZ* in CNS of wild-type embryos. Immunocytochemical staining of CNS with a β-galactosidase antibody is shown for *Ubx-lacZ* transgenes A to E (a to e). The anterior end is to the left. Magnification, ×400. (B) Effect of *trx*$^-$ mutation on *lacZ* expression. CNSs from both wild-type (a and c) and *trx*$^-$ mutant (b and d) embryos were doubly stained with antibodies against UBX (brown) and β-galactosidase (black). *trx*$^-$ embryos were identified by the loss of *Ubx* expression in a large fraction of cells in posterior neuromeres. The *trx*$^-$ embryo with relatively weaker *Ubx* but stronger *lacZ* staining is shown for better comparison of the β-galactosidase staining patterns. Higher magnification of the region corresponding to PS 6 to PS 8 is shown (c and d). The peripheral clusters of cells from PS 6 to PS 8 which are most strongly affected by *trx*$^-$ mutation are indicated by arrowheads. Magnifications, ×400 (a and b) and ×630 (c and d). (C) Effect of *Pc*$^-$ mutation on *lacZ* expression pattern. CNSs from both wild-type (a and b) and *Pc*$^-$ (c and d) embryos were stained with antibodies against UBX (a and c) or β-galactosidase (b and d). The expression of both *Ubx* and *lacZ* is extended anteriorly in *Pc*$^-$ mutant embryos (c and d). Magnification, ×200.

B fragment by transient assays correlates well with the study of *Ubx-lacZ* transgenes and indicates that this fragment contains a putative *trx* response element(s) (TRE).

As the expression of endogenous maintenance genes and their relative abundance may be critical for our analyses, the RNA products of *Pc*, *ph*, and *trx* in both haploid cells and SL-2 cells were examined by reverse transcription and PCR. As shown in Fig. 3, the relative abundances of these RNA products are quite different in these two cell lines. Compared with the level of *ph* products, the relative level of *trx* appears to be slightly lower in haploid cells than in SL-2 cells, while the relative level of *Pc* is substantially lower in haploid cells. Assuming that the stoichiometry is important for the formation of functional Pc-G complexes, it is conceivable that Pc-G can't function effectively in the absence of a sufficient amount of *Pc* products in haploid cells. This might be the reason that *trx*-dependent activity can be observed in haploid cells but not in SL-2 cells. It also seems possible that the enhancer activity of the B fragment could be partly contributed by the endogenous *trx*. In this context, the lack of such an activity in other upstream fragments would argue for the absence of TRE.

The central zinc-binding domain of *trx* is essential for transactivation. *trx* contains two conserved coding regions (Fig. 4A). The central region of *trx* contains nine cysteine-rich zinc-binding motifs that form three major clusters and a fourth one containing a single motif (51). The cysteine residues and some flanking sequences of clusters II and III are highly conserved in the human homolog of *trx* (28, 62, 79). The second conserved region resides in the C terminus (see the introduction). To examine the functional relevance of these regions, we have analyzed three large deletion constructs of *trx* in the transient assay system. As shown in Fig. 4B, deletion of the C-terminal half of the coding sequences including the last zinc-binding motif (trx-2) does not appear to significantly affect the activity of *trx*. However, further deletion of clusters II and III (trx-3) appears to have a deleterious effect on the activity of *trx*. Furthermore, deletion of clusters I, II, and III in trx-4 also inactivates the function of *trx*. These results clearly demonstrate that the central domain encompassing three major zinc-binding clusters of *trx* is essential for *Ubx* activation.

***Pc* repression is mediated through distal upstream sequences.** Since the mutant study described earlier demonstrated that the B fragment also contains putative *Pc* response elements (PRE), we wished to test whether *Pc*-dependent repression can also be reproduced. As shown in Fig. 5, the *lacZ* activity from Ubx-B was drastically reduced by cotransfection of wild-type *Pc* (i.e., Pc-1) in a titration experiment. The *lacZ* activity of Ubx-B was reduced to about 20% of its original

