Regulation of *Drosophila Adh* promoter switching by an initiator-targeted repression mechanism

Bing Ren and Tom Maniatis¹

Department of Molecular and Cellular Biology, Harvard University,
7 Divinity Avenue, Cambridge, MA 02138, USA

¹Corresponding author

The stage-specific expression of the *Drosophila* alcohol dehydrogenase (*Adh*) gene is achieved through the alternate activation of two tandem promoters. The proximal promoter is active primarily during late embryonic development and early larval stages, while the distal promoter is active in late third instar larvae and adults. Here, we provide evidence that this *Adh* promoter switch is regulated by a zinc finger repressor protein (AEF-1) that is expressed predominantly in adult flies and targets the initiator region of the proximal promoter. We propose that AEF-1 plays a critical role in *Adh* promoter switching by blocking interactions between a component of the general transcription machinery and the initiator region of the proximal promoter.

Keywords: *Adh*/AEF-1/core promoter/initiator/repression

Introduction

Expression of the *Drosophila Adh* gene is regulated in a temporal and tissue-specific manner, and this is accomplished in part by a promoter switch (Benyajati *et al.*, 1983). As shown in Figure 1, the *Adh* gene has two tandem promoters. The distal promoter is active in late third instar larvae and adults, and the proximal promoter is active during embryogenesis and early larval development. These two promoters are turned on during development by distinct transcriptional enhancers: the distal promoter by the *Adh* adult enhancer (AAE) and the proximal promoter by the *Adh* larval enhancer (ALE) (Corbin and Maniatis, 1989a,b, 1990). The AAE is active in third instar larvae and adults, while the ALE is active at all stages of development. Because of the persistent activity of the ALE, the proximal promoter must be repressed in adult flies.

At present, the mechanism by which the proximal promoter is repressed in adults is not understood. A promoter occlusion model was proposed, based on an analysis of the effects of rearrangements and deletions in the *Adh* regulatory region on promoter switching in transgenic flies (Corbin and Maniatis, 1989b). According to this model, transcription initiated from the upstream distal promoter in adults interferes with transcription from the downstream proximal promoter. More recently, Hansen and Tjian (1995) proposed that the *Adh* promoter switch is a consequence of differential recognition of the distal and proximal initiator elements by the TATA box-binding

protein (TBP)-associated factor 150 (TAF_{II} 150) and the general transcription factor TFIIA. This model is based on experiments showing that the distal promoter is more active than the proximal promoter *in vitro*, and that this is the result of a difference in the initiator (Inr) elements of the two promoters. However, this model was not tested directly *in vivo*, and the mechanism by which the proximal promoter is switched off in adults was not addressed.

In the course of studying a *Drosophila* transcriptional repressor protein AEF-1 (adult enhancer factor 1), we obtained evidence that the Inr element of the proximal promoter is a target for negative control by AEF-1. The AEF-1 protein was first identified as a DNA-binding factor that recognizes a region in the AAE (Falb and Maniatis, 1992b). Mutations in the AEF-1-binding site of the AAE resulted in a 5- to 10-fold increase in transcription from the *Adh* distal promoter in adult transgenic flies. In addition, *in vitro* binding studies showed that AEF-1 can block the binding of a potent transcriptional activator protein (*Drosophila* C/EBP) to the AAE (Falb and Maniatis, 1992a). The level of AEF-1 mRNA is highest in adult flies but low in late embryo and larval stages. Thus, one function of AEF-1 in adult flies is to decrease but not shut off the activity of the AAE. AEF-1-binding sites have been found in the enhancers of *Adh* genes of other strains of *Drosophila*, in the human *Adh* gene and in the *Drosophila* YP1 gene (Falb and Maniatis, 1992a). Thus, the role of the AEF-1 DNA element in regulating enhancer activity is highly conserved in evolution.

Here, we present evidence that AEF-1 also plays a key role in regulating the activity of the proximal promoter. Specifically, we show that AEF-1 binds with high affinity to the Inr region of the proximal promoter, and inhibits transcription both *in vivo* and *in vitro*. We also demonstrate that mutations in the proximal promoter that disrupt AEF-1 binding result in elevated proximal promoter activity in adult flies. Finally, when the Inr elements of the distal and proximal promoters were exchanged, the activity of the proximal promoter increased in adults while that of the distal promoter decreased. Thus, *Adh* proximal promoter activity in adult flies is modulated by AEF-1 through an initiator-targeted repression mechanism.

Results

AEF-1 binds specifically to the initiator region of the *Adh* proximal promoter

Previous studies of AEF-1 enhancer interactions established a DNA-binding site consensus sequence and identified nucleotides critical for binding (Figure 2A; Falb and Maniatis, 1992a,b; An and Wensink, 1995). We noticed that the Inr region of the proximal promoter bears significant sequence similarity to the AEF-1-binding site consensus sequence. In fact, as shown by gel mobility shift experi-

ments, recombinant AEF-1 binds with high affinity to the *Adh* proximal promoter (Figure 2B). The binding constant (K_d) of the AEF-1–proximal promoter interaction is $\sim 2 \times 10^{-10}$ M, compared with 1×10^{-10} M for the AEF-1–AAE interaction. By contrast, the affinity of AEF-1 for the distal promoter is 1000-fold less (Figure 2B). DNase I footprinting experiments with the proximal promoter revealed a 15 bp binding site located between –5 and +10 nucleotides from the transcription initiation site (Figure 2C; compare lanes 5 and 6 with lane 4). This footprint is not observed when base substitutions that deviate from the AEF-1 consensus sequence are introduced into this

site (lanes 1–3). We conclude that the binding of AEF-1 to the proximal initiator sequence is highly specific. Inr elements have been shown to be in close contact with TFIID, RNA polymerase II and other general transcription factors in the pre-initiation complex of many eukaryotic gene promoters (Roeder, 1996; Nikolov and Burley, 1997). Thus, when AEF-1 binds to the Inr element, it could sterically interfere with transcription initiation. In fact, as we will show, AEF-1 can specifically repress transcription from the *Adh* proximal promoter.

