A conserved regulatory unit implicated in tissue-specific gene expression in *Drosophila* and man

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The *Drosophila melanogaster* alcohol dehydrogenase (*Adh*) gene is expressed in a specific set of tissues during larval development and in adults. Expression in the adult fat body is controlled by the *Adh* adult enhancer (AAE). Previous studies identified a negative regulatory element in the AAE and a protein that binds specifically to this sequence [adult enhancer factor-1 (AEF-1)]. Here, we show that the AEF-1-binding site in the AAE and in two other *Drosophila* fat body enhancers overlaps a sequence recognized by the mammalian transcription factor CCAAT/enhancer-binding protein (C/EBP). Remarkably, these two proteins also bind specifically to overlapping sites in a liver-specific regulatory element of the human *Adh* gene. Cotransfection experiments in mammalian cells reveal that C/EBP stimulates the activity of the AAE by 50-fold, and this activity can be suppressed by AEF-1. In addition, AEF-1 prevents C/EBP binding in vitro, and displaces prebound C/EBP. Thus, a tissue-specific regulatory unit consisting of one positive and one negative regulatory element has been conserved between *Drosophila* and man.

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in tissue culture cotransfection assays, and AEF-1 is able to repress this activation. Binding studies with C/EBP reveal that it binds to sites in the *D. mulleri* Adh and yolk protein fat body enhancers, and in each case, this site is adjacent to an AEF-1-binding site. Furthermore, AEF-1 and C/EBP bind to overlapping sites in the human *Adh* promoter, which requires the C/EBP site for activation in liver cells (Stewart et al. 1990) and is subject to negative regulation in nonliver cells (Chin and Fournier 1987). Finally, binding studies with AEF-1 and C/EBP provide a molecular basis for the repression by AEF-1 of C/EBP-mediated activation: AEF-1 and C/EBP bind to the adjacent sites in a mutually exclusive manner, with AEF-1 playing the dominant role.

### Results

Mammalian C/EBP binds to sequences adjacent to AEF-1 sites in several fat body enhancers

The DNA-binding protein AEF-1 binds specifically to a sequence found in three different adult fat body enhancers ([Falb and T. Maniatis, unpubl.). Base substitutions introduced into the binding site in one of these enhancers, the AAE, result in a 5- to 10-fold increase in fat body-specific expression of a reporter gene introduced into *Drosophila* by P-element transformation ([Falb and T. Maniatis, unpubl.). Thus, the AEF-1-binding site functions as a negative regulatory element.

Inspection of the AAE sequence reveals a consensus binding site for the mammalian transcription factor C/EBP. This site is directly adjacent to the binding site for AEF-1 determined in methylation interference experiments ([Fig. 1B; D. Falb and T. Maniatis, unpubl.). Interestingly, the guanine residue at position -518 that interacts with AEF-1 in methylation interference assays is also a conserved residue in the enhancer core consensus site, to which C/EBP binds and activates transcription ([Landschulz et al. 1988; Friedman et al. 1989]).

To determine whether C/EBP binds to the site depicted in Figure 1B, DNase I footprinting assays were carried out with recombinant mammalian C/EBP protein. An 88-amino-acid carboxy-terminal fragment of C/EBP was produced in bacteria and incubated with an end-labeled fragment of the AAE. This polypeptide contains the leucine zipper DNA-binding domain of C/EBP, and its binding activity is indistinguishable from that of intact C/EBP ([Landschulz et al. 1989, Vinson et al. 1989]). Footprinting studies were also carried out with recombinant AEF-1 produced in bacteria. As shown in Figure 2A, C/EBP binds to the AAE (~506 to ~524), and the protected region overlaps the AEF-1 recognition site. There is also a low-affinity C/EBP-binding site upstream between ~566 and ~580, which C/EBP binds to weakly ([Fig. 7A, below]).