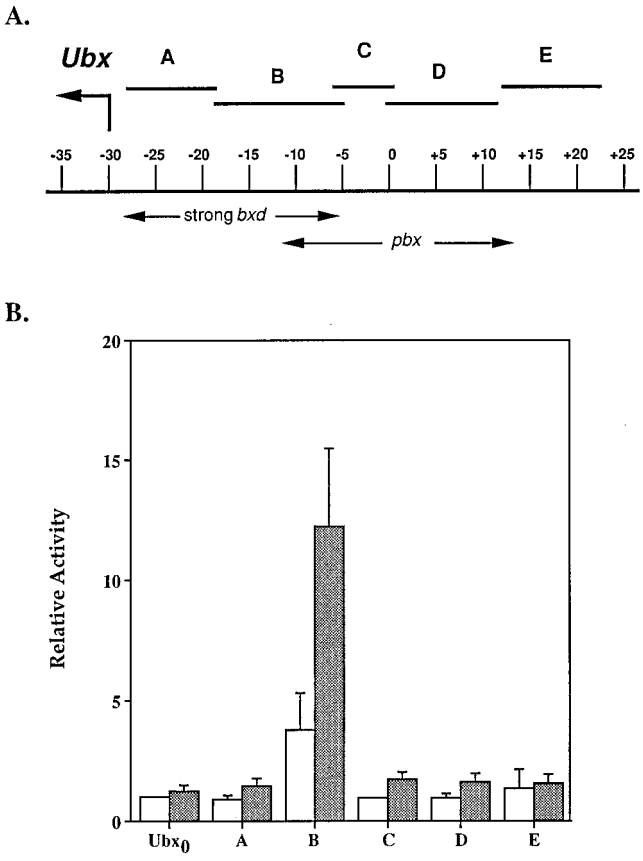

FIG. 2. Transactivation of *Ubx* promoter by *trx* in haploid cells. (A) Map of *Ubx* upstream sequences. The *Ubx* upstream region is shown as the central line. The coordinates (in kilobases) are based on the work of Bender et al. (3). The transcription unit of *Ubx* starts at approximately position −30 and is marked by an angled arrow. The *pbx* and strong *bxd* regions are also indicated. DNA fragments used for germ line transformation are shown as lines A to E above the central line. (B) Transactivation by *trx*. *Ubx-lacZ* constructs Ubx_0 and Ubx-A to -E (A to E) were cotransfected with Ubx-CAT in the presence of the expression vector pAct5CppA (open bars) or trx-1 (filled bars). An equal molar ratio was used for the reporter constructs *Ubx-lacZ* and Ubx-CAT, while a fivefold molar excess of either pAct5CppA or trx-1 was used. Transfection was carried out in duplicate for each construct. The β-galactosidase activities obtained from each *Ubx-lacZ* construct were normalized against the CAT activities from the internal control Ubx-CAT, and the average value for each duplicate was then calculated to obtain the relative activity, by using the activity of Ubx_0 without *trx* as the reference. Data presented are averages of relative activities obtained from three separate experiments. The relative activities were 1.000 (Ubx_0), 0.897 (A), 3.763 (B), 0.940 (C), 0.967 (D), and 1.327 (E) in the absence of *trx* and 1.230 (Ubx_0), 1.457 (A), 12.247 (B), 1.707 (C), 1.607 (D), and 1.513 (E) in the presence of *trx*. Error bars indicate standard deviations.

activity under maximal repression. It is interesting that the level of repressed activity is comparable to that of Ubx_0. Since the enhancer activity of the B fragment can be completely abolished by *Pc*, it is possible that the repressing effect of *Pc* is mediated primarily through the B fragment. However, the observation that Pc proteins are associated with the proximal promoters of *Ubx* and *Antennapedia* (58) would argue that the proximal promoter is also involved. To distinguish between these possibilities, similar titration experiments were carried out for Ubx_0. As shown in Fig. 5, Ubx_0 appears to be refractory to *Pc* even at the highest concentration of Pc-1. Therefore, the B fragment is primarily responsible for the *Pc*-mediated repression observed in this study. To further ascertain whether *Pc*-mediated repression is promoter specific, we also examined its effect on the promoter of the alcohol dehydrogenase gene.

TABLE 1. Transactivation of *Ubx* promoter by *trx*[a]

Construct	Result for expt:					
	1		2		3	
	−*trx*	+*trx*	−*trx*	+*trx*	−*trx*	+*trx*
Ubx_0	1.00	1.25	1.00	1.46	1.00	0.98
Ubx-A	0.69	1.45	1.00	1.78	1.00	1.14
Ubx-B	1.99	9.40	4.29	15.75	5.01	11.59
Ubx-C	0.98	2.02	0.96	1.73	0.88	1.37
Ubx-D	0.86	1.99	1.10	1.55	0.94	1.28
Ubx-E	0.78	1.90	2.26	1.58	0.94	1.06

[a] Data shown were collected from three sets of experiments used for Fig. 2. Each number represents the average of the relative activities for duplicate determinations. The specific activities of β-galactosidase and CAT were measured for each construct. The β-galactosidase activity was then normalized against the CAT activity. The average of the normalized activities from each duplicate determination was then used to calculate the relative activity, with the activity of Ubx_0 without *trx* as the reference.

It appears that this promoter is only slightly affected by *Pc* (activity reduced to ~88%). These results strongly suggest that *Pc* does not act as a general repressor.

The intact chromodomain is required for repression. One of the prominent structural features of the Pc protein is the highly conserved chromodomain (61, 63, 80). Recently, a second region of similarity has been found near the C-terminal end of *Pc* and the mouse M33 gene (63). To examine the functional significance of these domains, we tested the effects of three *Pc* deletion mutations on the *Ubx* promoter. These constructs were cotransfected with Ubx-B and a control plasmid, Adh-CAT. As shown in Fig. 6B, the wild-type *Pc* (Pc-1) reduces *lacZ* activity to 21% of the control level, while Pc-2, which has an internal deletion of the N-terminal 20 codons of the chromodomain, reduces *lacZ* activity only slightly (63%). In contrast, Pc-3 and Pc-4, which have different deletions of C-terminal sequences, reduce *lacZ* activity to 31 and 18%, re-

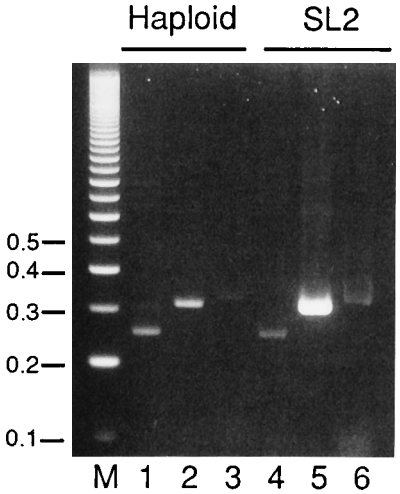

FIG. 3. Relative abundances of maintenance gene products in cultured cells. The amounts of RNA products of *ph* (lanes 1 and 4), *Pc* (lanes 2 and 5), and *trx* (lanes 3 and 6) in haploid cells (lanes 1 to 3) and SL-2 cells (lanes 4 to 6) were measured by reverse transcription and PCR. The primers that flank the introns of these genes were used for amplification of specific mRNAs. *ph* primers flank a 2.8-kb intron, *Pc* primers flank a 314-bp intron, and *trx* primers flank two small introns of 64 and 70 bp. The expected sizes of PCR products are 250 bp for *ph*, 310 bp for *Pc*, and 317 bp for *trx*. The molecular size marker used is a 100-bp ladder (M). Numbers on the left indicate sizes in kilobases.