Repression of the *Adh* proximal promoter by AEF-1

The inhibitory effect of AEF-1 on transcription was tested *in vivo* by transfecting *Drosophila* S2 cells with an AEF-1 expression plasmid, a Gal4 expression vector and various reporter plasmids containing five Gal4-binding sites upstream of a CAT reporter gene. When a CAT reporter driven by a minimal *Adh* distal promoter was transfected into S2 cells, a low level of repression was observed by co-transfected AEF-1 (Figure 3A, lanes 1–4). This repression, which was detected only at high levels of AEF-1 (lane 4), could be the consequence of low affinity binding of AEF-1 to the distal promoter (Figure 2B) or

Fig. 1. Schematic diagram of the structure of the *D.melanogaster* *Adh* regulatory DNA sequences. ALE, *Adh* larvae enhancer; AAE, *Adh* adult enhancer; D, distal promoter; P, proximal promoter.

Fig. 2. AEF-1 binds specifically to the initiator (Inr) region of the *Adh* proximal promoter. (A) Previously characterized AEF-1-binding sites and the *Adh* proximal initiator element (pie) sequence are listed. D.me, *Drosophila melanogaster*; D.mu, *Drosophila mulleri*. The shaded area indicates the consensus nucleotides necessary for AEF-1 binding. (B) Gel mobility shift assay showing the binding of AEF-1 protein to the *Adh* proximal promoter but not to the distal promoter. Radiolabeled DNA probes containing the *Adh* distal promoter (AdhD) or the proximal promoter (AdhP) were incubated with the indicated concentrations of recombinant AEF-1 protein (AEF-1p), and the protein–DNA complexes were separated from the free DNA probes by electrophoresis in a 4% polyacrylamide gel. (C) Footprinting analysis revealing the binding site of AEF-1 on the *Adh* proximal promoter. The AdhP probe used in (B) was incubated with 0 (lane 4) or increasing amounts of recombinant GST–AEF-1 fusion protein (30 ng in lane 5, 100 ng in lane 6), followed by DNase I treatment. The digestion products were resolved on a 6% polyacrylamide–urea gel. The *Adh* proximal promoter sequence is shown at the right, with the sequence that is protected by AEF-1 protein underlined. The TATA box is denoted by a box, the transcription start site by an arrow. Nucleotide substitutions (A→T) are made at the indicated positions to prepare a probe, AdhP(m). This probe was used in lanes 1–3 as control.

to non-specific effects due to overexpression of AEF-1. By contrast, when the proximal promoter was tested in a similar manner, significant repression was observed at the lowest level of transfected AEF-1 tested (Figure 3A, lanes 5–8). This effect was not observed when the proximal promoter contained mutations that prevent AEF-1 binding (Figure 3A, lanes 9–12).

AEF-1 repression of the proximal promoter was also observed in nuclear extracts from S2 cells (Figure 3B). Recombinant AEF-1 significantly decreased transcription

from the proximal, but not the distal, promoter (lanes 1–5), and no repression was observed with the mutant proximal promoter (lanes 6–8). Notably, these mutations also increased the expression level from the proximal promoter, suggesting the presence of low levels of AEF-1 in S2 cells (Figure 3A; compare lanes 5 and 9). AEF-1 not only represses activated transcription from the proximal promoter, but it also inhibits the basal level of transcription from this promoter to the same extent (B.Ren and T.Maniatis, unpublished data).

Fig. 3. AEF-1 specifically inhibits transcription from the *Adh* proximal promoter *in vivo* and *in vitro*. **(A)** S2 cells were transfected with the indicated reporter plasmids, a Gal4 expression vector and increasing amounts of AEF-1 expression vector. The reporter plasmids contain the bacterial CAT gene fused to an *Adh* distal promoter (AdhD), a proximal promoter (AdhP) or the proximal promoter with mutations [AdhP(m)], placed downstream of multiple Gal4-binding sites. In these and the following transfection experiments, the S2 cells were also co-transfected with a plasmid containing hsp82 promoter-driven *LacZ* as an internal control. At 48 h post-transfection, the cells were harvested for β -gal assay and CAT assay. The result from a typical thin-layer chromatography is shown. The fold repression of each reporter by co-transfected AEF-1 plasmid is listed at the bottom (the CAT activity of the control transfections, i.e. in lanes 1, 5 and 9, are set as 1). **(B)** *In vitro* transcription assay demonstrating the effect of recombinant GST-AEF-1 fusion protein on the *Adh* proximal promoter activity. The indicated DNA templates were pre-incubated with Gal4AH protein and 0 or increasing concentrations of GST-AEF-1 fusion protein for 15 min at room temperature. NTPs and S2 nuclear extract were then added and the reactions were allowed to continue for 1 h. The transcripts were analyzed by primer extension. The AdhD and AdhP transcripts are indicated by arrows to the left of the panel. At the bottom, the fold AEF-1 repression observed with each template is listed (the levels of transcription in reactions 1 and 6 are set as 1).

Binding of AEF-1 to the proximal Inr region is necessary and sufficient for repression

The results above show that AEF-1 binding to the proximal Inr region represses transcription. To prove that DNA binding is required for AEF-1 inhibition, *in vitro* transcription reactions were carried out in the presence of DNA oligonucleotide competitors. As shown in Figure 4A, a strong AEF-1-binding site oligo (AEF-1) efficiently relieved AEF-1 repression of the proximal promoter (compare lanes 5 and 7). By contrast, an oligo corresponding to the distal initiator element (DIE), which has a low affinity for AEF-1 (data not shown), did not affect repression by AEF-1 (lane 4). Surprisingly, a DNA oligo containing the sequence of the proximal initiator element (PIE) blocked transcription from the normal start site of the proximal promoter, and activated a cryptic downstream start site which is not repressed by AEF-1 (lanes 2 and 6). This is probably because the PIE oligo titrates away a component of the general transcription machinery. We conclude that AEF-1 binding to the proximal Inr element is required for repression of the proximal promoter.

