The Fab-7 element of the bithorax complex attenuates enhancer–promoter interactions in the *Drosophila* embryo

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Enhancers integrate positive and negative regulatory information to direct localized patterns of gene expression in the *Drosophila* embryo. Here we present evidence for the occurrence of *cis* regulatory elements that control the levels of gene expression by modulating enhancer–promoter interactions. For this purpose we have investigated the *Drosophila* bithorax complex (BX-C) because genetic studies suggest that the BX-C contains as much as 300 kb of *cis* regulatory information. A specialized DNA element, Fab-7, has been proposed to function as a boundary element that separates the *iab-6* and *iab-7* *cis* regulatory regions within the *Abd-B* domain of the BX-C. A 1.2-kb Fab-7 DNA fragment was placed between divergently transcribed *white* and *lacZ* test promoters and challenged with several defined enhancers expressed in the early embryo. These studies suggest that Fab-7 functions as an attenuator, which weakens gene expression by reducing enhancer–promoter interactions. Fab-7 selectively blocks distal enhancers in an orientation-independent fashion, and can function when located far from either the distal enhancer or target promoter. Fab-7 may be related to insulator DNAs, which flank genetic loci and functionally isolate neighboring genes. We propose that specialized DNA elements, such as the Fab-7 attenuator, might play a general role in controlling the levels of gene expression by modulating enhancer–promoter interactions.

[Key Words: *Drosophila*; Fab-7; enhancer; attenuator]

Received August 30, 1996; revised version accepted October 29, 1996.

The *Drosophila* bithorax complex (BX-C) contains three Hox-containing transcription units, which encode homeotic selector proteins that control the patterning of thoracic and abdominal body segments (Lewis 1978; Sanchez-Herrero et al. 1985; Lewis et al. 1995; Martin et al. 1995). One of these, *Abdominal-B* (*Abd-B*), controls the morphogenesis of posterior regions, including parasegments (PS) 10 through 14 (Karch et al. 1985; Tjong et al. 1985; Casanova et al. 1986). The gene is regulated by a series of complex *cis* regulatory regions, *iab-5* through *iab-9*, that restrict expression within specific parasegments (Karch et al. 1985; Celniker et al. 1989, 1990; DeLorenzi and Bienz 1990; Boulet et al. 1991; Sanchez-Herrero 1991; see Fig. 1). For example, the *iab-6* region is thought to contain a series of enhancers that mediate expression within different tissues of PS 11 (e.g., Vazquez et al. 1993).

Transcriptional repression is essential for the establishment and maintenance of parasegment-specific patterns of *Abd-B* expression. Previous studies have identified a 1-kb enhancer, IAB5, that directs expression

within PS 10, PS 12, and PS 14 (Busturia and Bienz 1993). Repression in anterior regions, PS 1 through 9, appears to be initiated by the *gap* proteins hunchback (hb), Kruppel (Kr), and knirps (kni). This repression is thought to be maintained in older embryos (and larvae) by proteins encoded by the *Polycomb* group (*Pc-G*; Wedeen et al. 1986; Busturia and Bienz 1993; Orlando and Paro 1993; Chan et al. 1994; Chiang et al. 1995; Zink and Paro 1995).

It has been proposed that boundary elements separate neighboring *cis* regulatory regions within the *Abd-B* domain (Gyurkovics et al. 1990; Galloni et al. 1993; Karch et al. 1994). These may function in a manner analogous to insulators, which functionally isolate neighboring genes by blocking interactions of *cis* elements with inappropriate target promoters (Kellum and Schedl 1991, 1992; Geyer and Corces 1992; Dorsett 1993). Previous studies have identified two potential boundary elements, Mcp and Fab-7 (Gyurkovics et al. 1990; Galloni et al. 1993; Karch et al. 1994). Mcp resides between *iab-4* and *iab-5*, whereas Fab-7 maps between *iab-6* and *iab-7* (summarized in Fig. 1). Mutations in Fab-7 result in the misregulation of *Abd-B* in PS 11, and a corresponding homeotic transformation of PS 11 tissues (sixth abdominal segment; A6) into PS 12 (A7; Gyurkovics et al. 1990; Galloni et al. 1993). *Abd-B* is normally expressed in an