In addition to the AAE, AEF-1 recognizes sequences in two other adult fat body enhancers, the *D. mulleri* Adh adult enhancer and the *yp1* gene fat body enhancer ([Falb and T. Maniatis, unpubl.). In light of this, we asked whether C/EBP also binds to sites in these regulatory elements.
Figure 2. AEF-1 and C/EBP bind to adjacent sites in three Drosophila fat body enhancers. AEF-1 and C/EBP proteins were produced in E. coli (see Materials and methods), and various amounts of bacterial extracts [indicated at top in μl] were used in DNase I footprinting assays with the four end-labeled probes. (A) Binding studies with the AAE. The AEF-1 (shaded oval)- and strong C/EBP (open rectangle)-binding sites are shown on a portion of the AAE sequence. A weak C/EBP site (-566 to -580) is indicated by the bracket next to the autoradiograph. Numbers above the sequence indicate the distance from the distal transcription start site. (B) Binding studies with the D. mulleri adult enhancer. AEF-1- and C/EBP-binding sites are shown on a portion of the adult enhancer sequence. Numbers above the sequence indicate the distance from the D. mulleri Adh-2 gene transcription start site. (C) Binding studies with the Drosophila yolk protein gene fat body enhancer. AEF-1- and C/EBP-binding sites are shown on a portion of the fat body enhancer sequence. Residues protected by double sex protein (DSX) [P. Wensink, pers. comm.] are bracketed. Numbers below the sequence indicate the distance from the yp1 transcription start site. (D) A comparison of C/EBP-binding sites. The regions protected from DNase I cleavage by C/EBP on the three regulatory regions are shown with the C/EBP enhancer core [Johnson et al. 1987] and consensus (S. McKnight, pers. comm.) sequences. The enhancer core sequence was obtained by comparing a number of viral enhancer sequences.
elements. As shown in Figure 2, B and C, C/EBP and AEF-1 do recognize adjacent sites in these enhancers. In the D. mulleri adult enhancer, the C/EBP footprint is separated by 3 bp from the AEF-1 site while the two sites overlap in the ypl enhancer. Figure 2D shows a comparison of the three Drosophila C/EBP-binding sites with the enhancer core and C/EBP consensus sequences. The AAE-binding site matches the enhancer core sequence, whereas the ypl enhancer site fits the C/EBP consensus site. Both of these Drosophila sites are recognized with equal affinity by C/EBP. The D. mulleri C/EBP-binding site matches the consensus sites less well and has a somewhat lower affinity for C/EBP.

AEF-1 and C/EBP bind to overlapping sites in the human Adh promoter

The human Adh gene is expressed at high levels in a tissue-specific manner in the adult liver (Ditlow et al. 1984). Recent cotransfection studies in human hepatoma cells demonstrate that C/EBP can activate transcription of the human Adh gene and that this activation is dependent on two C/EBP sites located within 50 bp of the transcription start site (Stewart et al. 1990). Furthermore, somatic cell fusion experiments revealed that the human Adh gene is under negative regulation in non-liver cell types (Petit et al. 1986; Chin and Fournier 1989). Given that the liver and fat body are functionally similar and that C/EBP and AEF-1 bind to adjacent sites in three adult fat-body-specific enhancers, we asked whether AEF-1 binds to human Adh regulatory sequences. As shown in Figure 3, C/EBP and AEF-1 bind to overlapping sites between –28 and –54 of the human Adh transcription start site. In this case, the C/EBP recognition site is similar to that of the CCAAT sequence (Fig. 5, below). Thus, the conservation of adjacent C/EBP- and AEF-1-binding sites found in three Drosophila fat body enhancers can be extended to a mammalian liver regulatory element that is under negative control in non-liver cells and whose function in liver cells is dependent on an intact C/EBP site. Furthermore, preliminary experiments indicate that an AEF-1-like binding activity is present in rat liver nuclear extracts (T. Miller and T. Maniatis, unpubl.).

