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A *Drosophila* GATA family member that binds to *Adh* regulatory sequences is expressed in the developing fat body

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SUMMARY

We have identified a *Drosophila* transcription factor that binds a sequence element found in the larval promoters of all known *alcohol dehydrogenase* (*Adh*) genes. DNA sequence analysis of cDNA clones encoding this protein, box A-binding factor (ABF), reveals that it is a member of the GATA family of transcriptional regulatory factors. ABF-binding sites within the *D. mulleri* and *D. melanogaster* larval *Adh* promoters function as positive regulatory elements and in cotransfection experiments, ABF functions as a transcriptional activator. In further support of a role for ABF in the regulation of *Adh* expression, ABF mRNA is expressed in the embryonic fat body, a tissue that contains high levels of *Adh* mRNA. Our studies demonstrate that the fat body develops from segmentally repeated clusters of mesodermal cells, which later expand and coalesce to form the mature fat body. These observations establish ABF as the earliest known fat body precursor marker in the *Drosophila* embryo. Together with the established role of GATA factors during mammalian development, these results suggest that ABF may play a key role in the organogenesis of the fat body.

Key words: *Drosophila Adh*, GATA transcription factor, fat body, mesoderm development

INTRODUCTION

The *Drosophila* genes that encode the enzyme alcohol dehydrogenase (Adh) provide well-characterized examples of tissue-specific transcriptional regulation during eukaryotic development. *Adh* is transcribed in the embryonic, larval and adult fat bodies of every *Drosophila* species examined, as well as in other tissues that vary from species to species (Sofer and Martin, 1987). These species-specific differences are controlled by *cis*-acting elements closely linked to the structural genes (Bayer et al., 1992; Brennan and Dickinson, 1988; Brennan et al., 1988; Corbin and Maniatis, 1989a; Fischer and Maniatis, 1986). In order to investigate the mechanisms that control the temporal and tissue-specific transcription of the *Adh* gene, previous studies have compared the *cis*-acting regulatory elements required for the expression of the *Adh* genes in two distantly related species, *Drosophila melanogaster* (Corbin and Maniatis, 1989a,b, 1990) and *Drosophila mulleri* (Fischer and Maniatis, 1986, 1988).

Adh transcripts in *D. melanogaster*, as in most other *Drosophila* species, are generated in a stage-specific manner from a single gene by the differential utilization of two tandem promoters (Savakis et al., 1986). Transcription is initiated from the proximal promoter in embryos and larvae, whereas the distal promoter is active for a brief period during mid-embryogenesis, in late larval stages and in adults. Each of these promoters is active in the fat body and in a limited number of other tissues, including the gut and reproductive organs.

Using germ line transformation, the *cis*-acting sequences necessary for the proper expression of the *D. melanogaster Adh* gene in vivo have been identified. The *Adh* adult enhancer (AAE), a 140 bp element located at about −500, drives expression of the distal promoter in adults, whereas the complex *Adh* larval enhancer (ALE) drives expression of the proximal promoter in larvae (Corbin and Maniatis, 1989a, 1990). The detailed dissection of the proximal promoter using both germ line transformation (Corbin and Maniatis, 1990) and a somatic transient assay (Shen et al., 1991) has identified two regions within the 400 bp immediately upstream of the proximal transcriptional start site that are necessary for wild-type transcription levels in all larval tissues.

By contrast, two distinct functional *Adh* genes, *Adh-1* and *Adh-2*, are present in *D. mulleri*. The *Adh-1* gene is expressed in embryos and larvae, whereas the *Adh-2* gene is expressed in adults. Although the organization of the locus is quite different, the *Adh* genes of *D. mulleri* are expressed in approximately the same pattern as those of *D. melanogaster*, and the *D. mulleri Adh* genes are expressed correctly when introduced into the germ line of *D. melanogaster* (Fischer and Maniatis, 1986).

In adults, expression of the *D. mulleri Adh-2* gene is

regulated by an enhancer located more than 2000 bp upstream of the start site of transcription (Fischer, 1987). In larvae, the expression of the *Adh-1* gene requires two enhancer elements – an upstream enhancer, box B, and an enhancer located 3′ of the gene. These enhancers, like those of the *D. melanogaster Adh* gene, behave as fat body-specific regulatory elements in association with a heterologous promoter. A promoter element, box A, is required for the proper expression in the full range of larval tissues. The identification of box A is based on the observation that linker substitution and deletion mutants that lie in the region between −91 and −60 of the *Adh-1* promoter result in lower levels of expression in vivo (Fischer and Maniatis, 1988). Unlike the larval enhancer elements, multiple copies of box A are inactive when placed upstream of a heterologous promoter (Fischer, 1987).