To identify the functional domains of AEF-1 required for proximal promoter inhibition, we carried out transient transfection experiments with various truncated forms or mutants of AEF-1. In these experiments, a CAT reporter gene driven by a minimal *Adh* proximal promoter downstream of five Gal4-binding sites and a Gal4 expression plasmid are co-transfected into the S2 cells as well as AEF-1 expression vectors. As shown in Figure 4B, the C-terminal DNA-binding domain of AEF-1 is sufficient for maximal levels of repression, and deletions of sequences critical for binding resulted in a loss of repression [AEF-1(f12)]. Similarly, amino acid substitutions in the DNA-binding domain (mAEF-1) that disrupt DNA-binding activity (data not shown) also abolished AEF-1 repression. The N-terminal region of AEF-1 [AEF-1(N)] contains glutamine-rich and alanine-rich regions, and has no effect on reporter gene activity. We conclude that the DNA-binding domain of AEF-1, which contains four Krüppel-like zinc finger motifs, is sufficient for repression of the proximal promoter, consistent with the hypothesis that AEF-1 represses proximal promoter activity by blocking access of the general transcription factors.

This model predicts that if an AEF-1 DNA-binding site is placed in the Inr region of a different promoter, AEF-1-dependent repression would be observed. To test this possibility, a hybrid promoter was constructed by combining the TATA box from the *Adh* distal promoter and the proximal Inr element. When this reporter was co-transfected with the AEF-1 expression plasmid into S2 cells, a 4-fold inhibition by AEF-1 was observed (Figure 4C), which is comparable with the inhibition of the wild-type proximal promoter. In contrast, little or no inhibition was observed with the promoter containing the distal TATA box alone or with the distal Inr element. Thus, the AEF-1-binding site can confer AEF-1-dependent repression on a heterologous promoter.

The Inr of the proximal promoter is required for *Adh* promoter switching

During fly development, the activity of the proximal promoter is first detected in late embryos, increases during early larval development, decreases during the late third

instar larval stage, and is switched off in adults. The decrease in proximal promoter activity is not due to repression of the ALE, since this enhancer is active in third instar larvae and adult flies (Corbin and Maniatis, 1989a). However, this decrease in activity does correlate with an increase in AEF-1 gene expression. Falb and Maniatis (1992a) showed that the level of AEF-1 mRNA is highest in adult flies and early embryos. Moreover, we have detected AEF-1 binding to the *Adh* proximal promoter Inr element with *Drosophila* adult nuclear extracts, but not the larval extracts (data not shown). Thus, it is possible that AEF-1 repression of the proximal promoter plays a critical role in *Adh* promoter switching. To test this possibility, an *Adh* gene sequence including the upstream enhancer sequences (both ALE and AAE) and the distal and proximal promoters was fused to the coding sequences of the *Escherichia coli* *LacZ* gene and introduced into flies by P-element transformation (Figure 5).

The construct designated AdhP contains the wild-type *Adh* promoter sequence, and was used as a control. Mutations that disrupt AEF-1 binding *in vitro* were introduced into the proximal Inr element to create the construct AdhP(m). In addition, the distal and proximal Inr elements were exchanged with each other in the construct designated AdhDsw (distal switch). Each of these constructs was stably introduced into the *Drosophila* germ line by P-element transformation. Total RNA was prepared from first instar larvae (when the AEF-1 mRNA level is low) and from adults (when the AEF-1 mRNA level is high) bearing a single integrated copy of the indicated constructs. To detect the distal and proximal promoter transcripts, three RNA probes (#1, #2 and #3) were designed so that they contain sequences complementary to the proximal promoter in AdhP, AdhP(m) or AdhDsw, and the downstream *LacZ* sequences (Table I). Proximal promoter transcripts from the different transgenes are detected by a 210 nucleotide (nt)-protected RNA fragment. The distal promoter transcripts are detected as spliced (178 nt band) and unspliced forms (274 nt band). The unspliced form was generated from these transgenes, presumably because the *LacZ* sequences replaced a splicing enhancer region located downstream from the *Adh* proximal promoter (Reed, 1996). As a control, RNA prepared from the parental strain yw was hybridized to the same RNA probes. A 44 nt fragment was generated from the endogenous *Adh* distal transcript which is normally spliced at the +33 position from the proximal promoter. Because the RNA probes used here differ in their proximal promoter initiator regions, the protection patterns generated from the endogenous *Adh* proximal promoter transcript are different in size, manifested by a 77 nt band for the probe complementary to wild-type, 71 nt for P(m) and 67 nt for Dsw.

As shown in the histogram in Figure 6A, in the first instar larvae, mutations in the proximal Inr region that prevent AEF-1 binding result in a moderate increase (2- to 5-fold) in proximal promoter expression with both constructs AdhP(m) and AdhDsw. Replacing the initiator element of the proximal promoter with that from the distal promoter does not change the promoter usage in first instar larvae. However, in adult flies, a significant difference was observed in activities of the wild-type and mutant proximal promoters (Figure 6B). While there was relatively little proximal promoter transcript observed with the wild-type

Fig. 4. DNA binding of AEF-1 to the initiator region is both required and sufficient for AEF-1 inhibition. **(A)** The effect of various oligonucleotide competitors on AEF-1 inhibition of the proximal promoter. *In vitro* transcription is carried out under the same conditions as in Figure 3B, with both template DNA AdhD-CAT and AdhP-CAT, that are shown on the left. Recombinant GST-AEF-1 protein and/or an excess of oligonucleotide competitors were included in the reactions when indicated. Arrows indicate the AdhD and AdhP transcripts. **(B)** A bar graph comparing the repression by various forms of AEF-1 on the AdhP reporter. S2 cell transfection experiments were carried out as in Figure 3A, with the AdhP reporter, a Gal4 expression plasmid and various AEF-1 expression vectors. The AEF-1 vectors used are illustrated to the left, including a full-length AEF-1 expression vector (AEF-1), the N-terminal domain of AEF-1 spanning the first 157 amino acid residues [AEF-1(N)], the DNA-binding domain of AEF-1 [AEF-1(C)], a full-length AEF-1 vector with two mutations in the DNA-binding domain (227L→G, 230H→A) (mAEF-1) and a truncated form of AEF-1 containing the first two zinc finger motifs (amino acids 153–253) [AEF-1(fg12)]. The vector pPac was used as control. The CAT activity here and in (C) was calculated by comparing the amount of converted forms with the unconverted form of chloramphenicol, and normalized against the β -gal activity. The averages from five independent transfection experiments are shown. **(C)** AEF-1 inhibition at heterologous promoters. S2 cells were transfected with CAT reporters containing multiple Gal4-binding sites and various synthetic promoters illustrated to the left of the bar graph. The *Adh* distal promoter TATA box (D-TATA) is combined with either the *Adh* distal initiator (D-Inr) or the proximal initiator (P-Inr) to drive CAT gene expression in these reporters. In addition, a Gal4 expression plasmid and either an AEF-1 expression vector (AEF-1) or the pPac plasmid (vector) are co-transfected into the cells.

