Promoter-proximal tethering elements regulate enhancer-promoter specificity in the *Drosophila Antennapedia* complex

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Insulator DNAs and promoter competition regulate enhancer–promoter interactions within complex genetic loci. Here we provide evidence for a third mechanism: promoter-proximal tethering elements. The *Scr-ftz* region of the *Antennapedia* gene complex includes two known enhancers, AE1 and T1. AE1 selectively interacts with the *ftz* promoter to maintain pair-rule stripes of *ftz* expression during gastrulation and germ-band elongation. The T1 enhancer, located 3′ of the *ftz* gene and ≈25 kb 5′ of the *Scr* promoter, selectively activates *Scr* expression in the prothorax and posterior head segments. A variety of *P* element minigenes were examined in transgenic embryos to determine the basis for specific AE1-*ftz* and T1-*Scr* interactions. A 450-bp DNA fragment located ≈100 bp 5′ of the *Scr* transcription start site is essential for T1-*Scr* interactions and can mediate long-range activation of a *ftz*/*lacZ* reporter gene when placed 5′ of the *ftz* promoter. We suggest that the *Scr*450 fragment contains tethering elements that selectively recruit T1 to the *Scr* promoter. Tethering elements might regulate enhancer–promoter interactions at other complex genetic loci.

Previous studies have identified two different mechanisms for regulating enhancer–promoter interactions within complex genetic loci: promoter competition and insulator DNAs (for reviews, see refs. 1 and 2). In the case of competition, a shared enhancer selectively interacts with the strongest of linked promoters (3, 4). This preferred interaction sequesters the enhancer so it is unavailable for interacting with weaker promoters. Insulator DNAs selectively block the interaction of a distal enhancer with a target promoter when the insulator is positioned between the two (reviewed in ref. 5). This block does not interfere with the activation of proximal genes by the same enhancer. Here we present evidence for a third mechanism of regulating enhancer–promoter interactions: promoter-proximal tethering elements.

The *Antennapedia* gene complex (ANT-C) is one of the two major *Hox* gene clusters in the *Drosophila* genome (summarized in Fig. 1). It is ≈500 kb in length and contains nine homeobox genes, including five homeotic selector genes that pattern the head and thorax (6, 7). The *Scr* selector gene is expressed in the anterior compartment of the first thoracic segment, as well as portions of the labial and maxillary head segments. This complex pattern of *Scr* expression depends, at least in part, on a distal enhancer, T1, that is located ≈25 kb 5′ of the *Scr* promoter (8). T1 is located downstream of the pair-rule gene *ftz*, which exhibits a seven-stripe pattern of expression distinct from the *Scr* gene. The *ftz* pattern is regulated by an intergenic enhancer, AE1, located between the divergently transcribed *Scr* and *ftz* genes (9, 10). Specific T1-*Scr* and AE1-*ftz* interactions are essential for the normal patterning of the early embryo. Misexpression of either gene disrupts segmentation and causes embryonic lethality (11, 12).

Previous studies have shown that AE1 prefers TATA-containing promoters (13). This observation suggests that AE1-*ftz* specificity is governed by promoter competition; the native *ftz* promoter is stronger than the *Scr* promoter. However, this type of simple competition mechanism cannot account for specific T1-*Scr* interactions. The distal T1 enhancer bypasses the strong proximal *ftz* promoter to activate the weak *Scr* promoter. Analysis of chimeric *Scr-ftz* promoter sequences identified a 450-bp DNA fragment that facilitates T1-*Scr* interactions. This fragment is located immediately 5′ of the *Scr* promoter and permits T1 to activate a *ftz-lacZ* reporter gene when placed 5′ of the *ftz* promoter. *Scr*450 does not foster AE1-*ftz* or AE1-*Scr* interactions. We propose that *Scr*450 contains "tethering" elements that selectively recruit the distal T1 enhancer. It is conceivable that tethering represents a common mechanism for regulating enhancer–promoter interactions within complex genetic loci.