Recent studies have sought to identify factors that play a role in the activity of these fat body-specific enhancers. All well-characterized fat body-specific enhancers contain a sequence that is recognized by box B-binding factor-2 (BBF-2), a *Drosophila* CREB/ATF transcriptional activator protein (Abel et al., 1992). In addition, adult fat body enhancers contain a regulatory unit which consists of a positive element that binds mammalian C/EBP and a negative element that binds *Drosophila* AEF-1, a zinc finger-containing transcriptional repressor (Falb and Maniatis, 1992). Studies using *Drosophila* tissue culture cells have localized an additional positive regulatory element within the AAE that shares sequence similarity with the steroid hormone response elements found in many mammalian genes and is recognized by FTZ-F1, a *Drosophila* member of the steroid hormone receptor superfamily (Ayer and Benyajati, 1992). One factor, *Adh* distal factor-1 (Adf-1), has been identified that interacts specifically with *Adh* upstream promoter elements (England et al., 1992).

In order to investigate the mechanisms responsible for the regulatory activities of the larval promoters of the *Adh* genes of *D. mulleri* and *D. melanogaster*, we have identified and characterized a factor that recognizes a sequence present within box A and the proximal promoter. This transcription factor, box A-binding factor (ABF), was initially cloned from a *D. melanogaster* cDNA library on the basis of its ability to bind specifically to the box A element of the *D. mulleri Adh-1* gene. ABF recognizes a sequence element, $^{T}/_{A}$GATAA, which is found in the larval promoter of all known *Drosophila Adh* genes, including box A and the proximal promoter. DNA sequence analysis of ABF cDNA clones reveals that it contains a single DNA-binding zinc finger similar to those found in the DNA-binding domain of the GATA family of transcriptional regulatory proteins (Orkin, 1992). The best characterized member of the GATA family, GATA-1, has been implicated in the transcriptional regulation of erythroid-specific genes (Martin and Orkin, 1990; Tsai et al., 1989) and is involved in the determination of red cell lineage in vertebrates (Pevny et al., 1991). Early in development, ABF is expressed in a dynamic pattern which begins in the presumptive endoderm and cephalic mesoderm and ends in the fat body. Later in embryonic development, when *Adh* begins to be expressed in the mature fat body, ABF is also expressed exclusively in this tissue.

MATERIALS AND METHODS

cDNA cloning and DNA sequencing

A 9- to 12-hour embryonic cDNA library in λgt11 (Zinn et al., 1988) was probed as described previously (Abel et al., 1992). For this screening, a multimerized probe prepared from an oligonucleotide encompassing box A and labeled by nick-translation (Sambrook et al., 1989) was used. This oligonucleotide includes the region from −60 to −91 of the *Adh-1* promoter with a *Bam*HI site added to the 5′ end and a *Bgl*II site at the 3′ end. The sequence is GATCCGCCGACCGCGCGGCCAGTGGTATTGATAAGACA (linker sequences underlined).

Other cDNA clones were isolated by screening the 9- to 12-hour embryonic cDNA library and ABF-specific embryonic cDNA clones using standard nucleic acid hybridization techniques (Sambrook et al., 1989). The ABF-specific cDNA clones were prepared using 0- to 12-hour embryonic poly(A)$^+$ RNA. All reactions were carried out as described (Thanos and Maniatis, 1992) with the following modifications. First-strand synthesis was primed using an oligonucleotide complementary to nucleotides 1184 to 1167 of the ABF sequence shown in Fig. 1A. After second-strand synthesis using RNaseH and DNA polymerase I, *Not*I-*Eco*RI linkers were added to the ends of the cDNA and the DNA fragments were cloned into the *Eco*RI site of λgt11. In screening these cDNA clones, a random-oligonucleotide primed DNA probe (Sambrook et al., 1989) prepared from a gel-purified *Eco*RI-*Nar*I fragment of λABF, extending from 846 to 1270 of the ABF sequence shown in Fig. 1A was used.

All cDNA clones were subcloned into the *Eco*RI of pSp72 (Promega). Deletions were generated using Exonuclease III or appropriate restriction sites and these double-stranded templates were sequenced with either Sp6 or T7 promoter primers using Sequenase (US Biochemical) or Taq polymerase (Stratagene). For some regions with high GC content, deoxyinosine mixes were also used for sequencing. Both strands of four independent, overlapping clones were sequenced in their entirety.

Bacterial protein production

For expression in *Escherichia coli*, the *Eco*RI fragment from λABF (starting at amino acid 189 of the sequence shown in Fig. 1A) was subcloned in frame into the translation vector pET3b (Rosenberg et al., 1987). As a control, λABF insert was subcloned in the reverse orientation in this plasmid. These plasmids were transformed into the bacterial strain BL21(DE3), grown to OD_{600}=0.4, induced with 0.4 mM IPTG, grown for an additional 2 hours and harvested by centrifugation. Total soluble bacterial protein was subsequently isolated (Desplan et al., 1985).