(AdhP) construct, significant levels (5- to 20-fold increase) of this transcript were observed with the constructs AdhP(m) and AdhDsw (compare the 210 nt band in lanes 6–8 and 10–12 with that in lanes 2–4). By contrast, the

distal promoter activity was only slightly affected in the AdhP(m) construct (compare the 274 nt and 178 nt bands in lanes 6–8 to those in lanes 2–4), and significantly reduced in the construct AdhDsw (lanes 10–12). As a

Fig. 5. Constructs and probes used in the P-element transformation experiments and the quantitative RNase protection analysis. The construct AdhP was made by fusing the wild-type *Adh* promoter sequences including ALE, AAE and the distal and proximal promoters to the bacterial *LacZ* gene. The initiator sequences of the distal and proximal promoters are listed on the top of the graph. The construct AdhP(m) is the same as AdhP except for the underlined base substitutions in the proximal initiator region. In construct AdhDsw, the initiator elements of the proximal and distal promoters were exchanged. These constructs were introduced into the *yw* strain of *D.melanogaster*. The number of transformed lines obtained and examined for each construct is listed to the right of the sequences. Transcripts expected from these transgenes are shown below the construct diagram: the proximal promoter transcript, the unspliced and spliced forms of the distal promoter transcripts.

Table I. Sizes of the RNA transcripts as detected by the RNase protection probes

probes		#1	#2	#3

P-element constructs		AdhP	AdhP(m)	AdhDsw
transgene transcripts	proximal promoter	210nt	210nt	210nt
	distal promoter	274nt 178nt	274nt 178nt	274nt n/a
endogenous <i>Adh</i> gene transcripts	proximal promoter	77nt	71nt	67nt
	distal promoter	44nt	44nt	44nt

result, the ratio between the proximal and distal promoter activity was significantly changed in adult flies with the AdhP(m) or AdhDsw construct (Figure 7). While the activity of the *Adh* proximal promoter in the wild-type construct (AdhP) is 4.4% compared with the distal promoter, the activity increases dramatically to 26% with the construct AdhP(m) ($P < 0.001$), and 102% with AdhDsw ($P < 0.001$). These observations show that the AEF-1 site in the Inr region of the *Adh* proximal promoter is required for repression of this promoter in adult flies. Interestingly, when the initiator of the *Adh* distal promoter was replaced by that of the proximal initiator (AdhDsw construct), the distal promoter activity decreased significantly (compare the 270 nt bands in lanes 10–12 with those in lanes 2–4 in Figure 6B). Thus, AEF-1 inhibition of the

proximal promoter can occur in a different promoter context.

Discussion

The *Drosophila Adh* gene is expressed during all stages of development and in a specific subset of larval and adult tissues. This complex pattern of gene expression requires two distinct transcription enhancers and two promoters. Examined in isolation, the larval enhancer is active in both larvae and adults. By contrast, the adult enhancer is active only in adults. In larvae, the distal promoter is bypassed, possibly because of the presence of a distal promoter-specific repressor. In adults, the combined activities of the ALE and AAE are directed toward the

Fig. 6. The proximal initiator element plays a critical role in the *Adh* promoter switch in adult flies. Quantitative RNase protection assays are performed to examine the effect of mutations or initiator switch in the *Adh* proximal promoter on its activity in first instar larvae and in adult flies. (A) Total RNA prepared from first instar larvae of the *yw* strain or three independent lines of the indicated transgenic flies was hybridized to the radiolabeled RNA probes indicated above the panel. Transcription from the *Adh* proximal promoter but not the distal promoter is observed from both transgenes (210 nt band) and the endogenous *Adh* gene (77, 71 and 67 nt bands). The levels of transcription from each P-element construct in larvae are quantitated by PhosphorImager (Fuji), normalized against the endogenous *Adh* expression, and are shown in the histogram below the panel. (B) As in (A) except that total RNA is prepared from adult flies. Transcription from the *Adh* proximal promoter is much reduced for the endogenous *Adh* gene (not shown) and for the transgene construct P (210 nt band). However, the proximal promoter activity persists in transgenes P(m) and Dsw (the 210 nt band). The levels of proximal promoter transcription are normalized against the amount of endogenous *Adh* distal promoter transcript (44 nt band) and are shown in the histogram below the panel.

distal promoter, while the proximal promoter is silenced. Previous studies demonstrated that repression of the proximal promoter in adults requires an active distal promoter (Corbin and Maniatis, 1989b). As diagrammed in Figure 8A, deletion of the distal promoter results in the activation of the proximal promoter in adults. However, this repression occurs only when the proximal promoter is located downstream from the distal promoter. A proximal promoter inserted upstream from the AAE, and thus not in the path of RNA polymerase initiated at the distal promoter, is active in adults (Figure 8A). Based on these observations, a transcriptional interference model was proposed for *Adh* promoter switching (Corbin and Maniatis, 1989b). In this model, RNA polymerase initiates transcription from the upstream promoter and destabilizes interactions between transcription factors and the downstream promoter (Bateman and Paule, 1988). Although this model is consistent with previous studies, it does not explain how mutations in the proximal Inr element increase the activity of the proximal promoter in adults, nor does it explain the high level of proximal promoter activity in adults when the Inr regions of the two promoters are switched. Thus, transcriptional interference cannot be the only mechanism for *Adh* promoter switching. Similarly, AEF-1 repression

alone is not sufficient for switching, since a proximal promoter introduced upstream from the AAE is active in adults. The proximal promoter must be located downstream from the distal promoter in order to be repressed. Thus, it seems likely that a combination of the two mechanisms is used.

An additional insight into the mechanism is provided by *in vitro* studies showing that the strength of the distal Inr element is significantly greater than that of the proximal Inr element (Hansen and Tjian, 1995). When an *Adh* construct containing the AAE and both the proximal and distal promoters was transcribed in extracts prepared from early stage *Drosophila* embryos, both promoters were active, but more transcription was initiated from the distal promoter. However, when the Inr elements of the two promoters were switched, transcription from the proximal promoter was higher than that observed with the distal promoter. The intrinsic strength of the distal Inr element therefore appears to be significantly greater than that of the proximal element.