Mutations in the C/EBP site of the D. melanogaster AAE reduce expression in vivo

To determine whether the C/EBP site in the AAE plays a role in regulating transcription in the Drosophila fat body, nucleotide substitutions were introduced into the two C/EBP-binding sites in the AAE, and the resulting mutated enhancers were tested in vivo. Illustrated in Figure 4A is a portion of the AAE containing the AEF-1- and C/EBP-binding sites. A linker scanning mutation in the strong C/EBP site [LsC/EBPw] was created by inserting the same EcoRV sequences used to make LsAEF-1 (D. Falb and T. Maniatis, unpubl.), resulting in a 5-nucleotide change. A 9-nucleotide substitution was introduced between –574 and –583 [LsC/EBPw], which changes residues in the upstream C/EBP site. Both LsC/EBPw and LsC/EBPg prevent C/EBP from binding to the respective mutated sites, yet neither mutation affects the binding of C/EBP to the other wild-type site (data not shown), demonstrating that C/EBP binds independently to the two sites in the AAE.

These mutant AAE fragments were fused to the hslacZ gene [Materials and methods] and introduced into the Drosophila genome on a P-element vector (Fig. 4B). Figure 4C shows that the LsC/EBPw mutation abolishes hs–lacZ expression in adult transformants. Interestingly, when the same nucleotide substitution is introduced 17 bp upstream in the AEF-1 site [LsAEF-1], hs–lacZ transcript levels increase 5- to 10-fold. Mutations in the upstream C/EBP site have no effect on expression, as hs–lacZ levels are the same in LsC/EBPw and AAE transformants.

A factor in ovarian extracts binds to the Drosophila C/EBP site

Having demonstrated that the C/EBP site in the AAE is necessary for Adh expression in Drosophila, we sought to identify and characterize Drosophila factors that recognize this site. To accomplish this, we performed gel retardation assays using an oligonucleotide probe containing the C/EBP site derived from the AAE and nuclear extracts prepared from Drosophila ovaries, an adult tissue where Adh is expressed from the distal promoter [Romano et al. 1988; Corbin 1989]. As shown in Figure 5, a single DNA–protein complex was observed, which cannot be competed by plasmid DNA [lanes 1–3]. Addition of unlabeled probe [lanes 4–6] or an AAE restriction fragment [lanes 10–12] to the binding reaction inhibits complex formation, demonstrating that this DNA–protein interaction is specific.

One interesting characteristic of mammalian C/EBP is

**Figure 3.** Binding studies with the human Adh regulatory region. AEF-1- and C/EBP-binding sites are shown on the human Adh promoter sequence. Numbers above the sequence indicate the distance from the Adh transcription start site.
that it binds with high affinity to the enhancer core homology and to the promoter CCAAT motif, even though these sites bear no obvious sequence similarity [Landschulz et al. 1988]. To determine whether the Drosophila factor shares this property, an oligonucleotide containing the CCAAT motif was used in the competition experiments. This oligonucleotide was used by Vinson et al. (1988) to detect C/EBP protein produced in recombinant bacteriophage. Addition of this oligonucleotide to the binding reaction inhibits complex formation [lanes 7–9], indicating that the Drosophila binding factor also recognizes sequences in both motifs. Recently, a Drosophila C/EBP homolog has been identified [D. Montell and A. Spradling, pers. comm.] and the corresponding cDNA cloned [D. Montell and P. Rorth, pers. comm.]. In collaboration with these investigators we have shown that this protein binds to the C/EBP site in the AAE [J. Olesen, D. Montell, and P. Rorth, unpubl.]. Experiments to determine the effect of this C/EBP homolog on Adh transcription are in progress.