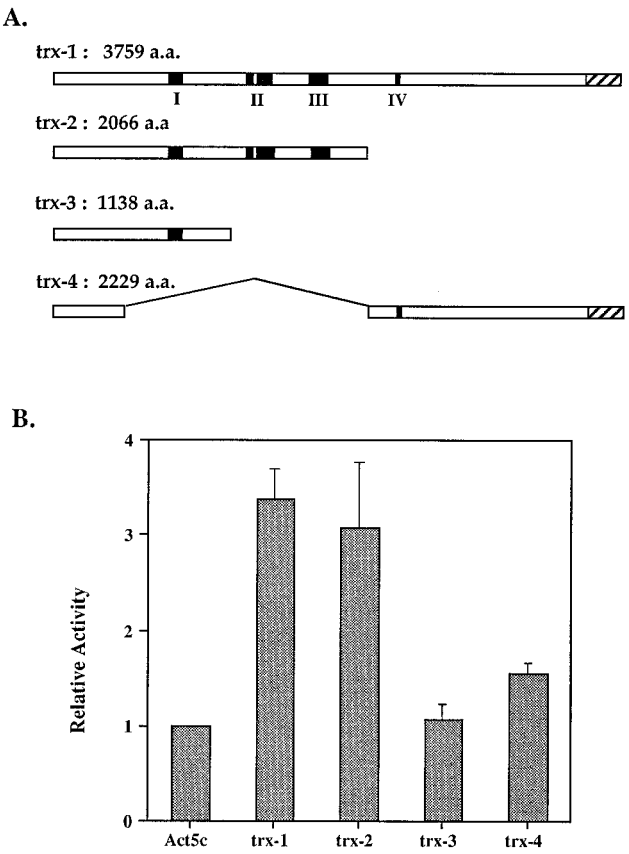

FIG. 4. Effects of *trx* mutations on transactivation. (A) Map of *trx* constructs. *trx* contains nine zinc-binding motifs which form four clusters as indicated by the black boxes I to IV. The clusters II and III correspond to the motifs 3 to 6 and 7 to 8, respectively. These two clusters are highly conserved in the human homolog. The C-terminal conserved sequence is indicated by a hatched box. The coding sequences of trx-2 and trx-3 terminate at codons 2067 and 1139, respectively. trx-4 contains an internal deletion from codons 536 to 2066. a.a., amino acids. (B) Transactivation by *trx* mutants. Ubx-B was cotransfected with a fivefold molar excess of various *trx* constructs in the presence of Ubx-CAT. For the control experiment (Act5C), the expression vector pAct5CppA was used to substitute for the *trx* construct. The relative activity was calculated for each *trx* construct as described in the legend to Fig. 2. The activity of Ubx-B without *trx* was used as the reference. The activities were 1.00 (Act5C), 3.38 (trx-1), 3.08 (trx-2), 1.08 (trx-3), and 1.55 (trx-4). Error bars indicate standard deviations.

spectively. Therefore, deletion of the chromodomain has a more deleterious effect on *Pc* than does deletion of the C-terminal sequences. This is consistent with the findings that several *Pc* mutant alleles contain DNA sequence alterations in the chromodomain and that a mutation in this domain results in the failure of Pc proteins to bind polytene chromosomes (53).

Competitive interactions between *trx* and *Pc*. On the basis of the antagonistic nature of genetic interactions between *trx* and *Pc*, it may be expected that the proteins interact in a competitive manner. To examine this possibility, a competition experiment was carried out. Ubx-B was cotransfected with a constant amount of trx-1 (in fivefold molar excess of Ubx-B) to provide a high level of *lacZ* expression. Increasing amounts of Pc-1 were added to challenge the *trx*-dependent activity. As shown in Fig. 7, addition of trx-1 stimulates *lacZ* expression about fourfold (compare the first and last bars). This high level of activity is reduced by *Pc* in a dose-dependent manner. At the highest dosage of Pc-1, the *lacZ* activity is reduced to a level slightly lower than that of Ubx-B alone (22.4 versus 28.6%). Thus, the *trx*-dependent stimulation can be completely abrogated by *Pc*. This result supports the view that *Pc* and *trx* may functionally compete with each other through the regulatory sequences residing in the B fragment.

Localization of response sequences of *trx* and *Pc*. To further define relevant sequences, we analyzed a series of mutants constructed with deletions within the B fragment (Fig. 8A). Results obtained with some representative mutants are summarized in Fig. 8B. It appears that a 5-kb B1 fragment located at the distal end of the B fragment can be stimulated by *trx* to a level similar to that of the B fragment, while the proximal 9.5-kb B2 fragment fails to respond. Thus, the B1 fragment contains most, if not all, TRE sequences within the B fragment. For B120, although the overall activity shows a slight reduction (~30%), the extent of stimulation by *trx* does not appear to be affected. It is unclear whether the reduction of the overall activity results from the loss of 1.5-kb 5′ sequences or the addition of 1-kb 3′ sequences. Nevertheless, the majority of the TRE sequences must be retained in the B120 fragment. Further deletion of the adjacent 1-kb DNA sequences again results in a slight reduction (~20%), indicating the presence of some portion of the TRE. In the remaining region, a 440-bp region delineated by B150 and B160 mutants appears to be most important, since deletion of this region results in a severe reduction of activity (B160), while the presence of this fragment alone (B151) is sufficient to restore 80% activity of this region. These results clearly indicate that the B151 fragment contains the TRE. It is interesting that the B151 fragment retains some fraction of the enhancer activity initially described for the B fragment. The colocalization of these two activities reinforces the idea that the enhancer activity is partly contributed to by the endogenous *trx*.