Gel retardation experiments and DNase I footprinting

For gel retardation assays, bacterial extracts were incubated with 15,000 cts/minute of the end-labeled box A oligonucleotide or an end-labeled probe prepared from the *Adh-1* promoter using conditions as described (Abel et al., 1992). Samples were electrophoresed on a 4% 0.5× Tris-borate/EDTA nondenaturing gel at 4°C.

DNase I footprinting experiments using a box A probe and bacterial extract were carried out as described (Heberlein et al., 1985) using the modifications of Abel et al. (1992). Maxam-Gilbert sequencing reactions were run on the 8% polyacryamide sequencing gel as markers.

Schneider cell transfections

For expression in *Drosophila* tissue culture cells, a clone containing the complete coding region of ABF was constructed using a unique *Mlu*I site within ABF. This full-length ABF was subcloned into the effector plasmid pP_{ac} (Krasnow et al., 1989). In this plasmid (pP_{ac}ABF), expression is driven by the *actin5C* promoter

and transcripts are terminated at *actin5C* polyadenylation sites. Reporter plasmids were derived from pD-33CAT (Krasnow et al., 1989) in which the sequence from −33 to +53 of the *D. melanogaster Adh* gene is fused to the bacterial gene CAT. pBoxA_6CAT was constructing by cloning a box A oligonucleotide flanked by *Bam*HI and *Bgl*II sites into the *Bgl*II site of the modified pD-33CAT vector (Abel et al., 1992).

Schneider line 2 cells were grown and transfected exactly as described in Abel et al. (1992). The plasmid *hsp82lacZ* (provided by A. Ephrussi), which contains the *Drosophila hsp82* promoter driving the expression of *lacZ,* was included as a control in all transfections. The amount of extract used in each CAT assay was normalized based on the β-galactosidase activity. CAT assays and β-galactosidase activities were carried out as described (Abel et al., 1992).

RNA preparation and northern analysis

For the isolation of embryonic RNAs, embryos were collected on yeasted grape juice plates from population cages at 6 hour intervals and aged at 25˚C. They were dechorionated in 50% Clorox. For larval stages, first instar larvae were harvested at ~36 hour of development and wandering third instar larvae were isolated. Adults were harvested 4 days after eclosion. Flies from all developmental stages were frozen on dry ice and stored at −80˚C.

Frozen flies were pulverized on dry ice with a mortar and pestle and quickly transferred to a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl and 0.1 M 2-mercaptoethanol. Total RNA was isolated by centrifugation using standard procedures and poly(A)$^+$ RNA was isolated by two passages over oligo(dT) cellulose (Sambrook et al., 1989).

For northern analysis, 4 μg of poly(A)$^+$ RNA from various developmental stages was fractionated on a 1% agarose gel containing 6.7% formaldehyde, transferred to Biotrans (ICN) nylon membrane, hybridized and washed under stringent conditions (Sambrook et al., 1989). The probe for this experiment was an *Ava*I-*Bst*XI fragment of the full-length ABF cDNA clone, which was labeled by random priming (Sambrook et al., 1989). The northern blot was also probed with a random-primed labeled *Eco*RI-*Hind*III fragment from the gene encoding ribosomal protein 49 (*rp49*; O'Connell and Rosbash, 1984).

Whole-mount embryo analysis

In situ hybridization to whole *Oregon R* embryos was carried out using the Genius DNA labeling and detection kit (Boehringer Mannheim) as described by Tautz and Pfiefle (1989) and modified by Michelson et al. (1990). The probe for this experiment was an *Acc*I-*Eco*RI fragment from the ABF cDNA clone and a *Bam*HI-*Xba*I fragment from the genomic *Adh* clone (Goldberg, 1980). Stained embryos were mounted in 90% glycerol so that they could be rotated for visualization from all perspectives during microscopy and photography.

RESULTS

ABF contains a DNA-binding domain similar to that of the GATA family of transcriptional regulatory proteins