**AEF-1 is a repressor of C/EBP-mediated transcription**

When mutations are introduced into the AEF-1 and C/EBP sites in the AAE, opposite effects are observed in adult transformants: AEF-1 mutations result in a 5–10-fold enhancement of expression, whereas mutations in the adjacent C/EBP site abolish expression. To determine the functions of these two proteins in regulating expression from the AAE, cotransfection experiments

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**Figure 4.** Analysis of base substitutions in the C/EBP sites of the AAE. (A) Diagram of AEF-1 [shaded oval]- and C/EBP [open rectangles]-binding sites in the AAE. Base substitutions in each of the binding sites are shown below the sequence, with the changed residues shown in boldface type. (B) Diagram of test constructs. Wild-type [AAE] and mutant [Ls constructs] enhancers were fused to the −43 hsp70-lacZ fusion gene [Lis et al. 1983] and then introduced into the Drosophila germ line on the C70TX P-element vector [Fischer et al. 1988]. The open rectangles represent AAE sequences; the solid, vertical bars indicate the site of mutagenesis in each case. The solid rectangle represents a 1-kb fragment of the α-tubulin coding region used as a control. The hsp70 sequences are shown as a solid line, the arrows mark the transcription start sites, and the shaded rectangle represents the lacZ coding region. (C) RNase protection analysis of wild-type and mutant constructs. hs-lacZ transcripts from two independent transformant lines of each of the indicated constructs were detected by quantitative RNase protection mapping. Total nucleic acid was isolated from adult transformants and hybridized simultaneously to the 32P-labeled, single-stranded probes SP6-hs1ac (Fischer et al. 1988) and SP6aTUB [Fischer and Maniatis 1986]. SP6-hs1ac protects a 408-nucleotide band from the fusion gene transcript (hsp70-lacZ) and a 265-nucleotide band from the endogenous Drosophila hsp70 gene transcript (Fischer et al. 1988). The SP6aTUB probe is complementary to Drosophila α-tubulin transcripts and protects a band of 70 nucleotides [tubulin] from RNase digestion. At least five lines of each construct were tested, although only two are shown. Protected bands are indicated.
were carried out in HeLa cells. We chose not to use Drosophila tissue culture cells, because gel retardation experiments revealed high levels of AEF-1-binding activity in nuclear extracts prepared from these cells [data not shown]. Furthermore, we chose HeLa cells because we assumed that they would not contain C/EBP, as they are not derived from hepatic or adipose tissue.

AEF-1 and C/EBP cDNAs were inserted into a mammalian expression vector, pCDNA [Seed 1987], creating vectors in which the cytomegalovirus enhancer drives expression of the linked cDNA. These reporters all include the −41 human β-globin promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. As shown in Figure 6, AEF-1 and C/EBP have little or no effect on the expression of a control reporter lacking upstream AAE sequences. However, when the AAE is included in the reporter plasmid, C/EBP enhances transcription by >20-fold. Transfection of the control (pCDNA) or AEF-1 expression vector has no effect on the AAE reporter. However, when the AEF-1 and C/EBP expression vectors are transfected together, a dramatic decrease in CAT activity is observed relative to that resulting from the transfection of C/EBP alone. Thus, AEF-1 can repress the transcriptional activation mediated by C/EBP. To test whether this repression is dependent on the presence of an intact AEF-1 site, a mutated AAE, LsAEF-1, was tested in the reporter plasmid. LsAEF-1 bears a 7-nucleotide substitution mutation, which prevents AEF-1 from binding in vitro, and leads to higher levels of expression in vivo [Fig. 4C; D. Falb and T. Maniatis, unpubl.]. As with the wild-type AAE reporter, C/EBP stimulates transcription from the mutant. However, transfection of the AEF-1 expression vector with the C/EBP expression vector results in a slight increase in expression rather than a decrease. Thus, repression by AEF-1 appears to be specific to the wild-type AAE and requires the presence of an intact AEF-1 site.

