The role of specific enhancer-promoter interactions in the *Drosophila Adh* promoter switch

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The *Drosophila melanogaster* alcohol dehydrogenase (*Adh*) gene is transcribed from two promoters active at different developmental stages. In this paper we show that the promoters are differentially stimulated by two enhancers, the *Adh* larval enhancer and the *Adh* adult enhancer. In early larval stages, the larval enhancer stimulates transcription from the proximal promoter; in late larval stages, the two enhancers act synergistically to stimulate transcription from the distal promoter; and in adults, the adult enhancer stimulates transcription from the distal promoter. To determine the basis for these enhancer-promoter interactions, we examined the effect of each enhancer on three different promoters. We found that the adult enhancer is stage specific and stimulates transcription from all three promoters. In contrast, the larval enhancer is potentially active in all stages and stimulates transcription from only two of the three promoters. These observations suggest that normal temporal expression of *Adh* depends on the stage-specific activity of the adult enhancer and the differential response of the proximal and distal promoters to the larval enhancer.

*Key Words:* Development; transcription regulation; gene expression; P element; tandem promoters

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Alternative promoters are sometimes used to express the same gene product at different stages of development or in different cell types (for review, see Schibler and Sierra 1987). For example, the *Drosophila melanogaster* alcohol dehydrogenase (*Adh*) gene is transcribed from two promoters that are active during different stages of *Drosophila* development (Benyajati et al. 1983; Savakis et al. 1986). The proximal promoter is active in embryogenesis and early larval development, is switched off during the late third-instar larval stage, and is switched back on at a low level in adults [Fig. 1A,B]. In contrast, the distal promoter is off during early larval development and is switched on briefly from late third-instar larval through early pupal stages. Both promoters are virtually inactive during the rest of pupal development. Just prior to eclosion, however, transcription from the distal promoter increases rapidly and remains at high levels throughout adult life. The same pattern of promoter switching occurs when a cloned 11.8-kb chromosomal DNA fragment containing the *Adh* gene is introduced into the *Drosophila* germ line (Goldberg et al. 1983).

Addional germ line transformation studies have identified two regulatory elements necessary for correct *Adh* gene expression during development (Posakony et al. 1985). The first, called the larval enhancer, is required for maximal levels of *Adh* expression in larvae and was localized between −600 and −69 bp of the distal promoter (Posakony et al. 1985). The second, called the adult enhancer, is required for maximal levels of *Adh* expression in adults and was localized between −660 and −69 bp of the distal promoter (Posakony et al. 1985).

In this paper, we show that the two *Adh* enhancers differ from one another in two important ways. First, the adult enhancer is stage specific, whereas the larval enhancer is not. When linked to a 'neutral' hsp70 promoter, the adult enhancer stimulates transcription only in third-instar larvae and adults. In contrast, the larval enhancer stimulates transcription in all developmental stages examined. Second, the adult enhancer can stimulate transcription from both *Adh* promoters (Posakony et al. 1985) and the hsp70 promoter, whereas the larval enhancer cannot directly stimulate transcription from the distal promoter. In a previous study we showed that the (downstream) proximal promoter is turned off in stages where the (upstream) distal promoter is active by transcriptional interference (Corbin and Maniatis 1989). Taken together, our results suggest that the stage-specific expression of the *Adh* promoters is controlled by the temporal specificity of the adult enhancer and by the differential response of the proximal and distal promoters to the larval enhancer.

**Results**

**Experimental strategy**

We identified the *cis*-acting DNA sequences required for stage-specific transcription from the distal and proximal
The DNA sequence between -5000 and -660 bp of the Adh gene. The sites of transcription initiation from the distal (D) and proximal (P) promoters are marked with arrows. The distal promoter is 713 bp upstream of the proximal promoter, and both transcripts encode the same protein product. The polyadenylation signal, AATAAA, common to the two transcripts and the translation start and stop codons of the Adh protein-coding region [solid boxes], ATG and TAA, are indicated. The structures of the distal and proximal transcripts are sketched below the diagram. Introns are marked by indentations. (B) The approximate levels of proximal [dotted line] and distal [solid line] Adh transcripts are plotted as a function of developmental stage (from Savakis et al. 1986). Adh mRNA concentrations are given in arbitrary units.