Materials and Methods

P-Transformation Assays. yw^{67} flies were used for all P-transformation assays. Fusion genes were introduced into the *Drosophila* germ line by using standard methods (14). Multiple transformants were generated for each construct, and at least three independent lines were examined by *in situ* hybridization. Embryos were collected, fixed, and hybridized with digoxigenin-labeled *CAT* and *lacZ* probes, as previously described (15, 16).

Preparation of Enhancers and Promoters. The AE1 enhancer is located ≈2.5 kb upstream of the *ftz* transcription start site (10). This 430-bp fragment was isolated from genomic DNA by conventional PCR methods and cloned into the *Xba*I site of a *p*-Bluescript vector, modified with *Asc*I sites flanking the polylinker. The T1 enhancer is located ≈4 kb downstream of the *ftz* coding region (8). This 3.8-kb fragment was isolated from the genome by PCR and cloned into the *Hin*dIII site of the *Asc*I modified *p*-Bluescript vector.

The initial *Scr* promoter used in this study is 1.1 kb in length and includes 555 bp of 5′ flanking sequence and 590 bp of 3′ sequence (17). *Scr* promoter deletions were generated by PCR. Two hundred twenty-five base pairs of 5′ flanking sequence was removed from the 1.1-kb promoter to generate *Scr*900. Another 225 bp of 5′ sequence was deleted to generate *Scr*700. The minimal core *Scr* promoter is just 80 bp in length and extends from −36 to +38. The *Scr* downstream promoter element (DPE) is located from +28 to +33 (GCACGT) and is a 6-for-6 match to the DPE consensus: (A/G/T)(C/G)(A/T)(C/T)(A/C/G)(C/T) (18). The mutagenized *Scr* chimera containing a TATA box was created by using a mutagenic oligonucleotide that converts the sequence TGATGCTCA (−31 to −23) to GTATAAAAG. To replace the *Scr* DPE with the corresponding sequence from *ftz*, a mutagenic oligonucleotide was used that changed GCACGT to ACATCG.

The *ftz* promoter used in Fig. 1 is ≈200 bp in length and

Abbreviation: DPE, downstream promoter element.

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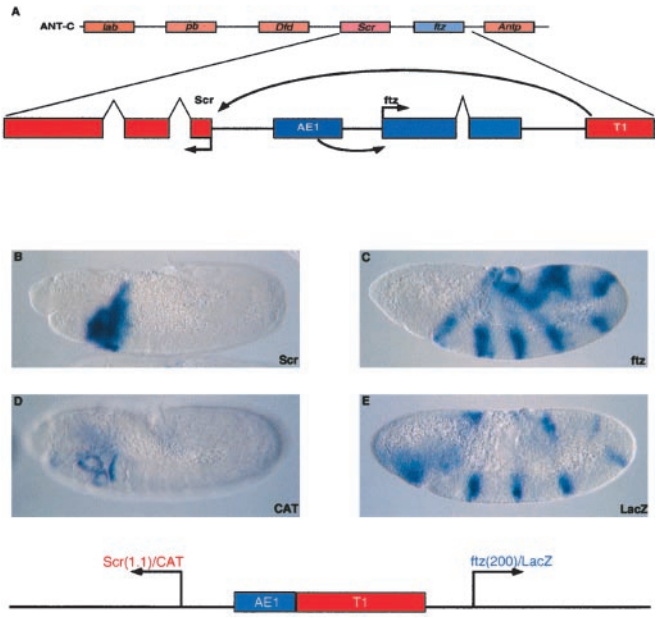