AEF-1 can displace C/EBP from its binding site in vitro

We have established that AEF-1 represses transcriptional activation by C/EBP from the AAE and that this repression is dependent on the AEF-1 site. This finding, coupled with the observation that the AEF-1 and C/EBP sites overlap, suggests that AEF-1 may repress transcription of the Adh gene by interfering with the binding of a transcriptional activator to the C/EBP site. To test this hypothesis, we asked whether these proteins could bind simultaneously to their sites in the AAE. DNase I footprinting studies were carried out using the two bacterially produced proteins [Fig. 7A]. The amounts of extracts necessary to just fill the respective sites [Fig. 2] were used in these order-of-addition experiments. AEF-1 and C/EBP were added to the binding reactions either alone [lanes 2, 3] or together [lanes 4, 5]. In both cases, when the two proteins are present in the binding reaction simultaneously, the AEF-1 pattern of protection is observed.
The relationship between AEF-1 and C/EBP for adjacent sites appears to be conserved between three adult fat body enhancers.

Discussion

We have shown that the mammalian transcription factor C/EBP binds to a site in the Adh adult enhancer and that mutations in this site result in lower levels of transcription in transgenic flies. In vitro binding experiments identify a Drosophila nuclear protein that binds to this site and possesses the divergent binding specificities of mammalian C/EBP. The C/EBP site overlaps a negative element that is recognized by the Drosophila protein AEF-1. These overlapping sites are conserved in two other Drosophila fat body enhancers and in the human Adh promoter, which is activated by C/EBP. C/EBP activates transcription from the AAE in cotransfection experiments, and AEF-1 represses this activation. In vitro binding experiments demonstrate that AEF-1 and C/EBP compete for binding to the adjacent sites in the three fat body enhancers and that AEF-1 can displace bound C/EBP from its sites.

A C/EBP site is necessary for AAE-directed transcription

Our studies of LsC/EBP transformants establish that an element in the AAE that includes the mammalian C/EBP consensus sequence is necessary for enhancer function. Mammalian C/EBP was initially identified in rat liver nuclear extracts as a factor that recognizes both viral enhancer and promoter sequences (Johnson et al. 1987). The cloning and characterization of a cDNA encoding this protein led to the discovery that it is expressed only in terminally differentiated cells in the liver, lung, and adipose tissue (Birkenmeier et al. 1989). In accord with these findings, a number of genes expressed in terminally differentiated liver and fat cells contain avid C/EBP-binding sites. Transient cotransfection studies revealed that C/EBP activates transcription of the liver-specific serum albumin (Friedman et al. 1989) and human Adh (Stewart et al. 1990) genes, and of the adipose-specific 422(ap2) and 5CD1 genes (Christy et al. 1989).

Given the functional similarity between the fat body and the liver, it is possible that factors necessary for the expression of the terminally differentiated genes in these tissues have been conserved between Drosophila and mammals. The discovery that the C/EBP site is necessary for Drosophila Adh expression and the identification of a Drosophila factor that shares the divergent binding specificities of C/EBP support this hypothesis.

Activation and repression of expression from the AAE

Tissue culture cotransfection assays demonstrate that C/EBP can activate transcription from the AAE and that this activation can be repressed by AEF-1. These data establish a link between the AEF-1 site defined in vivo
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Figure 7. AEF-1 prevents C/EBP from binding to the three Drosophila fat body enhancers. DNase I footprinting studies with D. melanogaster AAE (A); D. mulleri adult enhancer (B); and yolk protein fat body enhancer (C). AEF-1 and C/EBP proteins were produced in bacteria, and various amounts of bacterial extracts (indicated at top in μl) were used in footprinting assays with the three end-labeled probes. Order-of-addition experiments show binding reactions with AEF-1 and C/EBP alone (A and C, respectively) and together (arrows). The amounts of extract needed to just fill the respective sites on the three enhancers (determined in Fig. 2) were used in each experiment. The letter before the arrow indicates the protein that was added first; the letter after the arrow indicates the protein that was added subsequently. In each case, the first protein was incubated alone with the probe for 20 min, after which the second protein was added for 5 min before stopping the reaction. Shown beside each autoradiograph are the residues protected by AEF-1 (shaded ovals) and C/EBP (open rectangles) in the respective sequences. The hatched rectangle in A marks the weak C/EBP site.