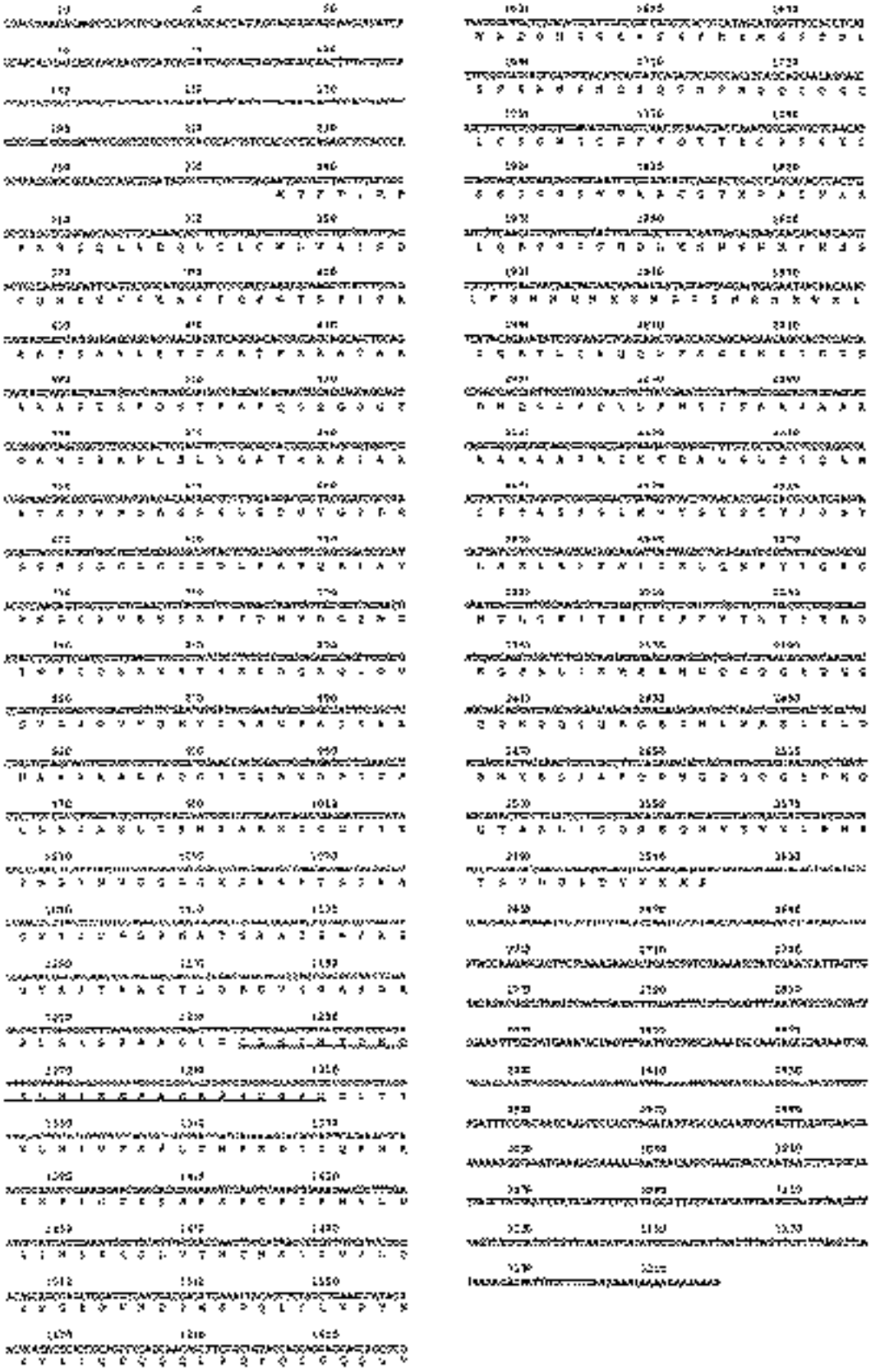
A cDNA clone encoding a protein that specifically binds to box A (−60 to −91 of the *D. mulleri* larval promoter) was isolated by screening a *D. melanogaster* 9- to 12-hour embryonic λgt11 cDNA expression library (Zinn et al., 1988) with a probe containing multiple copies of box A (Vinson et al., 1988). A *D. melanogaster* library was chosen because all studies of the *cis*-acting regulatory elements of the *D. mulleri* gene were carried out in *D. melanogaster* (Fischer and Maniatis, 1986, 1988). This stage of embryogenesis was chosen because it is when *Adh* is first expressed (Fischer and Maniatis, 1986; Lockett and Ashburner, 1989; Savakis et al., 1986) and the fat body begins to form (Rizki, 1978). An initial isolate (box A-binding factor; λABF) containing an insert of 2.4 kb encoded a protein that bound specifically to the box A probe and not to other control probes (data not shown).

Preliminary sequence analysis of λABF revealed that it lacked a translation initiation codon and was a partial cDNA clone. Further nucleic acid hybridization screens were performed in order to isolate cDNA clones containing the entire coding region of ABF. Several overlapping cDNA clones spanning 3.2 kb of sequence were isolated. The complete nucleotide sequence of these clones contains an open reading frame of 2337 bp, encoding a protein of 779 amino acids with a predicted molecular mass of 82 kD (Fig. 1A). The use of the AUG at nucleotide 282 is consistent with the size of the in vitro translation product (data not shown). This coding region is flanked by 5′ and 3′ untranslated regions of 283 and 402 bp, respectively. The overlapping cDNA clones together encompass nearly the complete mRNA because the length of the cloned region is comparable to that of the corresponding mRNA (Fig. 4).

Southern blot analysis of *Drosophila* genomic DNA at high stringency revealed a simple pattern of bands suggesting that ABF is encoded by a single gene (data not shown). The cytological location of the ABF gene was determined using in situ hybridization to polytene chromosomes prepared from the salivary glands of third instar larvae. ABF maps to the third chromosome at cytological position 89B (data not shown) near the dominant marker *Stubble* (Lindsley and Zimm, 1992).