Adh promoters by analyzing the effects of deletions or rearrangements on the expression of cloned Adh genes in transgenic flies. These genes were introduced into wild-type Adh embryos (ry\textsuperscript{606}) or Adh null embryos (Adh\textsuperscript{null}, cn; ry\textsuperscript{606}). Insertions that arose from injections in the ry\textsuperscript{606} strain were subsequently crossed into the Adh null background [see Methods; Rubin and Spradling 1982; Spradling and Rubin 1982]. We quantitated Adh transcripts from the two promoters of the introduced genes at various developmental stages by RNase protection experiments [Zinn et al. 1983]. The hybridization probes were designed to distinguish between transcripts from the introduced genes and those from the recipient Adh\textsuperscript{null} genes [Fig. 3C].

Identification of the larval enhancer

The DNA sequence between -5000 and -660 bp of the distal promoter contains one or more regulatory elements necessary for wild-type Adh expression in larvae, but not in adults [Posakony et al. 1985]. To study the role of this upstream sequence in more detail, we analyzed its effect on Adh expression at different developmental stages (for diagram of constructs, see Fig. 2). The presence of the upstream sequence resulted in a small [twofold] increase in the level of maternal Adh transcripts [Fig. 3A, lanes 1 and 2; 0- to 4-hr embryos]. However, in 4- to 16-hr embryos, when transcription is zygotic, the upstream sequence had little effect on transcription from either the proximal or distal promoter [Fig. 3A, lanes 3–8]. From late embryogenesis through the third larval instar, the upstream sequence significantly stimulated transcription from the proximal promoter [Fig. 3A, lanes 11 and 12; Fig. 3B, lanes 1–6]. In third-instar larvae and early pupae, the upstream sequence also enhanced transcription from the distal promoter [Fig. 3B, lanes 5–8]. In adults, however, the upstream sequence did not stimulate transcription detectably from either promoter (Fig. 3B, lanes 9 and 10). Thus, the combined effect of the upstream sequence on both Adh promoters is to stimulate transcription from late embryogenesis through larval/early pupal development.

To test whether the -5000-bp to -660-bp sequence is a transcriptional enhancer, we placed it at the 3' end of the Adh gene or in the reverse orientation at the 5' end of the gene (See Fig. 2). Transformants carrying these altered genes produced wild-type levels of Adh transcripts in larvae [Fig. 4, lanes 1–7] and in adults [Fig. 4, lanes 8–12]. To determine whether the upstream sequence could stimulate transcription from a heterologous promoter, we placed it upstream of a truncated hsp70 promoter which, in turn, was linked to the Adh-coding region [see Fig. 2]. In third-instar larvae, the upstream sequence stimulated transcription from the hsp70 promoter ~30-fold relative to controls [Fig. 5, lanes 5 and 6; for discussion of other time points, see below]. Thus, the -5000-bp to -660-bp sequence has properties characteristic of a transcriptional enhancer: It stimulates transcription in an orientation- and distance-independent fashion and from a heterologous promoter. We refer to this enhancer as the Adh larval enhancer, because it is required primarily in larvae. Our attempts to localize the larval enhancer show that it consists of at least two elements located between -5000 bp and -1845 bp [Corbin and Maniatis 1990].