Taken together, these studies of *Adh* promoter switching suggest that at least three mechanisms are involved in the preferential activation of the distal promoter in adults. First, the distal promoter is intrinsically stronger than the

Fig. 7. The activity of the proximal promoter in adult flies is significantly enhanced by mutations in the proximal promoter Inr element or by exchanging the Inr elements between the proximal and distal promoters. A bar graph demonstrating the average activity of the proximal promoter in adults of the indicated transgenic flies is shown. The activity was determined by comparing the amount of proximal promoter transcripts (210 nt in Figure 6B) with the amount of distal promoter transcripts (274 and 178 nt bands in Figure 6B) of each line, and averaging the results from all of the transgenic flies transformed with the same construct.

Fig. 8. Summary of the mechanisms that regulate the *Adh* promoter switch in adult flies. (A) The *Adh* proximal promoter activity in adult flies containing various P-element constructs is shown, with 'X' denoting no activity, and an arrow denoting elevated activity. These results were obtained from transgenic fly experiments in previous studies (Corbin and Maniatis, 1989a) and in this study. The open box represents the Inr of the proximal promoter; the gray box, the mutant Inr; the dark box, the Inr of the distal promoter. (B) Three mechanisms contribute to the *Adh* promoter switching: (i) the distal promoter is intrinsically stronger than the proximal promoter due to its strong Inr element; (ii) readthrough from the distal promoter interferes with the proximal promoter activity; and (iii) specific binding of AEF-1 to the proximal promoter Inr inhibits transcription from this promoter.

proximal promoter, leading to preferential use of the distal promoter. Second, transcriptional readthrough from the distal promoter interferes with pre-initiation complex assembly on the proximal promoter. Third, the preferential binding of AEF-1 to the proximal Inr further destabilizes interactions with the pre-initiation complex. Thus, the repression of the proximal promoter in adults may be the consequence of competition between the distal and proximal promoter, which is heavily skewed towards the distal promoter (Figure 8B).

An initiator-targeted repression mechanism

The AEF-1 repressor protein appears to have two different functions in the control of *Adh* gene expression. First, AEF-1 appears to modulate the activity of the AAE by interfering with the binding of a transcriptional activator protein (Falb and Maniatis, 1992a). *In vitro* studies show that AEF-1 binds specifically to a site in the AAE, and the binding of AEF-1 to this site prevents the binding of a C/EBP family activator to an overlapping site. In addition, mutations in the AEF-1-binding site in the AAE result in a 5- to 10-fold increase in distal promoter activity in adult transgenic flies (Falb and Maniatis, 1992a). This activity of AEF-1 appears to be highly conserved in evolution, since overlapping AEF-1- and C/EBP-binding sites are found in the fat body-specific enhancers of distantly related *Drosophila* species as well as in the human *Adh* gene (Falb and Maniatis, 1992a). The AEF-1 protein also plays an important role in regulating the level of *Drosophila* yolk protein gene expression in ovaries (An and Wensink, 1995). In this case, AEF-1 is thought to interfere with the binding of the female-specific doublesex protein and a *Drosophila* C/EBP protein to overlapping binding sites in the yolk protein gene enhancer.

Here we demonstrate a second function of AEF-1, the repression of the proximal promoter in adults. The ability of AEF-1 to bind to the proximal promoter correlates with its ability to repress transcription, and introduction of an AEF-1-binding site into the Inr region of another promoter renders that promoter sensitive to AEF-1 inhibition (Figure 4C). In addition, the binding of AEF-1 to the Inr element of the proximal promoter is sufficient for repression, as evidenced by the fact that repression is observed with only the DNA-binding domain of AEF-1 (Figure 4B). Thus, the mechanism of AEF-1 repression of the proximal promoter is likely to be the steric interference with the binding of general transcription factors to the Inr element.

Other eukaryotic transcription repressors have been found to target other regions of the core promoter. For example, the SV40 tumor antigen is thought to repress transcription from the SV40 early promoter by blocking the access of general transcription factors (Hansen *et al.*, 1981; Tjian, 1981). Similarly, the *Drosophila* P-element transposase (Kaufman and Rio, 1991), the cellular DNA-binding domain protein LBP-1 (Kato *et al.*, 1991) and the bovine papilloma virus E2 protein (Dostatni *et al.*, 1991) are repressors that are thought to interfere with the formation of the pre-initiation complex on DNA. In each case, the repressor prevents TFIID binding to the TATA box. The immediate early protein 2 (IE2) of human cytomegalovirus down-regulates its own expression by a mechanism that involves binding to a negative regulatory element located between the TATA box and the transcrip-

tion start site (Macias and Stinski, 1993; Wu *et al.*, 1993). IE2, however, does not disrupt TFIID complex binding to the promoter. Instead, it selectively impedes RNA polymerase II recruitment (Lee *et al.*, 1996). Finally, c-Myc can repress transcription from the basal promoters of the C/EBP α and albumin genes in a manner dependent on the Inr elements of these genes (Li *et al.*, 1994). However, the mechanism of this c-Myc-mediated repression is not clear. To date, AEF-1 is the only known repressor that acts by binding to the Inr element.

The core promoter in the regulation of gene expression

Core promoters are thought to be interchangeable, and their activities regulated by linked transcriptional enhancer elements. However, recent evidence suggests that core promoters can respond differentially to enhancers (Novina and Roy, 1996, and references therein). For example, the *Adh* distal promoter is not activated by the ALE, even though the ALE can act on the proximal promoter and on heterologous promoters (Corbin and Maniatis, 1989a). In addition, the core promoter of the *Drosophila decapentaplegic* (*dpp*) gene, in combination with its ventral enhancer, is not activated in the blastoderm embryo in response to the dorsal activator, whereas a heterologous hsp70 promoter can be activated by this enhancer in response to Dorsal morphogen (Schwyter *et al.*, 1995).