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Previous studies led to several proposals regarding the nature of Fab-7. Perhaps removal of Fab-7 results in the ectopic activation of *iab-7* enhancers in PS 11 where they are normally silenced by Pc-G repressors (Vazquez et al. 1993). According to this view, Fab-7 separates *iab-6* and *iab-7* into distinct chromatin (or loop) domains. Alternatively, it has been suggested that Fab-7 functions as a silencer element, which attenuates *Abd-B* expression in anterior regions, including PS 11 (Busturia and Bienz 1993). Evidence that Fab-7 is a boundary element is provided by the observation that a transgene inserted between Fab-7 and *iab-7* is activated by *iab-7* enhancers, but not by distal *iab-5* or *iab-6* regulatory elements, including the IAB5 enhancer (Galloni et al. 1993; see Fig. 1). In contrast, the notion that Fab-7 functions as a silencer, or Polycomb response element (PRE), is suggested by the analysis of transgenic fly strains containing a large (3.7-kb) Fab-7 DNA fragment attached to various heterologous enhancers and promoters. Fab-7 was shown to silence a *mini-white* gene and *lacZ* reporter; this silencer activity correlated with the binding of Pc protein to the integrated transgenes (Zink and Paro 1995). Fab-7 also

These results suggest that Fab-7 selectively blocks the interactions of distal enhancers. This block appears to be equally effective whether the 5'-3' orientation of the Fab-7 element is the same as the target gene (Fig. 2D) or is inverted (Fig. 2B). Fab-7 does not appear to block all distal enhancers to the same extent. For example, it at-

Figure 2. Fab-7 attenuates IAB5. Transgenic embryos were hybridized with either a digoxigenin-labeled *white* or *lacZ* antisense RNA probe. Hybridization signals were visualized by histochemical staining using alkaline phosphatase. All embryos are undergoing cellularization, about 150 min postfertilization; they are oriented with anterior to the left and dorsal up. (A) *white* expression pattern directed by the fusion promoter summarized in the diagram below the embryo. The synthetic gene complex contains two divergently transcribed genes, the leftward *white* gene and a rightward *lacZ* reporter gene (driven by a minimal

eve promoter sequence). Two different enhancers were placed between the *white* and *lacZ* transcription units; a 200-bp enhancer that directs expression of *hairy* stripe 1 (H1) as well as the 1-kb IAB5 enhancer from the *iab-5* cis regulatory region of *Abd-B*. The two enhancers are separated by a 1-kb spacer sequence. In this experiment, the spacer was obtained from a matrix attachment region (MAR) of the mouse μ light chain gene. An additive *white* expression pattern is observed consisting of a head stripe (directed by the H1 enhancer) and a band of staining in the presumptive abdomen (directed by IAB5). (B) Same as A except that the spacer sequence was replaced with the 1.2-kb Fab-7 element. The distal IAB5 enhancer is attenuated, so that there is only weak expression of the *white* reporter gene in the abdomen. In contrast, the proximal H1 enhancer is unaffected and directs a strong head stripe. (C) Expression of the rightward *lacZ* gene directed by the same fusion promoter shown in A. As for *white*, the fusion promoter directs an additive staining pattern consisting of a head stripe and abdominal band. (D) Same as C except that the spacer sequence was replaced with the Fab-7 element. The distal H1 enhancer is selectively blocked, so that there is only residual staining in head regions. The IAB5 enhancer continues to direct strong expression in the presumptive abdomen. (E) *lacZ* expression pattern directed by a fusion promoter containing the IAB5 enhancer and the 2xPE mesoderm-specific enhancer. The two enhancers are separated by the μ MAR spacer. An additive pattern is observed consisting of a band of expression in the abdomen (IAB5) as well as ventral staining in the presumptive mesoderm (2xPE). (F) Same as E except that the spacer sequence was replaced with Fab-7. The distal 2xPE enhancer is blocked, so that expression is lost in ventral regions. In contrast, IAB5 continues to direct strong expression in the presumptive abdomen.

tenuates, but does not abolish, interactions of the distal IAB5 enhancer (Fig. 2B), but results in a more complete block of the head stripe enhancer (Fig. 2D), as well as a second heterologous enhancer (2xPE; Jiang et al. 1991), which mediates expression in the presumptive mesoderm (cf. Fig. 2F and E).