Pc response was also examined in three deletion constructs. As shown in Fig. 8C, these constructs contain activities four- to fivefold greater than those of the basal construct Ubx_0. Upon the addition of *Pc*, their activities were reduced more than

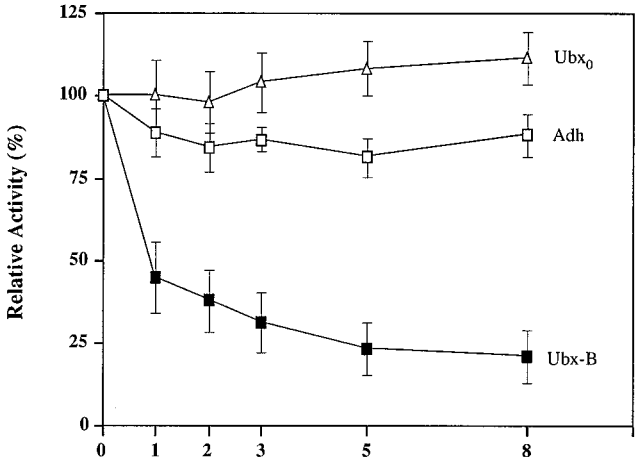

FIG. 5. Sequence dependence of *Pc* repression. Three promoters (Ubx_0, Ubx-B, and Adh) were tested for their responses to wild-type *Pc* (Pc-1) in a titration experiment. Both Ubx_0 and Ubx-B contain *lacZ* as the reporter, while Adh contains the CAT gene as the reporter. The molar ratios of Pc-1 to different reporters are indicated on the abscissa. The unrepressed activity of each reporter was used as the reference to calculate the relative activity of each reporter in the presence of Pc-1. At the highest dosage of Pc-1, the activity of Ubx_0 is slightly elevated (to ~110%), while the activity of Adh is slightly reduced (to ~88%). In general, the activities do not change significantly in the presence of Pc-1. However, the activity of Ubx-B shows a substantial reduction with increasing amounts of Pc-1. At the highest dosage of Pc-1, the activity of Ubx-B is reduced to 21%. The repressed activity of Ubx-B is generally comparable to that of Ubx_0. Error bars indicate standard deviations.

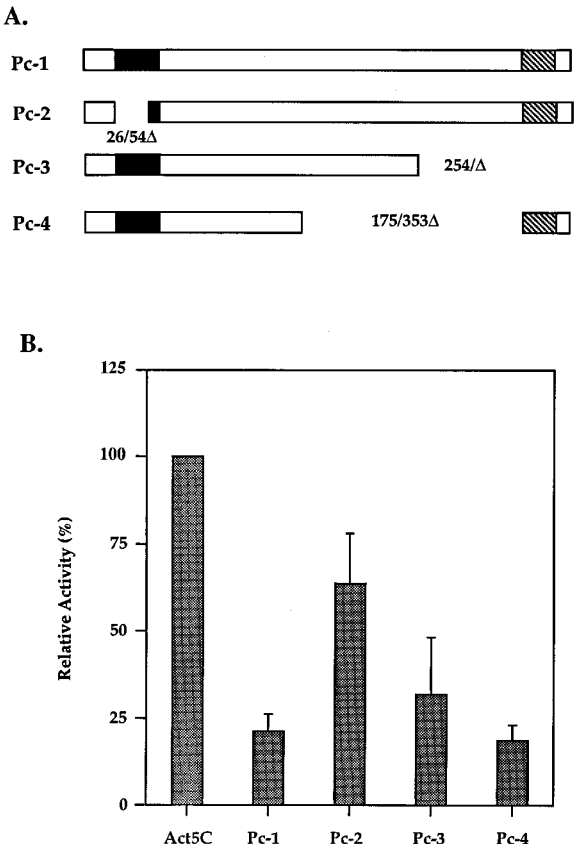

FIG. 6. Effect of *Pc* mutations on repression. (A) Structures of various *Pc* constructs. Two conserved regions are indicated. The chromodomain extends from codons 26 to 73 (black box), and the C-terminal conserved region extends from codons 351 to 380 (hatched box). Twenty-seven codons in the N-terminal part of the chromodomain are deleted in Pc-2, the C-terminal 136 codons are deleted in Pc-3, and Pc-4 has an internal deletion from codons 175 to 353, with three codons (H, G, and S) inserted at the junction. The C-terminal conserved region is nearly intact. (B) Repressing activity of *Pc* mutants. Ubx-B and the internal control Adh-CAT were cotransfected with various *Pc* constructs or Act5C vector in a molar ratio of 1:1:8. The β-galactosidase activity was normalized with the CAT activity for each set of transfection experiments. The unrepressed activity of Ubx-B was used as the reference to calculate the following relative activities of different *Pc* constructs: Pc-1, 21%; Pc-2, 63%; Pc-3, 31%; and Pc-4, 18%. Error bars indicate standard deviations.

twofold. In general, the extent of repression observed to occur in these constructs is smaller than that observed to occur in the B construct (compare Fig. 8C with Fig. 5). Despite the quantitative difference, these results clearly indicate the presence of the PRE in the smallest B151 fragment. Since a high level of enhancer activity is required for the repression assay, we were not able to determine whether PRE activity is also present in the B2, B142, and B160 fragments, which lack the enhancer activity.

The proximal promoter is essential for *trx*-dependent activation. The proximal region from positions −220 to −31 of the *Ubx* promoter is essential for expression of *Ubx* transgenes (44) and for promoter activity in an in vitro transcription assay (7, 8). This region contains multiple GAGA and zeste binding sites and a single NTF binding site (8). To test whether it is also essential for *trx*-dependent activation, several constructs containing sequence variations in this region were examined. As shown in Fig. 9, deletion of this region results in a drastic reduction in promoter activity and a failure to respond to *trx*. When this region was substituted with artificially generated tandem repeats of GAGA or zeste binding sites (44), small increases in activity were observed in the presence of *trx*. Substitution with tandem repeats of the NTF site did not improve the promoter. Thus, this region appears to be essential for the promoter activity and for *trx*-dependent activation. Substitution with tandem repeats of known binding sites is not sufficient to restore its activity.