Stage-specific activity of the enhancers

Sequences downstream of -660 bp are sufficient for wild-type transcription of Adh in adults [see above and Posakony et al. 1985] and contain the Adh adult enhancer (D. Falb and T. Maniatis, unpubl.). To determine whether either the larval or adult enhancer is stage specific outside the context of the Adh gene, we linked each enhancer to a truncated hsp70 promoter. The transcription pattern of these fusion genes should reflect the specificity of the enhancer, as the truncated promoter lacks tissue- and temporal-specific control elements [Lis et al. 1983; Garabedian et al. 1986; Hiromi and Gehring 1987; Fischer and Maniatis 1988].
**Figure 2. Structure of mutant Adh genes.** The structure of mutant genes [not to scale] used to identify and characterize Adh control regions is shown. The box labeled ALE (Adh larval enhancer) includes sequences between two XbaI sites at 660 bp and at approximately −5000 bp, except in genes ALE/hs/ADH, pD−660/−320, pD−660/−128, and pD−660/−60, where the ALE includes sequences between −4700 and −660 bp. The box labeled AAE (Adh adult enhancer) includes sequences from −660 to −128 bp. The 3′ end points of the modified Adh genes lie 640 or 1300 bp downstream of the polyadenylation signal. The presence or absence of sequences between 640 and 1300 bp, 3′ of the polyadenylation site, does not appear to affect expression from the introduced Adh genes (J. Posakony and T. Maniatis, unpubl.). The 5′ end points of D−5000 and pD−60 lie −5000 and 660 bp upstream of the distal promoter, respectively. In FLIP, the ALE is inverted relative to the direction of Adh transcription. In 3′ALE, the ALE is inverted and placed at the 3′ end of the coding region, 640 bp downstream of the polyadenylation site. The fusion gene ALE/hs/ADH contains the ALE linked to the hsp70 promoter (−68 to +198 bp) which, in turn, is linked to the Adh-coding region at +9 relative to the proximal promoter initiation site. The truncated hsp70 promoter contains one copy of the heat shock consensus sequence but is not induced by heat treatments (see Dudler and Travers 1984; Corbin 1989). The hs/ADH fusion gene was described previously [Fischer and Maniatis 1988] and contains −68 to +198 bp of the hsp70 gene fused to the Adh-coding region at +9 of the proximal promoter. The AAE/hs/lacZ gene contains, from 5′ to 3′, Adh sequences from −660 to −128 bp of the distal promoter and a truncated hsp70 promoter [from −43 to +265 bp of the transcription initiation site], fused in frame to the bacterial lacZ gene at amino acid 9 of the protein-coding sequence. AAE/hs/lacZ transformants were a gift from D. Falb. The ALE/P−386, D−660/−320, D−660/−128, and D−660/−60 genes lack sequences between −660 bp of the distal promoter and either −386 bp of the proximal promoter or −320, −128, or −60 bp of the distal promoter, respectively. ALE/P−386 and D−660/−60 were described previously [Corbin and Maniatis 1989]. The D−320, D−128, and D−60 genes lack sequences upstream of −320, −128, and −60 bp, respectively. All of the Adh inserts were cloned into the transformation vector, Carnegie 20, except pFLIP, which was cloned into the transformation vector pPI [a gift of J. Posakony]. The Adh deletion genes were inserted into the P element in the opposite orientation of the rosY [ry] gene, whereas the fusion genes were inserted into the P element in the same orientation as the ry gene. In most cases, several independent transformed lines were analyzed for each introduced gene: D−5000, 5 lines; D−660, 4 lines; FLIP, 2 lines; ALE/P−386, 2 lines; ALE/hs/ADH, 2 lines; hs/ADH1, 2 lines; AAE/hs/lacZ, 2 lines; ALE/P−386, 6 lines; D−660/−320, 3 lines; D−660/−128, 3 lines; D−660/−60, 3 lines; D−320, 3 lines; D−128, 3 lines, and D−60, 3 lines. Transcription from a given Adh construct did not generally vary by more than 3-fold between independent transformed lines, however, one D−5000 line produced levels of transcripts −12-fold lower than the average [data not shown]. This variability is presumably caused by the influence of nearby sequences or chromatin structure [Spradling and Rubin 1983].