Fig. 1. Summary of the *Scr-ftz* region of the ANT-C. (*A*) The diagram (*Upper*) shows the order of the five *Hox*-containing homeotic selector genes. The pair-rule gene *ftz* is also indicated. The second diagram presents an enlarged view of the *Scr-ftz* interval. The two genes are divergently transcribed, and the intron-exon organization of each gene is indicated. The AE1 enhancer is located in the intergenic region between *Scr* and *ftz* but selectively interacts with the *ftz* promoter to maintain the seven-stripe expression pattern in embryos undergoing germ-band elongation. *Scr* expression depends on the T1 enhancer, which is located 3′ of the *ftz* transcription unit. (*B*) *Scr* expression pattern in an 8- to 9-hr embryo undergoing germ-band retraction. Staining was visualized after hybridization with a digoxigenin-labeled *Scr* antisense RNA probe. Expression is detected in different tissues of parasegment 2, which includes the posterior-most regions of the head and the anterior compartment of the prothorax. (*C*) *ftz* expression pattern in a 4- to 5-hr elongating embryo. Staining is detected in seven stripes along the germ band. Staining was visualized after hybridization with a digoxigenin-labeled *ftz* antisense RNA probe. (*D* and *E*) Transgenic embryos were obtained from adults containing the *P* element transformation vector indicated in the diagram beneath the photomicrographs. Divergently transcribed *CAT* and *lacZ* reporter genes were placed under the control of the *Scr* and *ftz* promoter regions, respectively. The *Scr* promoter region is 1.1 kb in length and includes ≈555 bp 5′ of the transcription start site. The *ftz* promoter region is 200 bp and includes ≈100 bp of 5′ flanking sequence. The 430-bp AE1 enhancer and the 3.8-kb T1 enhancer were placed between the *CAT* and *lacZ* reporter genes. The leftward *CAT* gene is activated by the T1 enhancer in posterior head segments and the anterior compartment of the prothorax, similar to the endogenous *Scr* expression pattern (compare *D* with *B*). The *lacZ* reporter gene exhibits seven stripes of *ftz* expression, similar to endogenous *ftz* expression (compare *E* with *C*). *CAT* and *lacZ* expression patterns were visualized after hybridization with digoxigenin-labeled *CAT* or *lacZ* antisense RNA probes.

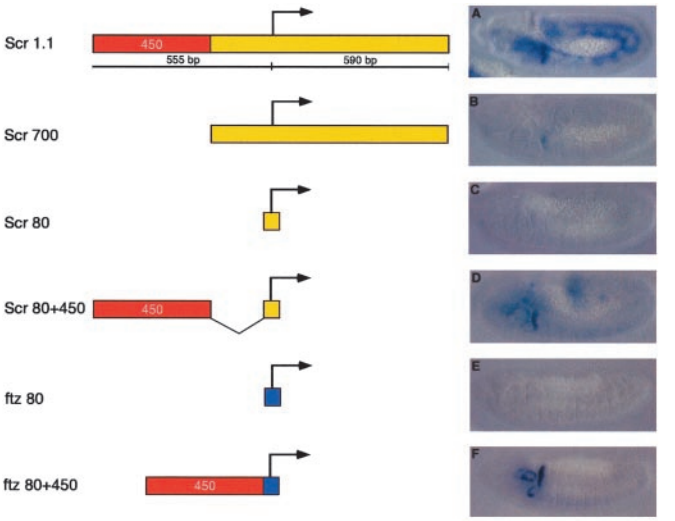

Fig. 2. Identification of promoter-proximal tethering elements. The T1 enhancer was placed 5′ of a series of truncated *Scr-lacZ* fusion genes. The ''full-length'' *Scr* promoter region is 1.1 kb in length and includes 555 bp of 5′ flanking sequence (*A*). Truncations that remove a 450-bp fragment exhibit diminished levels of expression (*B* and *C*). This 450-bp fragment is sufficient to stimulate expression from the minimal *Scr* core promoter (*Scr*80 + 450; *D*). Inserting this fragment immediately upstream of the heterologous *ftz* promoter is sufficient to drive strong T1 expression (*ftz*80 + 450; *F*) on an otherwise unresponsive promoter (*ftz*80; *E*).

extends from −107 to +91 (19). Subsequent experiments used a smaller *ftz* promoter that extends 5 bp upstream of the TATA box (TATATA) to 5 bp downstream of the DPE (ACATCG). For the *ftz* chimera lacking a TATA element, a mutagenic oligonucleotide was used to replace the sequence TATATA to GATGCT. Likewise, a mutagenic primer was used to replace the *ftz* DPE with the corresponding sequence from *Scr*. For the ftz^{Scr450} chimera, a hybrid primer (5′-GCCTTACTTGCTCG-TACTCGCTTTGCTATATATGCAGGATCTGCCG-3′) was used to fuse the *Scr*450 tethering element (−555 to −102 upstream of *Scr* +1) to the minimal *ftz* core promoter.