DISCUSSION

Reconstruction of regulation by *trx* and *Pc* in cultured cells. In this report, we have demonstrated that *Ubx* regulation by *trx* and *Pc* can be reconstructed in a *Drosophila* haploid cell line. The fidelity of this system is supported by the following observations. First, cotransfection of *trx* or *Pc* results in substantial stimulation or reduction of the activity of the *Ubx* promoter, respectively, when the *Ubx* promoter is linked to the B fragment from the *pbx/bxd* region. These observations are in agreement with the effects of trx^- and Pc^- mutations on the expression patterns of endogenous *Ubx* (2, 9, 10, 51, 86) and the *lacZ* reporter gene fused to the B fragment. Second, new polytene chromosome binding sites for trx and Psc proteins have been demonstrated to be present at the insertion sites of the B fragment and a smaller, 6.5-kb 2212H6.5 fragment, respectively (15, 16). The 2212H6.5 fragment overlaps extensively with the B1 fragment studied in this work and has been shown to respond to trx^- and Pc^- in imaginal discs and eyes, respectively (15). Thus, this fragment should contain both the PRE

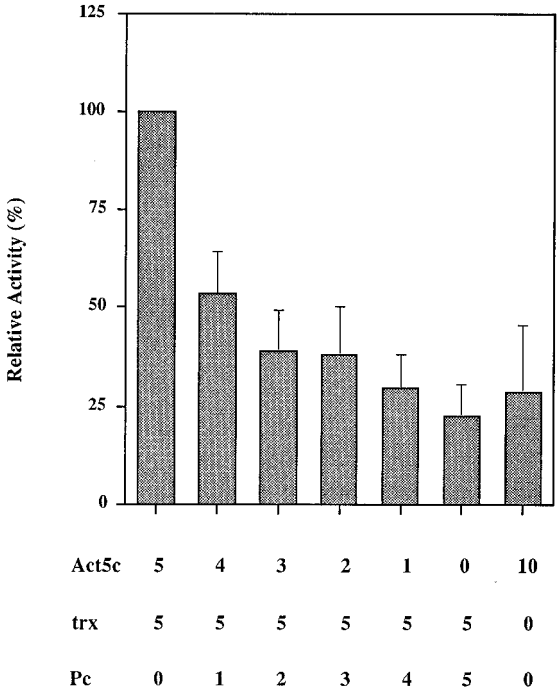

FIG. 7. Competitive interaction between *trx* and *Pc*. trx-1 and Ubx-B were cotransfected at a 5-to-1 ratio with increasing amounts of Pc-1. The Act5C vector was also added to maintain a constant molar quantity of Act5C promoter in each transfection experiment. The molar ratios among effectors and reporter are indicated below the abscissa. The 100% activity is taken from the experiment in which trx-1 was included but Pc-1 was omitted (first bar). The activity of Ubx-B alone is 28.6% (last bar). The addition of trx-1 provides approximately fourfold stimulation of *lacZ* expression. This high level of activity is substantially reduced by increasing the dosage of Pc-1. The relative activities are 53.5, 39.0, 37.7, 29.6, and 22.4% in the presence of Pc-1 (from 1 to 5, respectively). Error bars indicate standard deviations.