The distinct architecture of various core promoters may be one important reason for their different responses to different activators. Indeed, in the human U6 snRNA promoter, point mutations in the core promoter elements result in diametrically opposite responses to VP-16 and Sp-1 activation domains. Conversely, small changes in the activation domains can be distinguished at these core promoters (Das *et al.*, 1995). Recently, experiments carried out with yeast mutants that lack certain functional TAF genes have shed light on the molecular basis of the different core promoters. While the transcription of many genes is unaffected by the TAF mutations, some genes, particularly cell cycle-related genes such as CLN1, CLN2 and CLN3, require TAFs for expression. The DNA region that confers this TAF requirement was localized to the core promoter region of these genes through experiments involving a UAS-core promoter chimera between a TAF-independent gene and a TAF-dependent gene (Shen and Green, 1997). Thus, the core promoter appears to have far more regulatory capacity than previously envisaged.

Materials and methods

Plasmid construction and primers

Reporter constructs for S2 cell transfections were made as follows. For the construction of G5dproCAT (AdhD-CAT), a fragment containing sequences from -30 to +53 of *Adh* distal promoter was obtained by PCR and cloned into the *XbaI*-*SacI* site of G5e1bCAT (a gift from Wei Du), replacing the e1b TATA and the adjacent linker sequence. Similarly, a fragment of *Adh* proximal promoter (from -30 to +10) was cloned into the *XbaI*-*SacI* site of G5e1bCAT to construct G5pproCAT (AdhP-CAT). The mppro fragment was made by PCR with a 3' primer containing the indicated mutations in the proximal Inr elements, and then cloned into the *XbaI*-*SacI* site of the G5e1bCAT vector to make G5mpproCAT [AdhP(m)-CAT]. Constructs G5dtCAT, G5dtDieCAT and G5dtpieCAT contain the *Adh* distal TATA box, alone or in combination, of either the distal Inr or proximal Inr, and are used in Figure 4C. To construct G5dtCAT, two DNA oligos (5' GCTCTAGAGTATTTAAG-

GAGTGCGAAG 3' and 5' GCGGATCCTTCGAGCTCCTTAAATA 3') were first annealed and filled in by Klenow (New England Biolabs) at both ends, followed by digestion with *XbaI*-*Bam*HI. The resulting DNA fragment was cloned into the *XbaI*-*Bam*HI site of G5e1bCAT, replacing the e1b TATA box. For G5dtDieCAT, two DNA oligos (die-1, 5' GCGGTACCAAGTCATGCATTATTGTCT 3' and die-2, 5' GCGAGCTCAGACAATAATGCATGACTT 3') were annealed and filled in with Klenow, digested with *KpnI*-*SacI* and cloned into the *Bam*HI-*SacI* site of the G5dtCAT vector. For G5dtpieCAT, two DNA oligos (pie-1, 5' GCGGTACCTGGAAACCAACAATAAC 3' and pie-2, 5' GCGAGCTCGTTAGTTGTTGGTTCAG 3') were similarly processed and cloned into the G5dtCAT vector.

All AEF-1 expression plasmids for S2 cell transfections are in pPac vectors (Abel, 1993). The AEF-1, AEF-1(C) and AEF-1(N) expression vectors were provided by Ted Abel. Mutations in mAEF-1 (L227G, H230A) were obtained through random mutagenesis using PCR and selected based on the lack of DNA-binding ability of its protein product. AEF-1(f12) was constructed by removing the *XbaI*-*MseI* fragment from BsmycAEF-1(C) (plasmid 4364, a gift from Ted Abel), followed by cloning the H3-*NotI* fragment of the resulting vector into the *Bam*HI site of pPac.

Constructs for P-element transformation were made as follows: AdhP was constructed by cloning a 12 kb *Asp*718-*NotI* fragment from BsAdh β -gal (a gift from Ted Miller) into the *Asp*718-*NotI* site of the Caspr4 vector. For the AdhP(m) construct, site-directed mutagenesis was first performed with BsAdh β -gal as template using the MorphTM mutagenesis protocol (5'→3', Inc.) to make Bsmppro. Then the *Asp*718-*NotI* fragment from this plasmid was cloned into the Caspr4 vector. Similarly, AdhDsw was made by cloning the *Asp*718-*NotI* fragment from BsAdhdsw into the Caspr4 vector. The BsAdhdsw vector was constructed in three steps. First, DNA fragments containing the *Adh* distal promoter upstream sequences, the distal promoter or proximal promoter with switched Inr elements were obtained from template BsAdh(Sm) via PCR with three sets of primers: (i) T3 primer and 5' CTTGGACCTTCGAGCTC 3'; (ii) 5' GCGAAGGTCCAAGGAAACCAACAATAACCAGTGCA-GTTGTGTCAG 3' and 5' CAGTTCGTGTCGGCCCGTA 3'; and (iii) 5' CCGACACGAACTGTCATGCATTATTGTCTGGAGCCCTC-TTCCAA 3' and T7 primer. These fragments were annealed together, and the joined DNA fragment was amplified by PCR with T3 and T7 primers. Second, the final PCR product was digested with *Bam*HI and *XbaI*, and the 1.8 kb fragment was cloned into vector BsLacZ (a gift from Ted Miller) to make Bsdsw. Finally, a 4.4 kb *XbaI* fragment from BsAdh β -gal was cloned into the *XbaI* site of Bsdsw in an orientation that is similar to BsAdh β -gal. The orientation and sequences of this final plasmid, BsAdhdsw, were verified by restriction digestion and sequencing.

Recombinant AEF-1 protein production

GST-AEF-1 was purified as described (Abel, 1993). To purify AEF-1 protein, GST-AEF-1 was first dialyzed against factor Xa cleavage buffer (100 mM NaCl, 50 mM Tris-HCl, 1 mM CaCl₂, pH 8.0) overnight at 4°C, followed by complete digestion with a 1:100 ratio of factor Xa (Boehringer Mannheim) at room temperature. The GST protein fragment was removed by incubation with glutathione-agarose beads. The supernatant (containing AEF-1 protein) was concentrated with Bio-Max 10K filter (Ultrafree-15[®], Millipore) to 0.2 mg/ml and dialyzed against buffer A (20 mM HEPES, 100 mM KCl, 10% glycerol, 0.1 mM ZnCl₂, 1 mM β -mercaptoethanol, pH 7.6).