The examination of the amino acid sequence of the ABF protein and a search of the GenBank database revealed that ABF contains a single Cys-X_2-Cys-X_{17}-Cys-X_2-Cys zinc finger, a domain which is found in the GATA family of DNA-binding transcriptional regulatory proteins (for a review, see Orkin, 1992; Fig. 1B). The four members of the GATA multigene family in vertebrates all contain two zinc fingers, both of which are necessary to achieve specific, stable DNA binding. The carboxyl finger, however, appears to play a dominant role in DNA binding because mutation of the amino finger of GATA-1 reduces the stability and specificity of DNA binding but does not eliminate binding (Martin and Orkin, 1990; Yang and Evans, 1992). ABF contains a single zinc finger, which is most similar to the carboxyl finger of vertebrate GATA factors (Fig. 1B), specifically the carboxyl finger of hGATA-2 (Dorfman et al., 1992; Lee et al., 1991); in a stretch of 72 amino acids, these proteins are 71% identical. As shown in Fig. 1B, ABF is more closely related to the human multigene GATA family than it is to another known member of the GATA family in *Drosophila*, dGATAa (Winick et al., 1993). Studies of the regulatory factors involved in nitrogen metabolism in *Aspergillus* and *Neurospora* have previously identified two GATA family members, *areA* (Kudla et al., 1990) and *nit-2* (Fu and Marzluf, 1990), each of which contains a single finger. *areA* is the GATA family member that is most distantly related to ABF, for the DNA-binding domains of these proteins are only 46% identical. The similarity

A



between ABF and other GATA family members does not extend beyond the DNA-binding domain.

The *Drosophila opa* repetitive element, which consists of units of the nucleotide triplet CAX (Wharton et al., 1985), is found in several regions of ABF. The *opa* element is found in both coding and noncoding regions of many *Drosophila*

Fig. 1. ABF encodes an 82×10^3 M_r protein with a DNA-binding domain homologous to the GATA family of transcriptional regulatory proteins. (A) cDNA and predicted protein sequences of ABF. The ORF begins at nucleotide 282 and ends at nucleotide 2618, thus encoding a protein of 779 amino acids. The zinc finger, which is characteristic of the GATA family of transcriptional regulatory proteins, is underlined. (B) A comparison of the zinc finger of ABF with other members of the GATA family. The amino acid sequence of the C-terminal finger from each member of the human GATA family, hGATA-1 (Zon et al., 1990), hGATA-2 (Dorfman et al., 1992; Lee et al., 1991), and hGATA-3 (Ho et al., 1991; Joulin et al., 1991; Ko et al., 1991), the C-terminal finger of dGATAa (Winick et al., 1993), the single finger from *areA* (Kudla et al., 1990) and the single finger from *nit-2* (Fu and Marzluf, 1990) are shown. The identity of the protein is shown at left, and the numbers preceding and following each sequence correspond to the first and last amino acid residue, respectively. The number shown in parentheses is the percent identity in the region shown between that factor and ABF. Vertical lines indicate residues that are identical between ABF and hGATA-2; two dots indicate conserved residues. The cysteines are underlined. The consensus sequence derived from this group of proteins is shown at the bottom, where dots indicate nonconserved positions.

genes and it encodes the polyglutamine, polyasparagine and polyalanine stretches found in the ABF protein.

ABF binds to positive regulatory elements within *Adh* larval promoters

DNase I footprinting experiments were carried out in order to determine the region of box A recognized by ABF. As shown in Fig. 2A, recombinant ABF produced in bacteria protects a 13 bp region extending from −61 to −73 of the *Adh-1* promoter from DNase I cleavage. This region lies at the 3′ end of box A and includes a sequence, TGATAA, which fits the GATA family consensus sequence of WGATAR (Evans et al., 1990).

To explore whether ABF interacts with any other well-characterized *Adh* regulatory elements, we performed a gel shift analysis using probes from the *D. melanogaster* distal and proximal promoters, the *D. melanogaster* and *D. mulleri* adult enhancers, and the *D. mulleri* larval enhancer, box B. This analysis revealed that ABF specifically binds to the proximal promoter of *D. melanogaster* and to none of the other tested regulatory regions (data not shown). DNase I footprinting experiments demonstrate that recombinant ABF