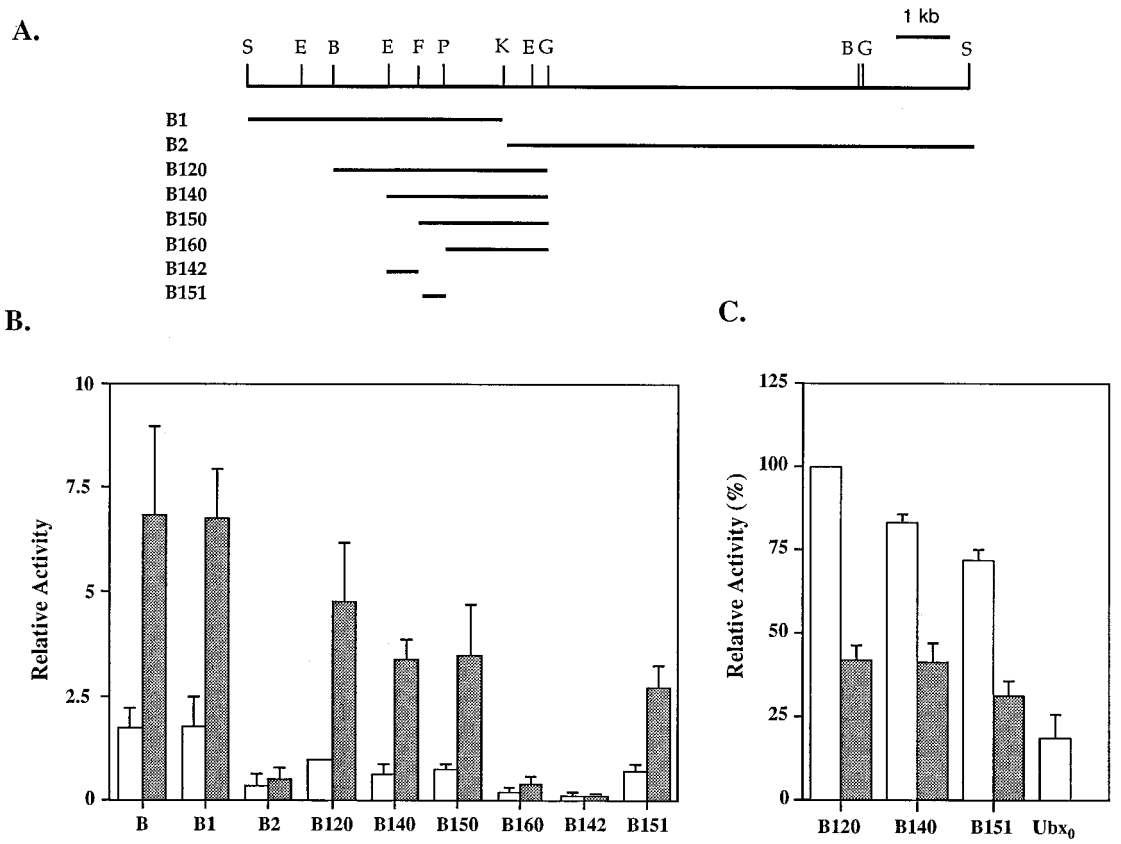

FIG. 8. Localization of response elements for *trx* and *Pc*. (A) Map of the B fragment and various fragments used for mapping of the TRE and the PRE. The direction of *Ubx* transcription is to the right. Relevant restriction sites are shown as follows: B, *Bam*HI; E, *Eco*RI; F, *Afl*II; G, *Bgl*II; K, *Kpn*I; P, *Pst*I; S, *Sal*I. (B) Localization of the TRE. Cotransfection was carried out for various deletion mutants with *trx* (filled bars) or without *trx* (open bars). Act-CAT was used as the internal control. The activity of B120 without *trx* was used as the reference to calculate the relative activity of each construct. Without *trx*, activities were as follows: B, 1.755; B1, 1.764; B2, 0.381; B120, 1.000; B140, 0.632; B150, 0.753; B160, 0.225; B142, 0.135; B151, 0.720. With *trx*, activities were as follows: B, 6.835; B1, 6.758; B2, 0.535; B120, 4.776; B140, 3.407; B150, 3.514; B160, 0.414; B142, 0.138; B151, 2.731. (C) Localization of the PRE. Cotransfection was carried out with *Pc* (filled bars) or without *Pc* (open bars). Adh-CAT was used as the internal control as described in the legend to Fig. 6. The activity of B120 without *Pc* was used as the reference to calculate the relative activity. Without *Pc*, percent relative activities were as follows: B120, 100; B140, 83.1; B151, 71.5; Ubx_0, 18.3. With *Pc*, percent relative activities were as follows: B120, 41.6; B140, 41.1; B151, 30.7. Error bars indicate standard deviations.

and the TRE. Our study of the B1 fragment and other, smaller fragments demonstrates that both *trx* and *Pc* can exert their effects through the same region. In addition, further analysis allowed us to map these response elements to a 440-bp region. Third, we demonstrate that the proximal region from position −220 to −31 is essential for proper function of *Ubx*. In the absence of this region, *trx*-dependent activation is severely affected. This region has been shown to be critical for expression of *Ubx-lacZ* transgenes and in promoter activity in cell-free transcription assays (7, 8, 44). It has also been suggested that the integrity of the proximal region is important to maintain long-range interactions with distal regulatory elements on the same chromosome, thereby preventing a *zeste*-independent transvection (50). Fourth, our study shows that an intact chromodomain is essential for *Pc*-mediated repression. The importance of this domain is again indicated by earlier studies of *Pc* mutations (53). It is worth noting that the requirement of an intact chromodomain can be alleviated by artificially tethering Pc proteins through the DNA binding domains of LexA (11) or Gal4 proteins (55). Whatever the true function of the chromodomain, this function appears to be preserved in our system and allows us to determine the sequences involved in *Pc* repression. It is also interesting that *Pc* is not inactivated by deletions of the C-terminal sequences (i.e., Pc-3 and Pc-4) in our assays. It has been shown that although deletion of the C-terminal sequences including the conserved C-terminal region results in inactivation (11, 55), further deletion up to the 272th codon restores the activity of *Pc* (11). It has been suggested that a repression domain may be located further upstream (11). It is very likely that such a domain is also preserved in Pc-3, a mutant which has a deletion up to the 254th codon. On the other hand, the conserved C-terminal region is implicated in the repressing activity in a different study (55). This region is almost intact in the Pc-4 mutant. When these data are taken together, it seems possible that two functionally similar domains are present and that the internal one may become functional when it is exposed by deletion. Clearly, a more systematic study is required for a better understanding of the functional domains of *Pc*. Lastly, the observation that high-level activity of the *Ubx* promoter supported by *trx* can be largely repressed by *Pc* in a dose-dependent manner is consistent with genetic studies that suggest an antagonistic interaction between *trx* and *Pc*. Furthermore, the functional role of the highly conserved central zinc-binding domain of *trx* has been implicated by the following observations: (i) an out-of-frame deletion (in the trx^{B11} allele) before this domain results in loss of function, but an in-frame deletion encompassing the zinc-binding cluster IV (in the trx^{E3} allele) has no drastic effect