Fab-7 can block the rhomboid NEE

Additional fusion promoters were examined to determine the efficacy of Fab-7 attenuator activity. Recent studies suggest that the 330-bp *rhomboid* (*rho*) neuroectoderm enhancer, NEE (Ip et al. 1992), is particularly resistant to blocking by the gypsy insulator (S. Barolo and M. Levine, in prep.; H. Cai and M. Levine, in prep.). A fusion gene that contains the 500-bp *even-skipped* (*eve*) stripe 3 enhancer (Small et al. 1996; Yan et al. 1996) and the *rho* NEE directs an additive staining pattern, including a central segmentation stripe and two lateral neuroectodermal stripes (Fig. 3A,C). Replacing the spacer DNA with two tandem copies of the 1.2-kb Fab-7 element results in the attenuation of NEE-mediated lateral stripes when monitoring the expression of the leftward *white* gene (cf. Fig. 3B and A). In contrast, the rightward *lacZ* promoter shows a selective block in stripe 3 expression (cf. Fig. 3D and C). A single copy of Fab-7 results in a similar, but less complete, block in enhancer–promoter interactions (data not shown). These results suggest that Fab-7 is somewhat less effective than the 340-bp *gypsy* insulator in blocking the NEE (Cai and Levine 1995; H. Cai and M. Levine, in prep.). In this

regard, two copies of Fab-7 appear comparable to one copy of *gypsy*.

Fab-7 works when positioned far from distal enhancers or target promoters

Fab-7 is located far from the *Abd-B* promoter, >35 kb downstream of the transcription start site (see Fig. 1 summary). Additional assays were conducted to determine whether Fab-7 could function when placed at a distance from the *white* and *lacZ* test promoters. As shown previously, the *rho* NEE mediates lateral stripes of gene expression when placed downstream of *lacZ*, >5 kb from the transcription start site (Fig. 3E; Gray and Levine 1996). Fab-7 blocks these lateral stripes when placed between the NEE and 3' end of *lacZ* (cf. Fig. 3F and E). The 5' H1 enhancer is unaffected, again suggesting that Fab-7 selectively blocks distal, not proximal, enhancers.

In an effort to assess Fab-7 function in the context of a more authentic arrangement of regulatory elements (see Fig. 1 summary), we positioned Fab-7 and IAB5 far from the *white* and *lacZ* test promoters. The IAB5 enhancer mediates efficient abdominal expression from both promoters when placed downstream of *lacZ* (Fig. 4A, C, and E). Fab-7 blocks these interactions when inserted between the IAB5 enhancer and 3' end of *lacZ* (cf. Fig. 4B and D with A and C, respectively). This block is comparable to that observed when both Fab-7 and IAB5 are located 5' of *lacZ* (see Fig. 2B). These results suggest that Fab-7 works equally well in blocking distal enhancers

Figure 3. Fab-7 attenuates heterologous enhancers. Transgenic embryos were stained and oriented as described in Fig. 2. (A) *white* expression pattern directed by a fusion promoter that contains the 500-bp *eve* stripe 3 enhancer and a 330-bp *rhomboid* (*rho*) lateral stripe enhancer (NEE). The two enhancers are separated by a 2.5-kb DNA fragment derived from the λ bacteriophage. This spacer sequence does not affect either enhancer, so that *white* is expressed in an additive pattern consisting of stripe 3 and lateral stripes. (B) Same as A except that the λ spacer sequence was replaced by two tandem copies of the 1.2-kb Fab-7 element. The *rho* NEE is selectively attenuated, so that only residual lateral stripes are observed. In contrast, the proximal stripe 3 enhancer is unaffected. (C) *lacZ* staining pattern directed by the same fusion gene as in A. The λ spacer does not affect either the distal stripe 3 enhancer or the proximal NEE, so that an additive staining pattern is observed. (D) Same as C except that the λ spacer was replaced with two copies of Fab-7. The distal stripe 3 enhancer is attenuated, but the proximal NEE is unaffected so that strong lateral stripes are observed. (E) *lacZ* staining pattern directed by the 200-bp *hairy* H1 enhancer and the *rho* NEE. The two enhancers were placed at opposite ends of the *lacZ* transcription unit, with H1 5' of *lacZ* and the NEE downstream of the gene. The 1-kb mouse μ MAR sequence was inserted between the 3' end of *lacZ* and the distal NEE. An additive staining pattern is observed, consisting of a head stripe (H1) and lateral stripes (NEE). (F) Same as E except that the 3' spacer sequence was replaced with one copy of the Fab-7 element. The distal NEE is attenuated, so that the lateral stripes are weak. In contrast, the 5'-H1 enhancer is unaffected and continues to direct an intense head stripe.

when located either close to or far from the target promoter. Additional assays suggest that Fab-7 also mediates efficient attenuator activity when located far from the distal enhancer. For example, Fab-7 blocks IAB5-*white* interactions when located between the *white* and *lacZ* test promoters (cf. Fig. 4F and E).