Construction of *P* Element Transposons. The *CAT*/*lacZ* P-transformation vector that was used for all of the experiments presented in this study is a modification of pCasPer, which contains divergently transcribed *white* and *lacZ* reporter genes (14). It was modified by insertion of a *CAT* reporter gene between *white* and *lacZ* (13).

Promoters were isolated as *Asc*I-*Bam*HI fragments and cloned into a unique *Bam*HI site located at the 5′ end of either the *CAT* or *lacZ* coding sequence present in *p*-Bluescript vectors. The *CAT* fusion genes were subsequently isolated as *Asc*I-*Not*I fragments and used to replace the *Asc*I-*Not*I *CAT* fragment in the pCasPer vector. The *lacZ* fusion genes were isolated as *Asc*I-*Xba*I fragments and used to replace the *Asc*I-*Xba*I *lacZ* fragment in the pCasPer vector. The AE1 and T1 enhancers were isolated as an *Asc*I fragment and cloned into the unique *Asc*I site located between the divergently transcribed reporter genes.

For the long-range tethering constructs, a 1.6-kb spacer from bacteriophage λ was isolated as an *Asc*I fragment and cloned into the unique *Asc*I site in pCasPer. The T1 enhancer was modified with flanking *Sbf*I sites and cloned into the unique *Pst*I site located downstream of the *lacZ* reporter gene.

Results and Discussion

The organization and expression of the *Scr-ftz* region of the ANT-C is summarized in Fig. 1. The *Scr* and *ftz* genes are divergently transcribed, and the two promoters are separated by a ≈15-kb intergenic region that contains the AE1 enhancer. AE1 selectively interacts with the *ftz* promoter and does not regulate *Scr* (10, 20). One of the major enhancers regulating *Scr* expression, T1, is located 3′ of the *ftz* transcription unit and maps ≈25 kb upstream of the *Scr* promoter (8). *Scr* expression is first detected at the onset of gastrulation and persists in the posterior head segments and prothorax during germ-band elongation, retraction, and segmentation (Fig. 1*B*). In contrast, *ftz* expression

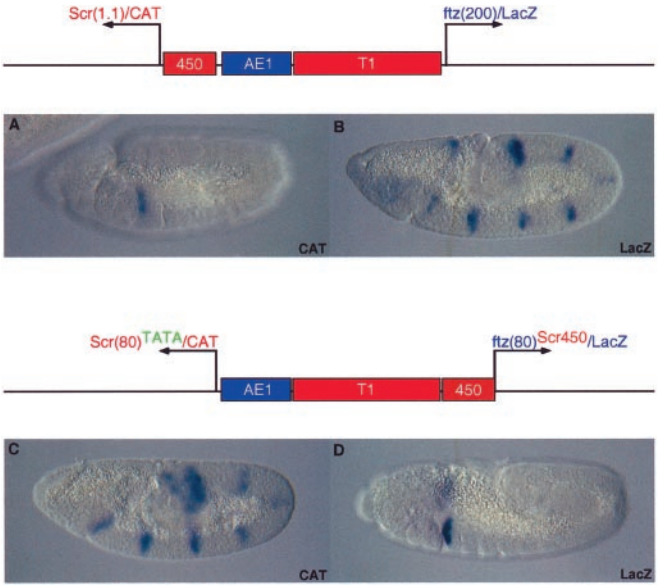

Fig. 3. Swapping regulatory specificity in the *Scr-ftz* region. Transgenic embryos express the *P* element minigenes indicated above the photomicrographs. The AE1 enhancer selectively activates the rightward *ftz/lacZ* reporter gene, and T1 activates the leftward *Scr/CAT* gene (*A* and *B*; also see Fig. 1 *D* and *E*). Regulatory specificity is reversed on modification of the *Scr* and *ftz* promoters (*C* and *D*). The *Scr*450 tethering fragment was removed from the 5′ region of *Scr* and attached to the minimal *ftz*80 promoter. In addition, the minimal *Scr* promoter was modified to include an optimal TATA element. The AE1 enhancer now strongly activates the leftward *Scr* promoter, and induces seven stripes of *CAT* expression along the germ band. Conversely, the T1 enhancer selectively interacts with the rightward *ftz/lacZ* fusion gene and activates *lacZ* expression in the prothorax and weakly in posterior regions of the head. AE1 expression of *Scr/CAT* depends on the presence of T1 between AE1 and *ftz/lacZ*. Removal of T1 results in greatly diminished *Scr/CAT* staining.