Gel mobility shift assay and DNase I footprinting assay

Approximately 10 fmol of DNA probe was incubated with the indicated amount of proteins in the presence of 200 ng of poly(dI-dC) and competitor DNA oligos as indicated for 15 min at room temperature in a 25 μ l reaction containing 10 mM HEPES-KOH (pH 7.6), 50 mM NaCl, 3 mM MgCl₂, 6 mM β -mercaptoethanol, 10% glycerol and 0.2 mM ZnCl₂. The mixture was then fractionated on a 4% non-denaturing polyacrylamide gel (0.5 $\times$ TBE and 5% glycerol) that was run at 4°C.

The AdhD, AdhP and AdhP(m) probes used in Figure 2B and C were prepared from G5dpro(s)CAT, G5pproCAT or G5mpproCAT with *Hind*III and *Pvu*II digestion, followed by labeling with [³²P]CTP. The correct DNA fragments were purified and isolated on a 4% non-denaturing polyacrylamide gel (1 $\times$ TBE and 5% glycerol).

DNase I footprinting assay was carried out as described (Thanos and Maniatis, 1995).

S2 cell transfections

Transfection of S2 cells was carried out according to the calcium phosphate method (Chen and Okayama, 1988). Cells were grown at

25°C in M3 medium (Sigma) supplemented with 12% fetal bovine serum (FBS) in 6-well dishes and split at 1×10^6 /ml concentration the day before transfection. Each transfection included 0.5 µg of the indicated reporter plasmid, expression plasmids as indicated in the figures, and 0.2 µg of hsp82LacZ. The total amount of DNA was adjusted to 6.0 µg with pPac vector. Forty eight hours after initial exposure to DNA, cell extracts were prepared and CAT enzyme activities were assayed (Gorman *et al.*, 1982). β-Galactosidase assays were performed as a control for transfection efficiency.

In vitro transcription assay

S2 cell nuclear extracts were prepared according to the protocol of Parker and Topol (1984) from 1 l of S2 cells that were grown to 5×10^6 /ml in 10 cm plates at 25°C. The final protein concentration was usually 5–8 mg/ml.

In vitro transcription was carried out in a 30 µl reaction. DNA template (0.5 µg) was pre-incubated with 0.5–1.0 µg of Gal4AH protein (a gift from Josh Brickman) and recombinant AEF-1 proteins when indicated for 15 min at room temperature before NTPs and 15 µl of nuclear extract were added. The final reaction contains 7.5 mM HEPES (pH 7.6), 100 mM potassium glutamate, 5 mM MgCl₂, 0.03 mM EDTA, 1.5 mM dithiothreitol (DTT), 3% glycerol and 0.8 mM each rNTP. After 1 h incubation at 25°C, the reaction was stopped by adding 100 µl of stop buffer (20 mM EDTA pH 8.0, 0.2 M NaCl, 1% SDS, 0.25 mg/ml glycogen) followed by 300 µl of 0.3 M sodium acetate. The mixture was then extracted with phenol/chloroform and precipitated with 2 vols of ethanol. The pellet was dissolved in 10 µl of 1× PE buffer (50 mM Tris, pH 8.3 at 42°C, 50 mM KCl, 10 mM MgCl₂, 10 mM DTT) and incubated with ~50 fmol of end-labeled CAT primer (5' GGACATTGAGCAACTGACTGAAATGCCTC 3') at 55°C for 20 min, followed by 10 min at room temperature. The primer extension reaction containing 1× PE buffer, 1 U of AMV reverse transcriptase (Pharmacia), 1 mM each dNTP, 0.5 mM spermidine and 3 mM sodium pyrophosphate was then assembled. After 30 min at 42°C, an equal volume of loading dye (98% formamide, 10 mM EDTA, 0.01% xylene cyanol, 0.01% bromophenol blue) was added to the reaction, and the products were fractionated on a 6% polyacrylamide/urea gel.

In Figure 4A, the PIE oligo is made by annealing pie-1 and pie-2 together, DIE by die-1 and die-2, and AEF-1 by two oligos (5' GATCCGACCGCAGCAACAACGATCA 3' and 5' GATCTGATCCTGTGTGTCTGGCGGTG 3').

P-element transformation and RNA analysis

Transformation vector AdhP, AdhP(m) or AdhDsw were injected into yw-67c23 strain embryos along with a helper plasmid pp25.7wc (Karess and Rubin, 1984) using standard procedures (Rubin and Spradling, 1982; Spradling and Rubin, 1982; Goldberg *et al.*, 1983). Injected flies were backcrossed individually to the parent strain, and transformants could be distinguished by their red eye color. These were then inbred to obtain homozygous lines.

Total RNA was isolated from the transformants as follows: 100–200 larvae or 50 flies were homogenized with a disposable plastic pestle in Eppendorf tubes in 1 ml of RNAsol B (Tel-Test, Inc.), then 70 µl of chloroform was added and the mixture was shaken vigorously for 15 s and stood on ice for 5 min. After centrifugation at 12 000 g (4°C) for 15 min, the aqueous phase was removed and precipitated with 1 vol. of isopropanol. The pellet was dissolved in 500 µl of distilled water and precipitated again with ethanol. The RNA protection probes were prepared as described (Melton *et al.*, 1984) from plasmid RAPP19, sp72mppro and sp72dsw. RAPP19, provided by Ted Miller, was constructed by cloning a *Sna*BI–*Fsp*I fragment from BsAdhβ-gal into the *Eco*RV site in vector sp73 (Promega). The other two plasmids were constructed similarly from Bsmppro or Bsdsw plasmid and cloned into the sp72 vector (*Sma*I site).

Acknowledgements

We are very grateful to Niranjana Pandey for providing the *Drosophila* larval nuclear extracts and adult nuclear extract. Special thanks go to Niranjana Pandey, Marc Walthelet, Tae Kook Kim, Bhavin Parekh, James Falvo, Ted Miller and other members of the Maniatis group for discussions and critical reading of the manuscript. We also thank Y. Kravtsov, S. Kunes, T. Miller, R. Targ and D. Caron for their technical help in the P-element transformation experiments, and T. Miller, T. Abel, Josh Brickman and W. Du for providing plasmids and proteins. We are also grateful to T. Abel for his contributions to the early stages of this

work. The work was funded by a grant from the National Institutes of Health to T.M.