Discussion

We have presented evidence that Fab-7 functions as an attenuator that selectively impedes the interaction of distal enhancers with target promoters. These results raise

the possibility that specialized DNA elements, such as attenuators, may be normal constituents of complex genetic loci. It has been proposed that Fab-7 functions as a boundary element or insulator, which separates *iab-6* and *iab-7* so that *iab-6* enhancers are not ectopically activated in PS 12 and *iab-7* enhancers are not activated in PS 11 (Galloni et al. 1993; Vazquez et al. 1993; Karch et al. 1994). Our studies do not exclude this model, but suggest that an additional function of Fab-7 is the quantitative modulation of IAB5-*Abd-B* interactions. This ensures that only low levels of *Abd-B* are expressed in anterior regions (PS 10) of the presumptive abdomen.

Figure 4. The Fab-7 insulator works when far from either the target promoter or distal enhancer. Transgenic embryos were stained and oriented as described in Figure 2. (A) *white* expression driven by the *hairy* H1 and IAB5 enhancers. The H1 enhancer is located 5' of both the *white* and *lacZ* transcription units whereas IAB5 is positioned downstream of *lacZ*. The μ MAR spacer sequence was placed between the 3' end of *lacZ* and the distal IAB5 enhancer. An additive *white* expression pattern is observed, consisting of a head stripe (H1) and a band of staining in the presumptive abdomen. (B) Same as A except that the 3' spacer sequence was replaced with the Fab-7 element. Expression from the IAB5 enhancer is blocked, but the 5'-H1 enhancer is unaffected so that *white* continues to be expressed in the limits of a head stripe. (C) *lacZ* expression driven by the H1 and IAB5 enhancers. This is the same fusion gene as in A, except that the rightward *lacZ* gene is being monitored. Strong staining is observed in both anterior (H1) and posterior (IAB5) regions. (D) Same as C except that the spacer was replaced with Fab-7. The distal IAB5 enhancer is blocked, so that expression is directed solely by the 5' H1 enhancer. (E) *white* expression pattern directed by the H1 and IAB5 enhancers. The fusion gene being analyzed is a variant of that shown in A-D. The H1 enhancer is 5', the IAB5 enhancer is 3' of *lacZ*, but the spacer sequence is positioned between the 5'-H1 enhancer and the *lacZ* transcription start site. An additive *white* expression pattern is observed consisting of the anterior head stripe (H1) and expression in the presumptive abdomen (IAB5). (F) Same as E except that the 5' spacer sequence was replaced with Fab-7. The distant IAB5 enhancer (now located ~5 kb from Fab-7) is selectively attenuated, so that only residual staining is observed in the abdomen. In contrast, the proximal H1 enhancer continues to direct *white* expression within the limits of a head stripe.

Mechanism of Fab-7 activity

Fab-7 may function in a manner analogous to insulator DNAs, including *scs*, *scs'*, and the *su(Hw)* insulator contained within the *gypsy* retrotransposon (Kellum and Schedl 1991, 1992; Geyer and Corces 1992; Dorsett 1993). *scs* and *scs'* flank the *hsp70* locus and are thought to prevent the heat induction of neighboring genes (Kellum and Schedl 1991). Previous transgenic expression assays have shown that these insulators can also block the interactions of distal enhancers with a target promoter in the context of synthetic gene complexes (Cai and Levine 1995; Scott and Geyer 1995).

At least two models can be envisioned for the Fab-7 attenuator, as summarized in Figure 5. We favor the notion that Fab-7 functions as an autonomous *cis* regulatory element that blocks the looping and/or scanning of distal enhancers to the target promoter (Fig. 5A). Perhaps Fab-7 mimics the core promoter by containing sites for looping proteins, which are required for long-range interactions of a distal enhancer with the promoter. Another way that Fab-7 might mimic the core promoter is by bending DNA. Fab-7 binding proteins might induce structural changes similar to those mediated by the TFIID complex on the core promoter (Oelgeschlager et al. 1996).