is detected before the completion of cellularization and exhibits seven stripes of expression during gastrulation and germ-band elongation (Fig. 1*C*). These distinct patterns of expression depend on selective AE1-*ftz* and T1-*Scr* enhancer–promoter interactions (Fig. 1*A*).

To identify the *cis*-regulatory elements responsible for this specificity, the *Scr* and *ftz* promoter regions were attached to divergently transcribed *CAT* and *lacZ* reporter genes (Fig. 1 *D* and *E*). The *Scr* promoter region is 1.1 kb in length and includes ≈555 bp of 5′ flanking sequence. The *ftz* promoter region includes just 200 bp and contains 105 bp of 5′ flanking sequence. The AE1 and T1 enhancers were placed between the two reporter genes. The T1 enhancer fails to activate the proximal *ftz-lacZ* gene but directs strong *CAT* staining in the maxillary and labial head segments, as well as the anterior compartment of the prothorax (Fig. 1*D*). In contrast, although closer to the leftward *Scr-CAT* gene, AE1 specifically interacts with the rightward *ftz-lacZ* gene to direct seven stripes of *lacZ* expression in embryos undergoing germ-band elongation (Fig. 1*E*). The *CAT* and *lacZ* staining patterns mimic the endogenous *Scr* and *ftz* expression patterns (Fig. 1 *D* and *E*; compare with Fig. 1 *B* and *C*), indicating that the *Scr* and *ftz* promoter sequences are sufficient to reproduce authentic regulatory specificity in the *Scr-ftz* region.

Core promoter sequences were altered to determine whether T1-*Scr* and AE1-*ftz* specificity depends on TFIID recognition elements, such as TATA and the DPE. The native *Scr* and *ftz* promoters are quite distinct. *ftz* contains a strong TATA element and a 5-of-6 match to the DPE consensus sequence. Conversely, *Scr* lacks TATA but contains a 6-of-6 match to the DPE consensus (18, 21). Chimeric *Scr-ftz* promoters were created to investigate the role of TFIID recognition elements. Core promoter elements from *ftz* were replaced with the corresponding regions from *Scr*. Similarly, constructs were built replacing the *Scr* core elements with those from *ftz*. However, none altered AE1-*ftz* and T1-*Scr* specificity (data not shown). These results are in contrast to previous studies where TFIID recognition elements direct enhancer targeting (13, 22) and suggest specificity is mediated by elements outside of the core promoter.