and the function of the protein encoded by the AEF-1 cDNA clone. Mutations in the AEF-1 site result in levels of expression 5- to 10-fold higher in Drosophila transformants, indicating that it functions as a negative element in Adh expression [Fig. 4, D. Falb and T. Maniatis, unpubl.], while the AEF-1 cDNA encodes a protein that can repress transcription through the AEF-1 site in tissue culture cells. Thus, AEF-1 protein exhibits the transcriptional regulatory functions predicted for a factor that binds specifically to this site.

Mechanism of transcriptional repression by AEF-1

Studies of P-element transformants established that the adjacent AEF-1 and C/EBP sites function as negative and positive elements, respectively (Fig. 4). One physiologi-
cal role of AEF-1 appears to be the modulation of the level of Adh transcription in the fat body, because mutations in the Adh-1-binding site result in 5- to 10-fold increases in expression in this tissue. Thus, negative regulators may not function solely by extinguishing gene expression in inappropriate tissues, but also by controlling the levels of transcription of certain genes in the appropriate tissues.

To gain insight into the molecular basis of the repression mediated by AEF-1, we carried out footprinting studies in the presence of bacterially produced AEF-1 and C/EBP [Fig. 7]. We found that AEF-1 could displace bound C/EBP from its binding sites in all three fat body enhancers. This observation is consistent with a mechanism in which positive and negative regulatory factors compete for binding to overlapping sites. This mechanism was proposed for the mutually exclusive binding relationship between the CCAAT-binding activator factor and displacement factor, which compete for binding to overlapping sites in the promoter of the sea urchin histone gene (Barberis et al. 1987). Likewise, positive and negative gene regulation is mediated in certain Drosophila genes by homeo box proteins that compete for common binding sites (Jaynes and O’Farrell 1988; Han et al. 1989; Small et al. 1991). Furthermore, PRDI-BF1, a negative transcriptional regulatory protein that binds to the human β interferon promoter, has been shown to repress by the same mechanism (Keller and Maniatis 1991). Repressors have also been shown to function by preventing formation of the initiation complex, in some cases blocking the binding of TFIID (Dostatni et al. 1991; Kato et al. 1991).

Our findings suggest that AEF-1 represses expression from the AAE by interfering with the binding of a C/EBP-like activator. This repression appears to occur through steric hindrance of C/EBP binding, as an intact AEF-1 site is necessary. The possibility that AEF-1 could also mediate active repression from a distance [Brand et al. 1985; Jaynes and O’Farrell 1991] has not been tested.

Evolutionary conservation of AEF-1 and C/EBP sites

The presence of overlapping AEF-1 and C/EBP sites in three adult fat body enhancers suggests that C/EBP activation coupled with AEF-1 repression may be a common mechanism involved in the generation of fat body-specific expression. In addition, the overlapping AEF-1 and C/EBP sites found in the human Adh promoter suggest that this mechanism has been conserved between Drosophila and mammals [Fig. 8]. Previous genetic studies in Drosophila have identified a locus that negatively regulates Adh expression (Birchler et al. 1990). These investigators reported that duplication of a segment of the 31DE-33B region results in a twofold decrease specifically in Adh expression at the level of transcription. This locus does not encode AEF-1, however, as cytogenetic mapping experiments have shown that AEF-1 maps to position 3L-78 (D. Falb and T. Maniatis, unpubl.). In addition, mammalian somatic cell fusion studies have identified genetic loci in mice that coordinately repress the expression of a number of liver-specific genes (Killary and Fournier 1984; Chin and Fournier 1987). One locus, the tissue-specific extinguisher-2 (Tse-2), found on chromosome 1 in mice, represses the expression of the mammalian serum albumin and the Adh genes, but not other liver-specific genes (Petit et al. 1986; Chin and Fournier 1989). Interestingly, the expression of the Adh (Stewart et al. 1990) and serum albumin (Friedman et al. 1989) genes is dependent on the presence of intact C/EBP-binding sites, and the promoters of both genes are activated by C/EBP in hepatoma cells. Although the cis-acting elements through which the Tse-2 repression of the Adh and serum albumin genes are not known, it is possible that Tse-2, like AEF-1, acts to repress C/EBP-mediated activation.