A second type of model invokes the organization of chromatin into separate nuclear domains or loops. For example, Fab-7 may work in conjunction with other boundary elements in the BX-C to organize *iab-6* and *iab-7* into separate domains (Fig. 5B; Vazquez et al. 1993). This would prevent enhancer hitchhiking, whereby interactions between *iab-6* enhancers and the *Abd-B* promoter would cause fortuitous interactions be-

tween *iab-7* enhancers and the promoter. In addition, separate loop domains would block cross-enhancer interactions whereby activators bound to *iab-6*, such as those encoded by the *trithorax* group, would interfere with Pc-G repressors in *iab-7*, resulting in the ectopic activation of *iab-7* enhancers in PS 11 (Vasquez et al. 1993). It is conceivable that Fab-7 associates with endogenous insulators flanking the integrated transgene to form loop domains (Corces 1995). The two models depicted in Figure 5 are not mutually exclusive in that the formation of loop domains might induce structural changes in Fab-7, similar to those detected at the core promoter (Oelgeschlager et al. 1996).

Multiple modes of Fab-7 regulation

Fab-7 plays an essential role in restricting *Abd-B* expression to posterior parasegments (Busturia and Bienz 1993; Galloni et al. 1993). It is conceivable that the full-length Fab-7 element functions as both an attenuator and PRE. As discussed earlier, a 3.7-kb DNA fragment that encompasses the core Fab-7 element was shown to repress a *bxd-Ubx* fusion promoter in a Pc-G-dependent fashion (Busturia and Bienz 1993). This fragment was also found to silence the expression of a mini-*white* gene and bind Pc proteins on polytene chromosomes (Zink and Paro 1995). Fab-7 mutations might disrupt a PRE that normally attenuates *Abd-B* expression in PS 11 and other anterior parasegments.

We cannot exclude the possibility that the core Fab-7 element resides near a PRE. However, several observations suggest that attenuator activity does not depend on Pc-G proteins. First, we have demonstrated that Fab-7 works as an attenuator in precellular embryos, which is well before the time when PREs silence the expression of homeotic selector genes in anterior parasegments (this usually occurs after germ-band elongation; Bienz and Muller 1995; Paro 1995). Second, Fab-7 activity is not affected by mutations in Pc-G genes, including *Pc* and the maternally expressed *extra sex combs* (*esc*) gene (J. Zhou, unpubl.). Finally, a well-characterized PRE, the 1.6-kb fragment from the *pbx* region of the *Ubx* domain (Zhang and Bienz 1992; Chan et al. 1994), does not function as an attenuator when placed between the H1 and IAB5 enhancers (P. Szymanski, unpubl.). These results indicate that attenuation and PRE-mediated silencing represent separate functions.

The demonstration that Fab-7 functions as an attenuator suggests that it can impede interactions between distal enhancers, such as IAB5, and the *Abd-B* promoter. In principle, IAB5 has the capacity to direct strong expression in PS 10, where *Abd-B* is normally expressed at low levels (Busturia and Bienz 1993). Fab-7 might be required to ensure weak expression in this region. It has been proposed that there may be another boundary element located between *iab-5* and *iab-6* (Vasquez et al. 1993). Redundancy of Fab-7 and a second attenuator could explain why Fab-7 mutants do not exhibit obvious homeotic transformations of PS 10 (A5) tissues (Gyurkovics et al. 1990; Galloni et al. 1993).

Figure 5. Models for Fab-7 function. (A) We propose that Fab-7 functions as an autonomous *cis* regulatory element that selectively blocks or impedes the interactions of distal enhancers with a target promoter. The mechanism for this directional repression is unknown, but it is possible that Fab-7 might bind looping proteins required for the interaction of distal enhancers with a target promoter. (B) It has been suggested that Fab-7 interacts with other boundary elements (X) to form a chromatin loop domain that separates the *iab-6* *cis* regulatory region from *iab-7*.

We propose that specialized DNAs such as Fab-7 might be relatively common constituents of complex genetic loci. Metazoan promoter regions can be modular, and contain a series of nonoverlapping enhancers (e.g., Goto et al. 1989; Harding et al. 1989; Howard and Struhl 1990; Riddihough and Ish-Horowicz 1991). These enhancers may be interlaced with specialized DNA elements. In the simplest view, enhancers integrate positive and negative regulatory information to determine the spatial limits of gene expression. Specialized DNAs, such as attenuators, might control the levels of expression by quantitatively modulating enhancer–promoter interactions.