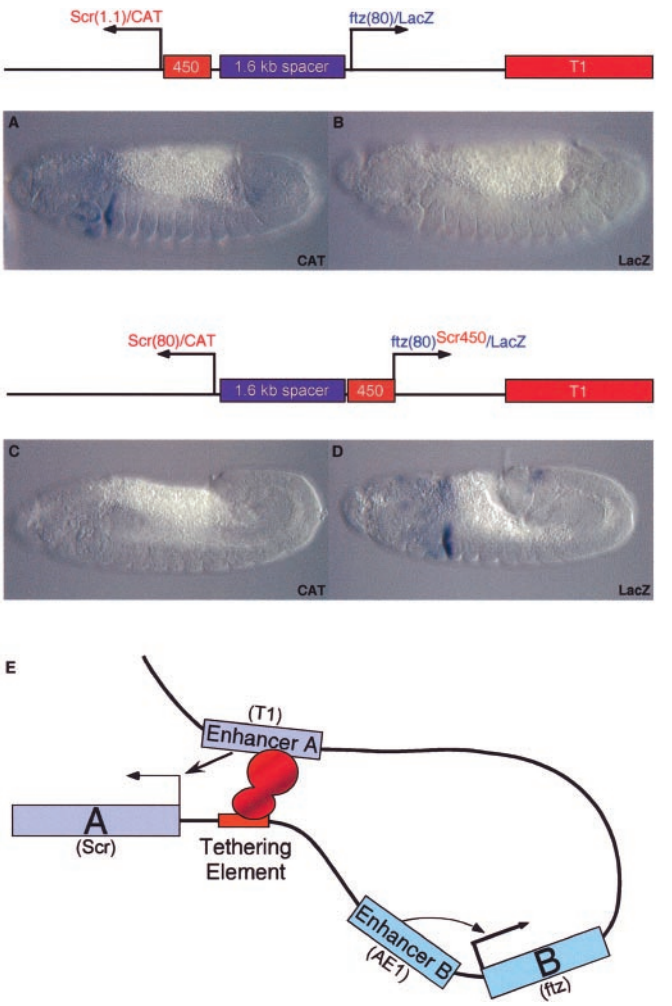

Fig. 4. *Scr* tethering elements mediate long-range enhancer–promoter interactions. Transgenic embryos express the *P* element minigenes indicated in the diagrams. In all cases, the T1 enhancer was placed 3′ of the *lacZ* reporter gene. Minimal *Scr* and *ftz* promoters are not activated by T1, which now maps ≈5 kb from the *ftz* promoter and more than 6 kb from *Scr*. The 3′ T1 enhancer activates the distal *Scr/CAT* reporter gene when 5′ tethering elements are included in the *Scr* promoter region (*A*). The proximal *ftz/lacZ* gene remains silent (*B*). Removal of the 5′ tethering elements from the *Scr* promoter region causes a loss in *CAT* expression (*C*). However, when the *Scr*450 fragment is placed 5′ of the *ftz* promoter, the 3′ T1 enhancer now activates the proximal *lacZ* reporter gene (*D*). *E* summarizes the model for enhancer tethering, whereby promoter-proximal elements selectively recruit a specific distal enhancer (Enhancer A, T1). Perhaps regulatory proteins bound to both the distal enhancer and the proximal tethering elements form homomeric complexes that stabilize enhancer-promoter loops. Enhancer B (AE1) is regulated through promoter competition, selectively interacting with the stronger promoter.

A series of truncated *Scr* promoter sequences were analyzed in an effort to identify the region(s) responsible for activation by T1 (Fig. 2). The most minimal *Scr* promoter identified that exhibited normal activation by T1 is just 530 bp in length. It includes a core 80-bp *Scr* promoter and a 450-bp DNA fragment from the 5′ flanking region (*Scr*80 + 450; see Fig. 2*D*). Only weak staining is obtained with the *Scr*700 promoter sequence (Fig. 2*B*), which lacks the 5′ 450-bp DNA fragment ("*Scr*450"), whereas a minimal core promoter ("*Scr*80") exhibits no T1 expression (Fig. 2*C*).

To determine whether *Scr*450 is sufficient to recruit the T1 enhancer to a heterologous promoter, *Scr*450 was placed upstream of a core *ftz* promoter that includes just 5 bp 5′ of TATA and extends 5 bp downstream of the DPE. The T1 enhancer activates this promoter and directs strong expression of the rightward *ftz-lacZ* reporter gene within the prothorax (Fig. 2*F*; compare with Fig. 2*E*). This observation suggests that *Scr*450 can selectively recruit the T1 enhancer to an adjacent promoter. AE1-*ftz* interactions are not augmented by the presence of *Scr*450 (see below).