The conservation of a simple regulatory unit consisting of AEF-1- and C/EBP-binding sites between Drosophila and man is an interesting evolutionary puzzle. Although the fat body and the liver perform similar functions, they originate from distinct germ layers in the early embryo. The fat body is mesodermal in origin, whereas the liver is derived from the endoderm. Another puzzle is the evolution of the Adh gene itself. Although the same regulatory unit is associated with the Drosophila and human Adh genes, structural comparisons between the two Adh proteins indicate that they have evolved independently. The mammalian Adh protein is similar to Adh in many organisms, including yeast, while Drosophila Adh is unrelated. Thus, the Drosophila and human Adh proteins appear to have arisen by convergent evolution. From one point of view it is therefore surprising that the genes encoding Drosophila and human Adh proteins share a common regulatory unit. However, from another perspective many functionally unrelated proteins that are expressed in the same tissues are encoded by genes containing common tissue-specific regulatory elements (Costa et al. 1989; Ruppert et al. 1990). This conservation is further supported by the identification of a Drosophila CREB/ATF family member, BBF-2, which appears to be involved in the regulation of gene expression in the fat body (Abel et al., this volume). Thus, two independent evolutionary forces appear to be at work: one driven by protein function, and the other by tissue-specific expression.

Materials and methods

Bacterial protein production

The full-length AEF-1 cDNA was constructed by combining two partial isolates in pSP72 (D. Falb and T. Maniatis, unpubl.). A 2815-bp fragment initiating at the putative AEF-1 translation start site (+330) was subcloned in-frame into the appropriate bacteriophage T7 expression vector (Studier and Moffatt 1986). This plasmid was then transformed into the bacterial strain BL21(DE3), which contains the T7 RNA polymerase gene under lacUV5 promoter control. Transformed bacteria were grown to OD<sub>600</sub> = 0.4, induced with 0.4 mM IPTG, grown for two hr, and harvested. Protein was purified from inclusion bodies (Gaul et al. 1987) and dialyzed against buffer containing 50 mm Tris (pH 7.9), 500 mm NaCl, 10% glycerol, and 1 mm PMSF.
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![Diagram: Conserved regulatory unit](https://example.com/diagram.png)

**Figure 8.** Evolutionary conservation of AEF-1 and C/EBP sites. Illustration of adjacent AEF-1- and C/EBP-binding sites. Gene-coding regions are shown as shaded rectangles; enhancer elements are shown as open rectangles. Lines are promoter and intergenic sequences, and arrows indicate transcription start sites. (D and P) The *D. melanogaster* distal and proximal transcription start sites, respectively. The binding sites for C/EBP (hatched box) and AEF-1 (solid oval) are shown. (Note: in our in vitro binding studies, AEF-1 and C/EBP do not bind simultaneously to adjacent sites.) The numbers below the drawings indicate the distances from the transcription start sites of the *D. melanogaster* distal promoter, the *D. mulleri* Adh-2 promoter, the *yp-1* promoter, the *yp2* promoter, and the human Adh promoter.

The 88-amino-acid DNA-binding domain of C/EBP was produced in *Escherichia coli* from the phage T7 expression plasmid pT5 (a gift of Steve McKnight) as described previously (Landschulz et al. 1989).