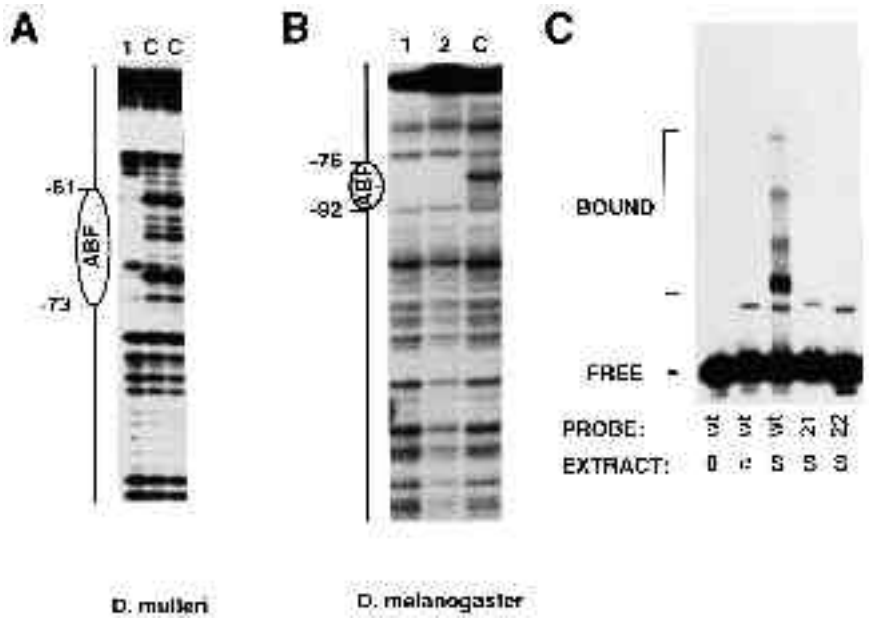


Fig. 2. ABF binds to box A and the proximal promoter. (A) DNase I footprinting studies of ABF binding to *D. mulleri* box A. (B) DNase I footprinting studies of ABF binding to the *D. melanogaster* proximal promoter. End-labeled probes containing each of these promoters were incubated with the indicated amount (1-2 μl, above each lane) of bacterial extract containing ABF fusion protein or 2 μl of control bacterial extract (C) and then treated with DNase I. The products were then separated on a sequencing gel. The residues protected by ABF are shown beside each autoradiogram as a shaded oval. (C) ABF binds less well to linker substitution mutants that reduce *Adh-1* expression in vivo. Gel retardation assay of ABF using mutant box A probes. Bacterial extract containing an ABF fusion protein (in the sense orientation (S) or in the antisense orientation (α)) was mixed with end-labeled probes prepared from the wild-type *Adh-1* promoter (WT) and from two linker substitution mutants (LS21 and LS22; Fischer and Maniatis, 1988). After incubation, complexes were separated by electrophoresis on a native gel. The free probe and the complexes specific for ABF are marked. Other bands that are not labeled are also seen in control bacterial extract, which does not contain ABF protein, and they thus represent the binding of endogenous bacterial proteins. The multiple bound complexes appear to be the result of proteolysis.

protects a region of the proximal promoter extending from −76 to −92 from DNase I cleavage (Fig. 2B). AGATAA, a sequence that fits the WGATAR consensus, lies within this region of the proximal promoter.

Thus two ABF-binding sites share a common sequence that fits the consensus for the GATA family of DNA-binding proteins. To demonstrate that ABF recognizes this sequence element within the footprinted region, we carried out a methylation interference experiment (Abel, 1993). This experiment revealed that ABF interacts with the guanine and adenine residues present in the sequence GATAAG, which lies at the 3′ end of box A in the region protected from DNase I cleavage by ABF. ABF also binds to the GATA element found in the erythropoietin receptor promoter (Zon et al., 1991; data not shown). Furthermore, recombinant mouse GATA-1 protein (Tsai et al., 1989) binds specifically to a box A probe (data not shown).

The binding site for ABF within box A lies in the region of two overlapping linker substitution mutations (LS21 and LS22) that lead to lower levels of expression in vivo (Fischer and Maniatis, 1988). A gel retardation assay was used to investigate the effect of these mutations on ABF binding. As shown in Fig. 2C, recombinant ABF binds at least one hundredfold less well to these two linker substitution mutants relative to wild-type ABF sequences. Thus, the ABF-binding site within box A is a positive regulatory element. Additional evidence of the importance of ABF-binding sites in *Adh* transcriptional regulation comes from germ line transformation experiments (Corbin and Maniatis, 1990) and somatic transient assays (Shen et al., 1991) which explored the effect of mutations within the *D. melanogaster* proximal promoter. Both of these studies demonstrated that deletion of the ABF-binding site within the proximal promoter leads to reduced levels of expression in vivo.

ABF is a transcriptional activator

The observation that ABF-binding sites function as positive regulatory elements within the larval promoters of the *D. mulleri* and *D. melanogaster Adh* genes suggested that ABF may function as a transcriptional activator protein. In order to investigate this function of ABF, we used transient cotransfection assays in *Drosophila* tissue culture cells. ABF was cloned into the *Drosophila* expression vector pP_{ac} (Krasnow et al., 1989), creating a vector (pP_{ac}ABF) in which the *Drosophila* actin promoter drives expression of sense ABF. A reporter plasmid was constructed which contained six copies of box A driving expression of the bacterial chloramphenicol acetyltransferase (CAT) gene. This effector plasmid was transiently cotransfected along with the reporter plasmid into *Drosophila* Schneider 2 cells. Cotransfection of ABF increases expression from a reporter ($pBoxA_6CAT$) containing the box A promoter element by about tenfold (Fig. 3). In the absence of cotransfected ABF, the box A promoter element exhibits some basal activity, consistent with the observation that ABF mRNA is present in this cell line (data not shown). ABF also activates transcription in HeLa cells where the basal activity of the reporter is low (data not shown).