To determine whether a regulatory swap in the activities of the AE1 and T1 enhancers can be achieved, *Scr*450 was removed from the *Scr* promoter, and the core elements were modified within *Scr*80 to include a consensus TATA element. The T1 enhancer no longer activates this promoter. Instead, it is efficiently activated by AE1 (Fig. 3*C*). The leftward *Scr-CAT* gene exhibits seven pair-rule stripes of expression, and the rightward *ftz-lacZ* gene, which contains *Scr*450, is expressed in the prothorax and posterior head segments (Fig. 3*D*). As mentioned earlier, the addition of TATA alone is not sufficient for AE1 activation of *Scr*. In this particular configuration of enhancers, T1 is located closer to the *ftz* promoter than AE1. When T1 is removed from this construct, AE1 activation of Scr^{TATA} is greatly diminished, suggesting that the T1 enhancer might possess slight enhancer blocking activity (data not shown). This blocking activity, coupled with the strengthening of *Scr* by inserting an optimal TATA element, permits AE1 to activate the *Scr/CAT* reporter gene. This result reinforces the idea that AE1 works through promoter competition (13). The enhancer blocking activity of T1 serves to weaken the *ftz* promoter, whereas adding TATA makes *Scr* stronger. The regulatory swap is nearly complete. T1 specificity is inverted; only expression from *ftz/lacZ* is observed. The modifications above, however, do not permit a complete swap in AE1 activity, as both promoters are activated by AE1 (data not shown).

As discussed earlier, the T1 enhancer is located ≈25 kb 5′ of the *Scr* promoter. Additional experiments were done to determine whether *Scr*450 can recruit T1 over long distances to different target promoters (Fig. 4). T1 was placed downstream of divergently transcribed *CAT* and *lacZ* reporter genes that contain minimal *Scr* and *ftz* core promoters, respectively. The two promoters were separated by a 1.6-kb spacer DNA from bacteriophage λ. The T1 enhancer fails to activate either the distal *CAT* gene or the proximal *ftz-lacZ* gene (Fig. 4 *B* and *C*). In this configuration, T1 maps ≈6 kb 5′ of the *Scr-CAT* reporter gene. Modification of the distal reporter gene to include the *Scr*450-bp fragment results in strong activation of *Scr-CAT* within posterior head segments and the prothorax (Fig. 4*A*). The proximal *ftz-lacZ* gene remains silent (Fig. 4*B*). However, insertion of *Scr*450 5′ of the *ftz-lacZ* reporter gene results in the selective activation of *lacZ* expression in the prothorax (Fig. 4*D*). Preliminary results show that long-range tethering can be achieved with the *Scr*80 + 450 promoter, similar to that seen for *Scr*1.1 (data not shown). These results suggest that *Scr*450 is sufficient to recruit the distal T1 enhancer to a target promoter.

We propose that the promoter-proximal *Scr*450 DNA fragment contains tethering elements that specifically recruit the distal T1 enhancer to the *Scr* promoter but do not influence the activities of other enhancers located in the ANT-C, such as AE1 (summarized in Fig. 4*E*). It is conceivable that specific T1-*Scr*450 interactions depend on the homotypic association of common proteins bound to both the enhancer and promoter-proximal DNA. A similar mechanism might be used by other complex loci. For example, GATA and other transcription factors are known to bind within the distal locus-control region (LCR) as well as promoter-proximal regions of globin genes (23, 24). Perhaps GATA–GATA interactions facilitate long-range interactions between enhancers contained within the LCR and globin promoters.

Previous tissue culture assays have identified a number of regulatory proteins that bind to promoter-proximal DNA sequences located just 5′ of the core promoter. Some of these proteins are unable to function over long distances, but instead they stimulate transcription only when bound near the core promoter (25–27). We suggest that these proteins might not function as "classical" activators, which recruit transcription complexes (28). Instead, they might augment transcription indirectly by functioning as tethers for distal enhancers. In this regard, we note that the promoter-proximal protein Sp1 can mediate the formation of DNA loops via homotypic interactions when bound to both distal and promoter-proximal binding sites (29).

Tethering elements represent a flexible and specific mechanism for regulating enhancer–promoter interactions in complex genetic loci. For example, an insulator DNA located between AE1 and the *Scr* promoter would block both the heterologous AE1 enhancer as well as the cognate T1 enhancer. It was recently proposed that core promoters can possess distinct regulatory activities, whereby DPE-containing promoters interact with some enhancers, while TATA-containing promoters interact with others (22, 30). However, this mechanism might not play a critical role in regulating AE1-*ftz* and T1-*Scr* interactions. Genetic studies are consistent with the possibility that tethering elements are used by other *Hox* loci, including the *Drosophila Abd-B* locus (31).

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