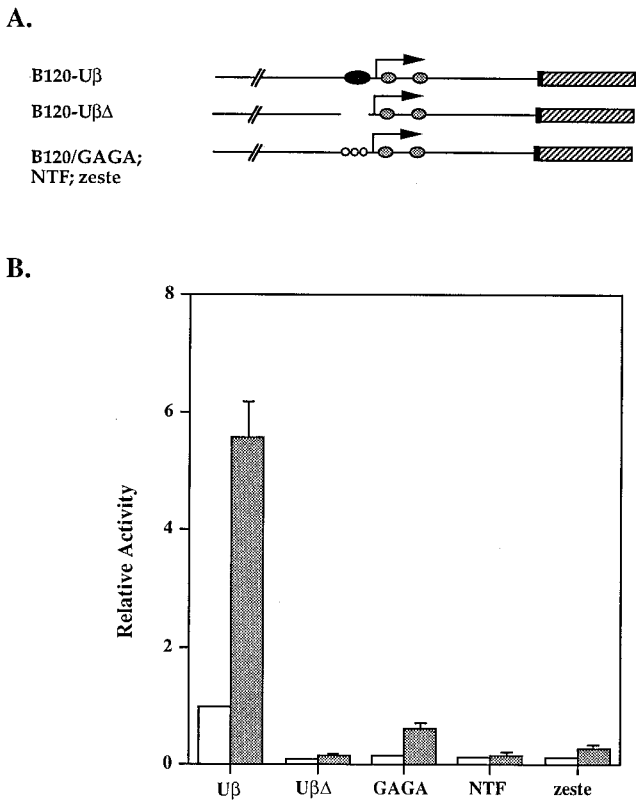

FIG. 9. The proximal sequences are essential for *trx* response. (A) The structure of the proximal sequences of the *Ubx* promoter in reporter constructs. The region from positions −220 to −31, which contains multiple GAGA and zeste binding sites and a single NTF site, is indicated by a black oval, while two clusters of homeoprotein binding sites in the downstream region are indicated by stippled ovals. The start site and the direction of transcription are indicated by angled arrows. The coding region of *Ubx* is indicated by a black box, while the *lacZ* reporter gene is indicated by a hatched box. The size of *lacZ* is not to scale. The proximal sequences from positions −220 to −31 are deleted in B120-UβΔ. The same region is replaced by tandem repeats of GAGA, NTF, or zeste binding sites (open circles). (B) B120-Uβ and its derivatives were cotransfected with Act-CAT in the absence (open bars) or presence (filled bars) of *trx*. The unstimulated activity of B120-Uβ was used as the reference to calculate the relative activities. With *trx*, relative activities were as follows: B120-Uβ, 1.000; B120-UβΔ, 0.097; B120-GAGA, 0.161; B120-NTF, 0.121; B120-zeste, 0.139. Without *trx*, relative activities were as follows: B120-Uβ, 5.568; B120-UβΔ, 0.158; B120-GAGA, 0.621; B120-NTF, 0.179; B120-zeste, 0.298. Error bars indicate standard deviations.

on *Ubx* expression (51); and (ii) disruptions of the human homolog of *trx* caused by chromosomal translocations are frequently found in this domain (28, 62, 79).

Genetic studies have suggested that complex mechanisms are involved in *Ubx* regulation by maintenance genes (6, 40, 59, 60). The apparent formation of protein complexes by members of the Pc-G (25) and their possible roles in chromatin organization (58) strongly suggest that these proteins act in concert to repress homeotic genes. The synergistic effects of genetic interactions observed for members of the trx-G also suggest that these proteins act in cooperative fashions to overcome the effect of the Pc-G (21, 40, 41, 71). It is thus surprising to find that *Ubx* regulation by *trx* and *Pc* can be properly reconstructed in cultured cells by the addition of a single gene at a time. However, a mass action model proposed to explain the dosage effects of various modifiers on position effect variegation (PEV) predicts that at equilibrium the formation of a complex is proportional to the nth power of the concentration of each constituent protein (47). Depending on the number of molecules for each subunit in the complex, very dramatic changes can be effected by changing the concentration of any given constituent subunit. Since PEV has been suggested to share a mechanistic similarity with the Pc-G function (60, 75), it may be expected that the addition of one component could have a significant impact. Furthermore, given the low relative abundances of both *trx* and *Pc* products in haploid cells, it is conceivable that the effects of transfected *trx* and *Pc* may become more substantial. Another unusual aspect of the haploid cell line is its apparent ability to function in the absence of early spatial cues. In the embryo, the products of *trx* and *Pc* are widely distributed, but their effects on *Ubx* are spatially restricted. It has been suggested that the product of a gap gene, *hunchback* (*hb*), may function to recruit Pc-G complexes to the target sequences (6, 87). However, it has been shown that the introduction of high-affinity hb binding sites does not significantly change the ability of the PRE to establish maintenance (15). In addition, despite the absence of hb binding sites, the *white* gene is efficiently repressed by fragments containing the PRE we define here (15). Thus, a different mechanism may exist for the action of the Pc-G. The fact that both *trx* and *Pc* function appropriately in the haploid cell line indicates that this cell line can preserve some important aspects of *Ubx* regulation by maintenance genes. This novel property should be useful for detailed mechanistic study of the molecular functions of these genes.

Implication of TRE and PREs. Although genetic studies indicate that *Ubx* is regulated by *trx* and *Pc*, it might be argued that the regulation is mediated indirectly through secondary genes. However, the association of trx proteins and several Pc-G proteins with specific *Ubx* upstream sequences on polytene chromosomes or formaldehyde cross-linked chromosomes (15, 16, 19, 58) has provided strong evidence that *Ubx* is under direct regulation of these maintenance genes. We have defined the response sequences for both *trx* and *Pc* through transient-assay studies. Considering that the transfected target sequences are more readily accessible to the effectors than is the chromosomal DNA under the conditions of the transient assay, it is conceivable that the observed effects are to a large extent mediated through direct interactions, instead of being secondary effects through activation or repression of other chromosomal target genes. However, this does not necessarily imply that trx or Pc proteins are directly responsible for sequence-specific binding or that they act alone. For *Pc*, it has been suggested that other factors in Pc-G protein complexes may be directly involved in DNA binding (60). How trx proteins may act remains to be determined. Despite the uncertainty about the details of these interactions, the ability of this system to respond properly to both *trx* and *Pc* and to identify sequences similar to those found in other studies has led us to conclude that the region defined here contains authentic response elements for both genes.

In the 50-kb upstream region, the B fragment is the only fragment that shows substantial stimulation by both endogenous and transfected *trx*. The 440-bp B151 fragment appears to be the region within the B fragment that retains the same property. Although we can't exclude the possibility that there are some scattered TREs in other regions, our results strongly suggest that the B151 fragment contains the major TRE. On the basis of the results that B120 and B151 constructs are repressed to similar extents, we conclude that the B151 fragment also contains a substantial fraction of the PRE. In our study of *Pc* repression, only fragments with high levels of enhancer activity can be analyzed. It is therefore difficult to determine whether there are other PREs in inactive fragments.

However, since the B120 construct is repressed to a lesser extent than is the B fragment, it is possible that some PREs are present in other regions of the B fragment. This might be related to the observation that although several smaller overlapping fragments within the 2212H6.5 fragment show variable degrees of PEV on eye pigmentation, none of them can mediate complete anterior repression in embryos (15).

The observations that the addition of a DNA fragment from the *pbx* region can confer anterior repression upon an otherwise segmentally repetitive pattern generated by a short *bxd* fragment (56) and that Pc proteins are detected in extended areas of *Ubx* in cross-linked chromosomes (58) strongly suggest the presence of PREs in the regions outside the B fragment. Thus, it appears that a majority of TRE is located within a rather small region, while PRE may be more broadly distributed. Given the antagonistic nature of *trx* and *Pc* and their competitive interaction observed in our study, such a configuration may be important for the mechanism of *Ubx* maintenance. We suggest that the "on-off" state may be maintained through effective interactions of trx or Pc proteins with their response elements in the upstream region. In the simplest view, their interactions with the response elements could be mutually exclusive. Alternatively, the two types of interactions could occur simultaneously. However, a secondary interaction with factors situated in the proximal promoter may be crucial for the promoter activity (see below for further discussion).