Materials and methods

P-element transformation and in situ hybridization

P-element transformation vectors containing *lacZ* and *white* reporter genes were introduced into the *Drosophila* germline by injection of *yw67* embryos as described in Small et al. (1992). In situ hybridizations were performed as described (Tautz and Pfeifle 1989; Jiang et al. 1991), using digoxigenin–UTP-labeled antisense RNA to *lacZ* or *white*. Multiple transgenic lines for each construct were generated, and four to six independent lines were independently stained.

Construction of *lacZ* reporter genes

The 1.2-kb Fab-7 DNA fragment was obtained by PCR amplification from *Drosophila* genomic DNA. *Bam*HI restriction sites were introduced into the primers. A final *Bam*HI fragment was recovered that spans nucleotide positions 84,594 to 83,423 within the complete BX-C sequence (Lewis et al. 1995; Martin et al. 1995). A 1-kb MAR spacer sequence was also obtained by PCR amplification using primers homologous to the 3' matrix attachment region near the 3' intronic enhancer of the Ig μ chain gene (Forrester et al. 1994). The 2.5-kb λ spacer was obtained by digesting total λ genomic DNA with *Bst*YI.

The fusion genes shown in Figures 2A–D were prepared by inserting a 200-bp *Spe*I–*Bam*HI fragment from the *hairy* stripe 1 enhancer (kindly provided by G. Struhl, Columbia University, New York, NY) into the C4PLZ vector (see Cai and Levine 1995). A 1-kb *Sal*I DNA fragment from the IAB5 enhancer (Busturia and Bienz 1993) was then inserted in the *Sph*I site, located between the *hairy* stripe 1 enhancer and *lacZ* transcription unit. The 1-kb spacer or the 1.2-kb Fab-7 element was subsequently cloned in the unique *Bam*HI site between the *hairy* stripe 1 and IAB5 enhancers.

The fusion genes shown in Figures 2E and F were prepared by replacing the stripe 1 enhancer with a 500-bp *Not*I–*Bam*HI fragment containing two copies of the proximal enhancer from the *twist* promoter region (2xPE; see Jiang et al. 1991).

The *eve* stripe 3-*rho* NEE fusion gene shown in Figures 3A–D was prepared by inserting a 500-bp *eve* stripe 3 enhancer (Small et al. 1993) into the *Xba*I site of the pBluescript II SK plasmid vector (Stratagene). Subsequently, a 330-bp *Sty*I–*Xho*I DNA fragment containing the NEE *rho* enhancer (Ip et al. 1992) was cloned into the *Eco*RI site. The enhancers were excised as a *Not*I–*Eco*RV fragment and ligated into the *Eco*RI site of the –42 pCaSpeR vector (Small et al. 1992). Either one or two tandem copies of the Fab-7 element was inserted in the *Bam*HI site located between the *eve* stripe 3 and NEE enhancers.

The fusion genes shown in Figures 3E and F were prepared by inserting the 200-bp *Spe*I–*Bam*HI *hairy* stripe 1 enhancer into

the C4PLZ vector. A 700-bp (full-length) *rho* NEE was inserted into a *Stu*I site at the 3' of the *lacZ* transcription unit. The MAR spacer sequence or the Fab-7 fragment was inserted in a unique *Bgl*II site located between the distal NEE and 3' end of *lacZ*.

The fusion genes shown in Figures 4A–D were prepared by inserting the 1-kb IAB5 enhancer in the 3' *Stu*I site in place of the *rho* NEE. Finally, those shown in Figures 4E and F were prepared by inserting either the spacer or Fab-7 fragment into the unique *Bam*HI site located 5' of the *white* and *lacZ* transcription units.

Acknowledgments

We thank Mariann Bienz for providing IAB5 Fab-7 DNA, and Gary Struhl for his fine unpublished work on the *hairy* stripe 1 enhancer. We thank David Arnosti, Bill McGinnis, and Kees Murre for critically reading the manuscript. We also thank Liezelle de la Pena for technical assistance, and Christian Burks for help with computational analyses. This work was funded by a grant from the National Institutes of Health (GM34431) and done in part under the auspices of the Department of Energy with funds from the UC/LANL CULAR program (UCDRD #9624).

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Note added in proof

Similar results have been obtained by Hagstrom et al. (this issue).

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Genes Dev. 1996, **10**:

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