**DNase I footprints**

DNase I footprinting experiments were carried out as described in Heberlein et al. [1985], with the following modifications. Seven microliters of 13.3% polyvinyl alcohol and 1 μg of poly[d(I-C)] were added to the 50-μl binding reaction. After incubation at 0°C for 20 min, 50 μl of a solution of 5 mM CaCl2, 10 mM MgCl2, was added followed by the addition of 4 μl of DNase I diluted to 2 μg/ml in cold H2O. The DNase I digestion was stopped after 1 min at 22°C with 180 μl of stop solution (0.1% SDS, 20 mM EDTA, 500 mM NaCl, 20 μg/ml of salmon sperm DNA) and 200 μl of phenol–chloroform, and then ethanol-precipitated. The pellet was resuspended in 2 μl of 0.1 M NaOH, followed by 4 μl of denaturing loading dyes. The DNAs were subsequently analyzed on an 8% polyacrylamide sequencing gel.

**Mutagenesis**

Linker mutants were obtained by site-directed mutagenesis (Taylor et al. 1985; Amersham) of the 140-bp AAE. The three targeted sites were mutagenized with synthetic oligonucleotides containing wild-type flanking sequences and the central mutagenic sequences shown in Figure 4. Mutagenized clones were verified by deoxyxynucleotide sequencing. Mutant AAE fragments were subcloned into pSP72, joined to the pSP73lacI (Fischer et al. 1988) BglII site, and inserted into the P-element transformation vector C70TX (Fischer et al. 1988) on an Xbal fragment.

**Establishment of transformed Drosophila lines**

Transformation vectors and the helper plasmid pp25.7wc (Kress and Rubin 1984) were injected into *y*Δ106 embryos using standard procedures (Rubin and Spradling 1982; Spradling and Rubin 1982, Goldberg et al. 1983), and the resulting transformants were inbred to obtain homozygous lines.

**RNA analysis**

Total nucleic acid was purified from 3-day-old adult flies as described previously [Fischer and Maniatis 1986]. Continuously labeled, single-stranded RNA probes were prepared as described previously [Melton et al. 1984] from the plasmids pSP6-hslac (Fischer and Maniatis, 1986; Fischer et al. 1988), and pSP6aTUB (Corbin and Maniatis 1990). Quantitative RNase mapping experiments were carried out as described previously [Zinn et al. 1983], except that hybridizations were performed at 43°C without prior incubation at 85°C, and RNase digestions were carried out at 30°C for 30 min.
Ovarian nuclear extract preparation

Ovarian follicles were isolated, and nuclear extracts were prepared as described previously (Shea et al. 1990), except that only early stage follicles [1–10a] were used.

Gel retardation experiments

Ovarian nuclear extract (5-10 μg protein) was incubated with −1 ng of 32P-end-labeled DNA fragment, 3 μg of poly(dI·dC)/poly(dI·dC), and competitor DNA in buffer that was 4 mM Tris (pH 7.5), 75 mM NaCl, 5% glycerol, 0.5 mM DTT, and 1 mM EDTA. The 25-μl-binding reaction was carried out at 22°C for 30 min, and fractionated on a 6% nondenaturing polyacrylamide gel (containing 5% glycerol) that was prerun and run at 4°C.

Mammalian cell transfections

Transfection of HeLa cells was carried out according to the calcium phosphate method (Chen and Okayama 1988). Each transfection included 3 μg of reporter plasmid, 15 μg of effector plasmid, and 5 μg of pCMVβGal transfection control (a gift of Dimitris Thanos). pCMVβGal contains the cytomegalovirus enhancer and promoter driving expression of the bacterial lacZ gene. pCDNA I (Invitrogen), the expression vector, contains the cytomegalovirus enhancer driving expression of the cDNA lacZ gene. p-Galactosidase assays were performed to control for transfection efficiency.

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