Transcriptional analysis of the fusion genes showed that the adult enhancer is stage specific [Fig. 6]. In first-instar larvae, the adult enhancer did not stimulate transcription from the hsp70 promoter [lane 1]; in second-instar larvae, it stimulated transcription very weakly [lane 2; a faint band is visible after prolonged exposure but cannot be seen in this reproduction]; and in third-instar larvae it stimulated transcription to relatively high levels [lane 3]. The adult enhancer did not stimulate transcription in pupae, [lanes 4 and 5], but it stimulated transcription to very high levels in adults [lanes 6 and 7]. These data suggest that the transcription factors that regulate the adult enhancer are themselves activated in a stage-specific fashion. Note that the peaks in activity of the fusion gene coincide with the peaks in activity of the distal Adh promoter [Fig. 3B]. This correlation suggests that the adult enhancer could stimulate transcription from the distal promoter in third-instar larvae, as well as in adults.

In contrast to the adult enhancer, the larval enhancer stimulated transcription from an hsp70 promoter at all developmental stages examined [Fig. 5]—albeit to different levels at different stages [for further details, see legend to Fig. 5]. This result is surprising because the larval enhancer stimulates transcription only in embryonic and larval stages in its normal context [Fig. 3A,B]. These results show that the ability of an enhancer to stimulate transcription can be influenced by the linked promoter. We therefore examined the effect of each enhancer on transcription from each Adh promoter at various developmental stages [see below].

**The larval enhancer and proximal promoter are sufficient for wild-type transcription in early larval stages**

Adh transcripts initiate exclusively at the proximal promoter in early larval stages [Fig. 1B; Savakis et al. 1986].
Figure 3. The -5000-bp to -660-bp sequence stimulates transcription from both the distal and proximal promoters in larvae. (A) Preparations of total nucleic acids from embryos of representative lines transformed with pD-660 (-lanes) or D-5000 (+ lanes) were analyzed by quantitative RNase mapping (see Methods). Embryos of the D-660.1 and D-5000.7 transformant lines were collected at 4-hr intervals, as indicated above the lanes. The 0- to 4-hr collections measure maternal RNA, whereas the remaining collections measure zygotic RNA levels [Savakis et al. 1986]. The 20- to 24-hr preparations contained some newly hatched first-instar larvae, as expected in our culture conditions. Each sample was hybridized simultaneously to the 32P-labeled, single-stranded RNA probes, SP6αTUB and SP6MEL. The SP6αTUB probe is complementary to α-tubulin transcripts and protects a band of ~70 nucleotides from RNase digestion. The SP6MEL probe is complementary to Adh transcripts and is described in C. Following RNase digestion, the products were fractionated on a 5% denaturing polyacrylamide gel and visualized by autoradiography. Transcripts from the introduced Adh genes are marked by solid arrows, whereas transcripts from the Adh\textsuperscript{f6} gene of the recipient line are marked by shaded arrows (see C). Transcripts from the distal promoter of the Adh\textsuperscript{f6} gene are too faint to detect in the exposure shown. Similar data were obtained from independent lines transformed with the same P-element construct. (B) Preparations of total nucleic acids from larvae, pupae, and adults of representative lines transformed with D-5000 (+ lanes) or pD-660 (-lanes) were analyzed by quantitative RNase mapping, as in A, except that only the SP6MEL probe was used [described in C]. Preparations were from D-5000.7 and D-660.1 transformants during the following stages: first instar [lanes 1 and 2], second instar [lanes 3 and 4], mid-third instar [lanes 5 and 6], pupae [lanes 7 and 8], and adult males [lanes 9 and 10]. Similar data were obtained from independent lines transformed with the same P-element construct. (C) Expected products from RNase mapping experiments using the Adh-specific probe, SP6MEL. The transcribed portion of the Adh\textsuperscript{f} gene is shown. Solid boxes indicate exons; open boxes, introns. The extent of the complementary single-stranded RNA probe, SP6MEL, is indicated by the shaded bar. The two bracketed drawings show the RNase-resistant hybrids that form between the probe and the proximal and distal transcripts of the transformed (Adh\textsuperscript{f}) and endogenous (Adh\textsuperscript{f6}) Adh genes. Transcripts from the Adh\textsuperscript{f} gene give rise to three fragments after RNase digestion: one of 320 nucleotides, common to transcripts from the proximal and distal promoters; one of 168 nucleotides, specific to transcripts from the proximal promoter; and one of 134 nucleotides, specific to transcripts from the distal promoter. Transcripts from the endogenous Adh\textsuperscript{f6} gene are not spliced properly due to a 4-bp substitution plus 6-bp deletion near the end of the first coding exon and are unstable, present at only 5–10% the steady-state level of wild-type Adh transcripts [Benyajati et al. 1982]. Transcripts from the distal Adh\textsuperscript{f6} promoter yield fragments of 135 and 372 nucleotides; transcripts from the proximal promoter, fragments of 169 and 372 nucleotides. For simplicity, most of the figures presented in this paper show only a subset of the fragments protected with this probe.
Our results with the hsp70 promoter constructs suggest that the proximal promoter is stimulated entirely by the larval enhancer at these stages (Figs. 5 and 6). We showed that the larval enhancer and proximal promoter are sufficient for wild-type Adh transcription in larvae, by fusing the larval enhancer to the proximal promoter at -386 bp (see Fig. 2). In larvae transformed with the resulting constructs, the proximal promoter was active at wild-type levels (Fig. 7, cf. lanes 1–5, with 6–7).