Temporal and spatial pattern of ABF expression

The *D. melanogaster* proximal promoter is active from mid-

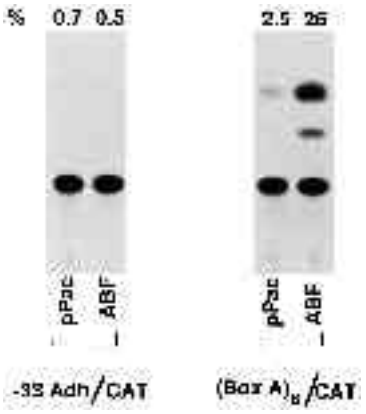


Fig. 3. ABF activates transcription in transient cotransfection studies in *Drosophila* tissue culture cells. S2 cells were transfected with 0.25 μg of a reporter plasmid containing 6 copies of box A driving the expression of CAT($pBoxA_6CAT$) and 5 μg of an effector plasmid containing ABF (pP_{ac}ABF). As a control, the reporter plasmid (−33AdhCAT) lacking any promoter sequences and the effector plasmid (pP_{ac}) lacking an insert were used. The plasmid *hsp82lacZ* was used as a control for transfection efficiency. The amount of extract used in each CAT assay was normalized based on the β-galactosidase activity. The reactions were separated on TLC plates and autoradiograms of these TLC plates are shown. The numbers above each lane represent the percent conversion of ^{14}C-labeled chloramphenicol to monoacetylated forms. Results similar to those shown have been observed in four independent experiments.

embryogenesis through the third larval instar (Savakis et al., 1986). Similarly, the *D. mulleri Adh-1* gene, when introduced into the *D. melanogaster* genome using P-element mediated transformation, is expressed in embryos and at higher levels in larvae (Fischer and Maniatis, 1986). To compare the expression of the gene encoding ABF with the expression of these *Adh* genes, a northern blot containing poly(A)$^+$ RNA prepared from various developmental stages was hybridized to a probe prepared from the ABF cDNA clone. As shown in Fig. 4, the ABF mRNA is 3.5 kb in length and is present at varying levels throughout develop-

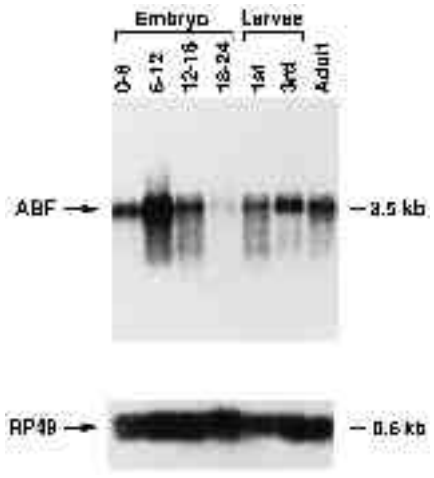


Fig. 4. ABF is expressed at varying levels throughout development. Poly(A)$^+$ RNAs were isolated from 0- to 6-hour, 6- to 12-hour, 12- to 18-hour, and 18- to 24-hour embryos, first and third instar larvae and adults. These were run on an agarose gel, transferred to a nylon membrane and probed with radioactive DNA probes prepared from ABF and RP49 (O'Connell and Rosbash, 1984). The bands corresponding to these RNAs as well as their size in kb are shown.

ment. The highest levels are seen during 6 to 12 hours of embryogenesis, a time when *Adh* transcription begins in the embryo.

A previous study, which used in situ hybridization to characterize in detail the temporal and spatial expression pattern of the *D. melanogaster Adh* gene during embryogenesis, established that *Adh* transcription begins 10.5 hours after fertilization (Lockett and Ashburner, 1989). This expression, which occurs from both the proximal and distal promoters, is confined to the fat body. At 16 hours, expression occurs solely from the proximal promoter and is seen in the gut in addition to the fat body. To explore the spatial and temporal expression pattern of ABF and to compare this pattern with that of *Adh*, ABF mRNA was localized in *Drosophila* embryos of various developmental stages using a whole-mount in situ hybridization technique (Tautz and Pfeifle, 1989).

The analysis of the spatial pattern of ABF expression during embryogenesis (Figs 5, 6) reveals that expression is dynamic and appears to occur in two phases. In the early phase, ABF transcripts are present in a variety of tissues that arise from different germ layers. In the late phase, which begins after the completion of germ band extension, ABF expression is observed only in the developing fat body.