Long-range interactions for promoter activation. The finding that the TRE is located about 20 kb upstream of the natural transcription start site raises an interesting question about how trx proteins can activate the promoter over a long distance. Our observation that *trx* fails to stimulate the *Ubx* promoter bearing a deletion from positions −220 to −31 demonstrates that mere interaction with the TRE in the distal upstream region is not sufficient for trx proteins to activate the promoter. It may be essential for trx proteins to interact directly or indirectly with certain factors that are poised in the proximal region. If such an interaction is prevented by DNA sequence alteration or physical inaccessibility (i.e., blocked by Pc-G complexes), trx proteins may not function effectively. Since substitution of the proximal region with GAGA or zeste binding sites results in a partial restoration of the activity, it is possible that both GAGA and zeste proteins are involved in such an interaction. It is interesting that these genes may be considered members of the trx-G, on the basis of their functional similarities (40) and the identification of *Trithorax-like* as the GAGA-encoding gene (24, 76). It is also relevant to point out that biochemical studies have shown that GAGA proteins can disrupt nucleosome assembly on the *Hsp70* promoter (81), while zeste proteins can form homo-oligomers that bind physically unlinked DNA molecules together (5). These properties are consistent with a model that GAGA proteins act to destabilize an unfavorable chromatin structure through a looped DNA structure.

Our results also suggest that factors other than GAGA or zeste proteins may play an important role in the proximal region, since neither of them is sufficient for a complete restoration of the promoter activity. Our recent finding that *female sterile homeotic* (*fsh*), a member of trx-G, can activate the *Ubx* promoter through the same proximal region (41a) suggests that *fsh* is a potential candidate. This view is supported by genetic studies that demonstrate a synergistic interaction between *trx* and *fsh* (21, 71). Thus, unlike *trx*, several members of the trx-G may exert their effects primarily through the proximal promoter. We propose that these proteins are poised at the proximal promoter to receive the proper regulatory signal from the distal region. Recently, an enhanced *zeste*-independent transvection has been described for *Ubx* mutants defective in the proximal promoter (50), indicating that the proximal promoter may be essential to maintain long-range interactions with distal regulatory elements in a normal chromosomal context. It will be interesting to know whether the interaction proposed here is related.

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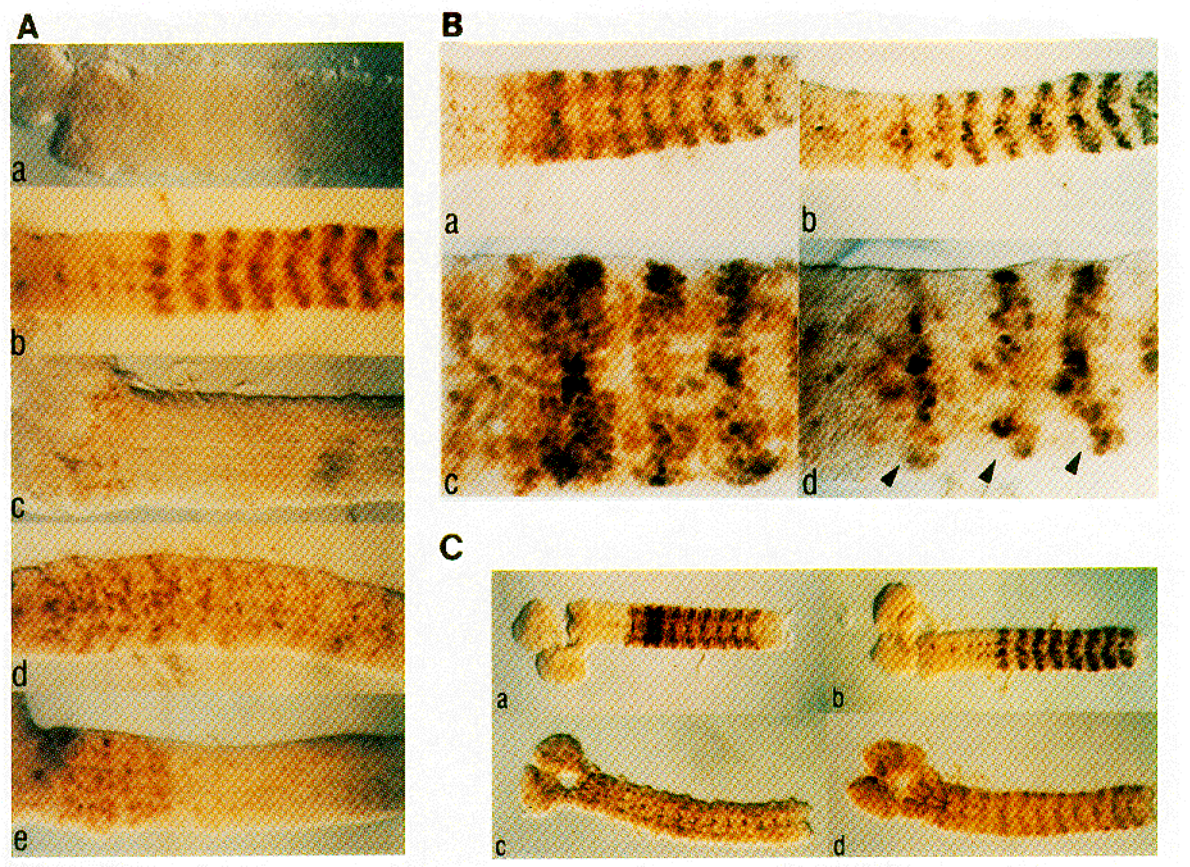
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