**The larval enhancer does not act directly on the distal promoter in larvae**

Our experiments with the hsp70 promoter showed that both enhancers are active in third-instar larvae (Figs. 6 and 7). As shown above, the larval enhancer is required for wild-type transcription from the distal promoter at this stage, because transcription levels decrease when the larval enhancer is deleted (Fig. 3B, lanes 5 and 6). To test whether it is sufficient to stimulate the distal promoter, we placed the larval enhancer directly upstream of the TATA box at -60 bp, see Fig. 2). This construct did not produce detectable amounts of transcription from the distal promoter in third-instar larvae (Fig. 8, lanes 6–10), suggesting that additional regulatory elements are required. To localize these additional elements, we placed the larval enhancer at -128 and -320 bp of the distal promoter. The addition of these sequences did not increase transcription from the distal promoter (lanes 11–20). Thus, in third-instar larvae, sequences between -320 and -660 bp are necessary for wild-type transcription from the distal promoter. These data suggest that the adult enhancer and/or a closely associated upstream promoter element acts synergistically with the larval enhancer to stimulate transcription from the distal promoter in larvae.
The larval enhancer does not act on the distal promoter in adults

The larval enhancer stimulates transcription in adults when placed immediately upstream of either the hsp70 or proximal Adh promoters (Fig. 5, Posakony et al. 1985). We wondered whether the larval enhancer could also stimulate transcription from the distal promoter in adults. The larval enhancer is not required in adults (Fig. 3B), but its ability to stimulate the distal promoter may be masked if the adult enhancer is sufficient for maximal transcription. To test this possibility, we deleted the adult enhancer and examined the effect of the larval enhancer on transcription from the distal promoter.

Transcription from the distal promoter decreased when the adult enhancer was deleted, as predicted (Fig. 9). Placing the larval enhancer upstream of these truncated genes did not increase the transcription levels (Fig. 9, cf. lanes 13–15 with 16–18; 7–9 with 10–12; 1–3 with 4–6). Thus, the larval enhancer cannot substitute for the adult enhancer in adults. Taken together with the observation that the larval enhancer is not sufficient to stimulate the distal promoter in third-instar larvae, these data suggest that the larval enhancer does not act directly on the distal promoter at any stage of development.

Discussion

We have shown that stage-specific Adh expression is regulated by interactions between two promoters and two enhancers. The enhancers differ from one another in two important respects. First, the adult enhancer is active only in late larvae and adults, whereas the larval enhancer is potentially active in all stages. Second, the adult enhancer can directly stimulate transcription from both Adh promoters, whereas the larval enhancer cannot.