References

- Abel, T. (1993) Transcription factors that bind to *Drosophila* *Adh* regulatory elements. Ph.D. thesis. Harvard University, Cambridge, MA, USA.
- An, W. and Wensink, P.C. (1995) Integrating sex- and tissue-specific regulation within a single *Drosophila* enhancer. *Genes Dev.*, **9**, 256–266.
- Bateman, E. and Paule, M.R. (1988) Promoter occlusion during ribosomal RNA transcription. *Cell*, **54**, 985–992.
- Benyajati, C., Spoerl, N., Haymerle, H. and Ashburner, M. (1983) The messenger RNA for alcohol dehydrogenase in *Drosophila melanogaster* differs in its 5' end in different developmental stages. *Cell*, **33**, 125–133.
- Chen, C.A. and Okayama, H. (1988) Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques*, **6**, 632–638.
- Corbin, V. and Maniatis, T. (1989a) The role of specific enhancer–promoter interactions in the *Drosophila* *Adh* promoter switch. *Genes Dev.*, **3**, 2191–2120.
- Corbin, V. and Maniatis, T. (1989b) Role of transcriptional interference in the *Drosophila melanogaster* *Adh* promoter switch. *Nature*, **337**, 279–282.
- Corbin, V. and Maniatis, T. (1990) Identification of *cis*-regulatory elements required for larval expression of the *Drosophila melanogaster* alcohol dehydrogenase gene. *Genetics*, **124**, 637–646.
- Das, G., Hinkley, C.S. and Herr, W. (1995) Basal promoter elements as a selective determinant of transcriptional activator function. *Nature*, **374**, 657–660.
- Dostatni, N., Lambert, P.F., Sousa, R., Ham, J., Howley, P.M. and Yaniv, M. (1991) The functional BPV-1 E2 trans-activating protein can act as a repressor by preventing formation of the initiation complex. *Genes Dev.*, **5**, 1657–1671.
- Falb, D. and Maniatis, T. (1992a) A conserved regulatory unit implicated in tissue-specific gene expression in *Drosophila* and man. *Genes Dev.*, **6**, 454–465.
- Falb, D. and Maniatis, T. (1992b) *Drosophila* transcriptional repressor protein that binds specifically to negative control elements in fat body enhancers. *Mol. Cell. Biol.*, **12**, 4093–4103.
- Goldberg, D.A., Posakony, J.W. and Maniatis, T. (1983) Correct developmental expression of a cloned alcohol dehydrogenase gene transduced into the *Drosophila* germ line. *Cell*, **34**, 59–73.
- Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.*, **2**, 1044–1051.
- Hansen, S.K. and Tjian, R. (1995) TAFs and TFIIA mediate differential utilization of the tandem *Adh* promoters. *Cell*, **82**, 565–575.
- Hansen, U., Tenen, D.G., Livingston, D.M. and Sharp, P.A. (1981) T antigen repression of SV40 early transcription from two promoters. *Cell*, **27**, 603–613.
- Karess, R.E. and Rubin, G.M. (1984) Analysis of P transposable element functions in *Drosophila*. *Cell*, **38**, 135–146.
- Kato, H., Horikoshi, M. and Roeder, R.G. (1991) Repression of HIV-1 transcription by a cellular protein. *Science*, **251**, 1476–1480.
- Kaufman, P.D. and Rio, D.C. (1991) *Drosophila* P-element transposase is a transcriptional repressor *in vitro*. *Proc. Natl Acad. Sci. USA*, **88**, 2613–2617.
- Lee, G., Wu, J., Luu, P., Ghazal, P. and Flores, O. (1996) Inhibition of the association of RNA polymerase II with the preinitiation complex by a viral transcriptional repressor. *Proc. Natl Acad. Sci. USA*, **93**, 2570–2575.
- Li, L.H., Nerlov, C., Prendergast, G., MacGregor, D. and Ziff, E.B. (1994) c-Myc represses transcription *in vivo* by a novel mechanism dependent on the initiator element and Myc box II. *EMBO J.*, **13**, 4070–4079.
- Macias, M.P. and Stinski, M.F. (1993) An *in vitro* system for human cytomegalovirus immediate early 2 protein (IE2)-mediated site-dependent repression of transcription and direct binding of IE2 to the major immediate early promoter. *Proc. Natl Acad. Sci. USA*, **90**, 707–711.
- Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.*, **12**, 7035–7056.

- Nikolov,D.B. and Burley,S.K. (1997) RNA polymerase II transcription initiation: a structural view. *Proc. Natl Acad. Sci. USA*, **94**, 15–22.
- Novina,C.D. and Roy,A.L. (1996) Core promoters and transcriptional control. *Trends Genet.*, **12**, 351–354.
- Parker,C.S. and Topol,J. (1984) A *Drosophila* RNA polymerase II transcription factor contains a promoter-region-specific DNA-binding activity. *Cell*, **36**, 357–369.
- Reed,R. (1996) Initial splice-site recognition and pairing during pre-mRNA splicing. *Curr. Opin. Genet. Dev.*, **6**, 215–220.
- Roeder,R.G. (1996) The role of general initiation factors in transcription by RNA polymerase II. *Trends Biochem. Sci.*, **21**, 327–335.
- Rubin,G.M. and Spradling,A.C. (1982) Genetic transformation of *Drosophila* with transposable element vectors. *Science*, **218**, 348–353.
- Schwyster,D.H., Huang,J., Dubnicoff,T. and Courey,A.J. (1995) The *decapentaplegic* core promoter region plays an integral role in the spatial control of transcription. *Mol. Cell. Biol.*, **15**, 3960–3968.
- Shen,W. and Green,M.R. (1997) Yeast TAF_{II}145 function as a core promoter selectivity factor, not a general coactivator. *Cell*, **90**, 615–624.
- Spradling,A.C. and Rubin,G.M. (1982) Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science*, **218**, 341–347.
- Thanos,D. and Maniatis,T. (1995) Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. *Cell*, **83**, 1091–1100.
- Tjian,R. (1981) T antigen binding and the control of SV40 gene expression. *Cell*, **26**, 1–2.
- Wu,J., Jupp,R., Stenberg,R.M., Nelson,J.A. and Ghazal,P. (1993) Site-specific inhibition of RNA polymerase II preinitiation complex assembly by human cytomegalovirus IE86 protein. *J. Virol.*, **67**, 7547–7555.

Received September 18, 1997; revised and accepted December 10, 1997