At present, we do not understand why the larval enhancer fails to stimulate the distal promoter. The simplest explanation is that the proteins bound at the distal
promoter. First, the hsp70 and proximal Adh promoters, which both respond to the larval enhancer, do not appear to share any common regulatory sequences other than the TATA box motif, TATAAAATA. In contrast, the nonresponsive distal promoter contains a different TATA box motif, TATTTAA. Second, the distal promoter remains unresponsive to the larval enhancer even when upstream promoter sequences are added, whereas the proximal Adh promoter remains responsive even when promoter sequences upstream of -80 are deleted. Furthermore, the larval enhancer also fails to stimulate transcription from a truncated sgs-3 promoter (TATA box sequence TATAAAAAG), whereas control enhancers do stimulate transcription (V. Corbin and R. Cohen, unpubl.). These data suggest that the TATA box, rather than an upstream promoter element, is important for interactions between the larval enhancer and the linked promoter. We therefore propose that different TATA factors bind to the proximal and distal Adh promoters or that the same factors bind differently to the two TATA motifs. In either case, only the proximal promoter factor(s) can interact with the factors bound at the larval enhancer.

This interpretation is supported by two different kinds of observations. First, in vitro DNA binding studies suggest that enhancer and TATA-binding proteins interact. In the presence of enhancer-binding proteins, the transcription factor IID (TFIID) binds more extensively to the TATA box (Sawadogo and Roeder 1985; Horikoshi et al. 1988a, b). Second, in vivo studies suggest that certain classes of TATA- and enhancer-binding proteins cannot

promoter cannot interact functionally with the proteins bound at the larval enhancer. Several lines of evidence suggest that the restrictive proteins act, either directly or indirectly, through the TATA box of the distal promoter. First, the hsp70 and proximal Adh promoters, which both respond to the larval enhancer, do not appear to share any common regulatory sequences other than the TATA box motif, TATAAAATA. In contrast, the nonresponsive distal promoter contains a different TATA box motif, TATTTAA. Second, the distal promoter remains unresponsive to the larval enhancer even when upstream promoter sequences are added, whereas the proximal Adh promoter remains responsive even when promoter sequences upstream of -80 are deleted. Furthermore, the larval enhancer also fails to stimulate transcription from a truncated sgs-3 promoter (TATA box sequence TATAAAAAG), whereas control enhancers do stimulate transcription (V. Corbin and R. Cohen, unpubl.). These data suggest that the TATA box, rather than an upstream promoter element, is important for interactions between the larval enhancer and the linked promoter. We therefore propose that different TATA factors bind to the proximal and distal Adh promoters or that the same factors bind differently to the two TATA motifs. In either case, only the proximal promoter factor(s) can interact with the factors bound at the larval enhancer.

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interact. For example, certain combinations of TATA motifs and enhancer elements fail to stimulate transcription in yeast [Chen and Struhl 1989]. Moreover, the adenovirus E1A gene product selectively stimulates mammalian and viral promoters containing a specific TATA box sequence [Simon et al. 1988]. Of the enhancer–promoter combinations tested here, only the larval enhancer/distal promoter combination seems unable to stimulate transcription. Thus, in the context of the wild-type Adh gene, the larval enhancer stimulates only the proximal Adh promoter, leaving the distal promoter dependent on the (stage-specific) adult enhancer for stimulation.

An alternative explanation for our results is that both enhancers can stimulate transcription from the distal promoter but that the larval enhancer failed to do so because the distal promoter lacked an essential upstream promoter element in our constructs. This hypothetical element would facilitate interactions between the distal promoter and any enhancer and, together with the distal promoter TATA box, would provide a functional target for enhancer interactions. Because the adult enhancer can stimulate the distal promoter when sequences between −387 and −128 bp are deleted [U. Heberlein and R. Tjian, pers. comm.], this element would map upstream of −387 and downstream of the adult enhancer (currently localized between −660 and −450 bp; D. Falb and T. Maniatis, unpubl.). By this view, the distal and proximal promoter TATA boxes again appear to be functionally distinct, because the proximal, but not the distal, TATA box is apparently sufficient to respond to the larval enhancer.

Another explanation for our results is that the larval enhancer preferentially interacts with the proximal promoter and therefore fails to interact with the distal promoter in our constructs. Such competition for an enhancer occurs in the chicken ε-globin locus, where an enhancer located between the adult β-globin and the embryonic ε-globin genes preferentially interacts with the β-globin promoter in adults [Choi and Engel 1988]. Although an analogous mechanism cannot be ruled out for the Adh locus, we note that in the constructs examined here, the proximal promoter is active only at very low levels in adults, probably because low levels of readthrough transcription from the distal promoter prevent the proximal promoter from becoming fully active [Corbin and Maniatis 1989]. Under these circumstances, it seems unlikely that the proximal promoter could effectively compete with the distal promoter.

A model for Adh promoter switching

A model for the stage-specific regulation of the two Adh promoters is presented in Figure 10. During larval development, transcription from the proximal promoter is stimulated by interactions between factors bound to the larval enhancer and to elements near the transcription start site. These factors presumably interact through a DNA looping mechanism [Ptashne 1986]. The distal promoter is quiescent at this stage, probably because the transcription factors that bind to the adult enhancer are absent or inactive.

In late third-instar larvae, transcription from the distal promoter is stimulated by the synergistic action of both enhancers. We propose that the larval enhancer stimulates transcription from the distal promoter indirectly at this stage, by acting through the adult enhancer. For example, factors bound at the larval enhancer could facilitate the binding of factors to the adult enhancer. The latter factors would then interact directly with the
Promoter is severely curtailed by transcription interference. Just prior to eclosion, the activity of the distal promoter continues to increase and remains high throughout adult life. Curiously, the larval enhancer is not required for normal levels of transcription from either promoter at this stage. The adult enhancer apparently stimulates the distal promoter to maximal levels without the help of the larval enhancer. Possibly the larval enhancer is not required at this stage because the adult enhancer factors are more active or abundant in adults than in late third-instar larvae. As in late third-instar larvae, the activity of the proximal promoter is reduced to low levels by transcription interference (Corbin and Maniatis 1989).

In conclusion, we propose that the developmental switch from the proximal to distal promoter is controlled by three parameters: (1) the differential abilities of the two promoters to respond to the larval enhancer; (2) the stage-specific activity of the adult enhancer; and (3) transcriptional interference. Thus, both the inherent properties of Adh control elements and their arrangement are important components of the Adh promoter switch.

**Methods**

*Establishment of transformed Drosophila lines*

Transformation vectors and the helper plasmid pp25.7wc [Kasress and Rubin 1984] were injected into ryso6 or Adheso, cr; ryso6 embryos, using standard procedures (Rubin and Spradling 1982); Spradling and Rubin 1982; Goldberg et al. 1983). Insertions arising from injections into ryso6 embryos were crossed into the Adhso, cr; ryso6 background, as described [Fischer and Maniatis 1988]. Chromosomal linkages were assigned and homozygous lines were selected as described [Goldberg et al. 1983; Fischer and Maniatis 1988]. Most lines discussed in the paper are homozygous. Insertions that were homozygous lethal were kept over the balancer chromosome, TM2 [Lindsley and Grell 1968].

**Transformation vectors**

All Adh fragments originated from plasmid sAF2, which contains the Adh allele and flanking DNA (Goldberg 1980). Plasmids were constructed using standard methods (Maniatis et al. 1982) and are described briefly below. All of the modified Adh genes shown in Figure 2, except pFLIP, were cloned into Carnegie 20 [Rubin and Spradling 1983]. The basic structure of the modified Adh genes is shown in Figure 2. pD−660 was made by inserting an Xhol linker into the Xbal site at +2510 [relative to the distal start site] in clone sAF2 and ligating the 3.2-kb Xbal–Xhol fragment into the Xbal and Sall sites of pC20X, a derivative of Carnegie 20 [Rubin and Spradling 1983]. pD−660 thus contains Adh sequences from −660 to +2510 bp, relative to the distal cap site and has a unique Xbal site at −660 bp. pD−5000 was made by inserting the 4.4-kb Xbal fragment of sAF2 [containing sequences between approximately −5000 and −660 bp] into the Xbal site of pD−660 and screening for insertions in the wild-type orientation. The plasmid pF-386 was made by inserting an Xhol linker into the Hpal site of pD−660, cutting with Xbal, and ligating under dilute conditions to delete Adh sequences 5’ to −386 of the proximal promoter. The 4.4-kb Xbal fragment was inserted into the
Corbin and Maniatis

XhoI site of pP - 386 to make pALE/p - 386, which harbors an internal deletion for Adh sequences between -660 bp of the distal promoter and -386 bp of the proximal promoter.

The transformation vectors pD - 320, pD - 128, and pD - 60 were made by ligating the DraI-XhoI, FspI-XhoI, and SalI-XhoI fragments, which contain Adh sequences from -320, -128, and -60 to about +3100, respectively, into the P-element vector, Carnegie 20 or a simple derivative. To make the plasmids pD - 660-320, pD - 660/-128, and pD - 660/-60, the DraI-XhoI, FspI-XhoI, and SalI-XhoI fragments described above were subcloned into pSP73 (provided by D. Melton). The inserts were then isolated as BglII-XhoI fragments and ligated with Carnegie 20 (cut with HpaI and SalI) and a 4.1-kb HincII-BamHI fragment containing the larval enhancer (sequences from about -4800 to -660) from p4.4. Plasmid p4.4 was made by inserting the 4.4-kb XhoI fragment of sAF2, which contains the larval enhancer, into the XhoI site of pSP64.

Plasmid R688X was made by inserting a BglII linker into the Asp718 site of R68HX (a gift of J. Fischer), which contains the hsp70 gene from -68 to +198 bp, fused to the Adh-coding region at +9 of the proximal transcript. Adh sequences extended ~1300 bp past the polyadenylation site. Plasmid pALE/hsp/ADH was made by ligating the BglII-XhoI fragment of R688X to the 4.1-kb HincII-BamHI fragment of p4.4 and the 10.7-kb HpaI-SalI-digested Carnegie 20 vector. The AAE/hsp/lacZ transformants were a gift of D. Falb. The hsp/ADH transformants were a gift of J. Fischer (Fischer and Maniatis 1986). Both are described in the legend to Figure 2.

pFLIP was made by modifying pTARP, which contains the 11.8-kb SacI fragment of AdhP1 within the P-element vector pPL-1 (both gifts of J. Posakony). First, pTARP was partially digested with XbaI and ligated to the -5000 to -660 bp Adh fragment, such that the -5000 to -660 bp fragment was inserted at +2510 in the 3' to 5' direction relative to the remainder of the Adh gene. Next, the ry* gene, contained on an 8.1-kb SalI-cutter genomic DNA fragment, was ligated into the unique XhoI site to give pFLIP.

RNA analysis

Total nucleic acid was purified from staged Drosophila embryos, larvae, pupae, and adults, as described (Fischer and Maniatis 1986). Embryos were collected every 4 hr and allowed to age at 25°C for varying times. Most of the embryos were of the correct developmental stage, as judged by bright-field microscopy. Larvae were loosely staged by collecting embryos for ~12 hr and letting them age for the appropriate amount of time: First-instar larvae were collected 36 ± 6 hr after egg laying (ael), second instar, 53 ± 6 hr ael; third instar, 78 ± 6 hr ael. For experiments where carefully staged third-instar larvae were needed, larvae were isolated <4 hr after the second- to third-instar molt and allowed to age for the appropriate amount of time, as indicated in the figure legends. Adult samples were collected 4 days after eclosion.

Quantitative RNase mapping experiments were carried out as described [Zinn et al. 1983], except that hybridizations were performed at 37°C without prior incubation at 85°C, and RNase digestions were carried out at 25°C for 30 min.

Hybridization probes

Continuously labeled, single-stranded RNA probes were prepared as described [Melton et al. 1984], except that BSA was added to a concentration of 60 mg/ml. The plasmids pSP6MEL and SP6-hsplac were provided by J. Fischer (Fischer and Maniatis 1986; Fischer et al. 1988). The plasmid pSP6βTUB was made by inserting an ~400-bp XhoI-SalI fragment of genomic DNA from pDMTe1 (a gift of P. Wensink) into the XhoI and SalI sites of pSP65.

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V Corbin and T Maniatis

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