ABF transcripts are first detected after cellularization at ~3 hours postfertilization (Fig. 5A). These transcripts are initially found in four regions of the cellular blastoderm: the anterior and posterior midgut primordia, the primordium of the cephalic mesoderm (the more anterior ventral staining in Fig. 5A), and the vitellophages, which are located within the yolk in the center of the embryo. ABF appears first at the posterior of the embryo and this domain is similar to the posterior portion of the expression patterns of the gap gene *tailless* (*tll*) and *huckebein* (*hkb*), and the homeotic gene *fork head* (*fkh*)(reviewed in Skaer, 1993). These genes are also present in other regions of the embryo that do not appear to correspond to the regions in which ABF is expressed. Temporally, *tll*, *hkb* and *fkh* are detected in stage 4 embryos prior to cellularization before ABF expression appears. Recently, a *Drosophila* homolog of the mouse transcription factor HNF-4, termed HNF-4(D), has been identified (Zhong et al., 1993). HNF-4(D) is a maternal mRNA that is initially present uniformly throughout the egg and becomes localized to the terminal regions of the embryo prior to cellularization. ABF, in contrast, is not detected until cellularization is complete.

As gastrulation begins, ABF transcripts continue to be detected in these areas and are transiently observed in the dorsalmost region of the embryo that gives rise to the amnioserosa (Fig. 5B,C). Expression in the amnioserosa is in a series of five stripes which extend from about 25 to 55% of egg length. This pattern resemble the refined pattern of *zerknüllt* expression (the 'mohawk'; Rushlow et al., 1987), but the region of ABF expression is more restricted in its anterior-posterior extent. The expression of ABF in the amnioserosa at this stage is similar to the expression pattern of dGATAa (Winick et al., 1993), but ABF is more restricted in its lateral extent. During germ band extension (Fig. 5D-F), ABF transcript levels decline in the anterior and posterior midgut and the amnioserosa, but expression continues to be observed in the cephalic mesoderm. The bilaterally symmetric plates of the cephalic mesoderm are clearly visible in Fig. 5F.

Immediately before the onset of germ band retraction, ABF-expressing mesodermal cells appear in segmentally repeating clusters. This pattern is reminiscent of the mesodermal expression of *nautilus* in clusters of unfused myogenic cells at the same developmental stage (Michelson et al., 1990), although the ABF-positive cells appear to be deeper within the mesoderm and are found in lateral but not medial positions. As the mesodermal expression of ABF appears in the germ band, that in the cephalic region begins to decay and midway through germ band retraction is no longer detected (Fig. 6A,B). During shortening of the germ band, however, the segmental groups of ABF-expressing cells persist, with a particularly large cluster observed in the posterior segments (Fig. 6B). After shortening is completed, the ABF-positive clusters expand and coalesce into a continuous sheet along the lateral wall of the embryo (Fig. 6C,D). This sheet of cells resides between the gut and the somatic muscles and is periodically interrupted by the emergence of tracheal branches (Fig. 6D). These anatomical features are characteristic of the maturing fat body (Hartenstein and Jan, 1992; Rizki, 1978; Rizki and Rizki, 1978). That ABF is expressed in the fat body at this stage is further supported by the coincident expression pattern of *Adh* (Fig. 7E), a known marker of the fully differentiated fat body (Lockett and Ashburner, 1989). It remains to be determined if all fat body cells express both of these markers. Expression of the *Drosophila* homolog of HNF-4 is also seen in the mature fat body in addition to a variety of other tissues (Zhong et al., 1993). Given this final localization and the apparent evolution of ABF expression within the germ band mesoderm, we infer that the earlier segmental clusters of ABF-positive cells represent fat body precursors. The segmental origin of the larval fat body, despite its final form as an apparent contiguous mass of cells that lacks morphologically distinct boundaries, has been suggested previously based on its altered development in homeotic mutants (Rizki and Rizki, 1978).

DISCUSSION

ABF is a *Drosophila* GATA family member

In this paper, we describe the cloning and characterization of ABF, a novel *Drosophila* member of the GATA family of transcriptional regulatory proteins. Given its membership in this family and the identification of another *Drosophila* member of the GATA family, dGATAa (Winick et al., 1993), we propose that ABF be called dGATAb. Unlike its mammalian counterparts, ABF contains a single zinc finger which is most similar to the C-terminal finger found in two-finger GATA proteins. Single-finger GATA proteins have also been identified in *Aspergillus* (Kudla et al., 1990) and *Neurospora* (Fu and Marzluf, 1990). ABF binds to positive regulatory elements found in larval *Adh* promoters and activates transcription. The DNA-binding domain of ABF is most similar to mammalian GATA proteins and, indeed, ABF binds to the GATA site present in the erythropoietin receptor promoter. Reciprocally, mGATA-1 binds to box A.

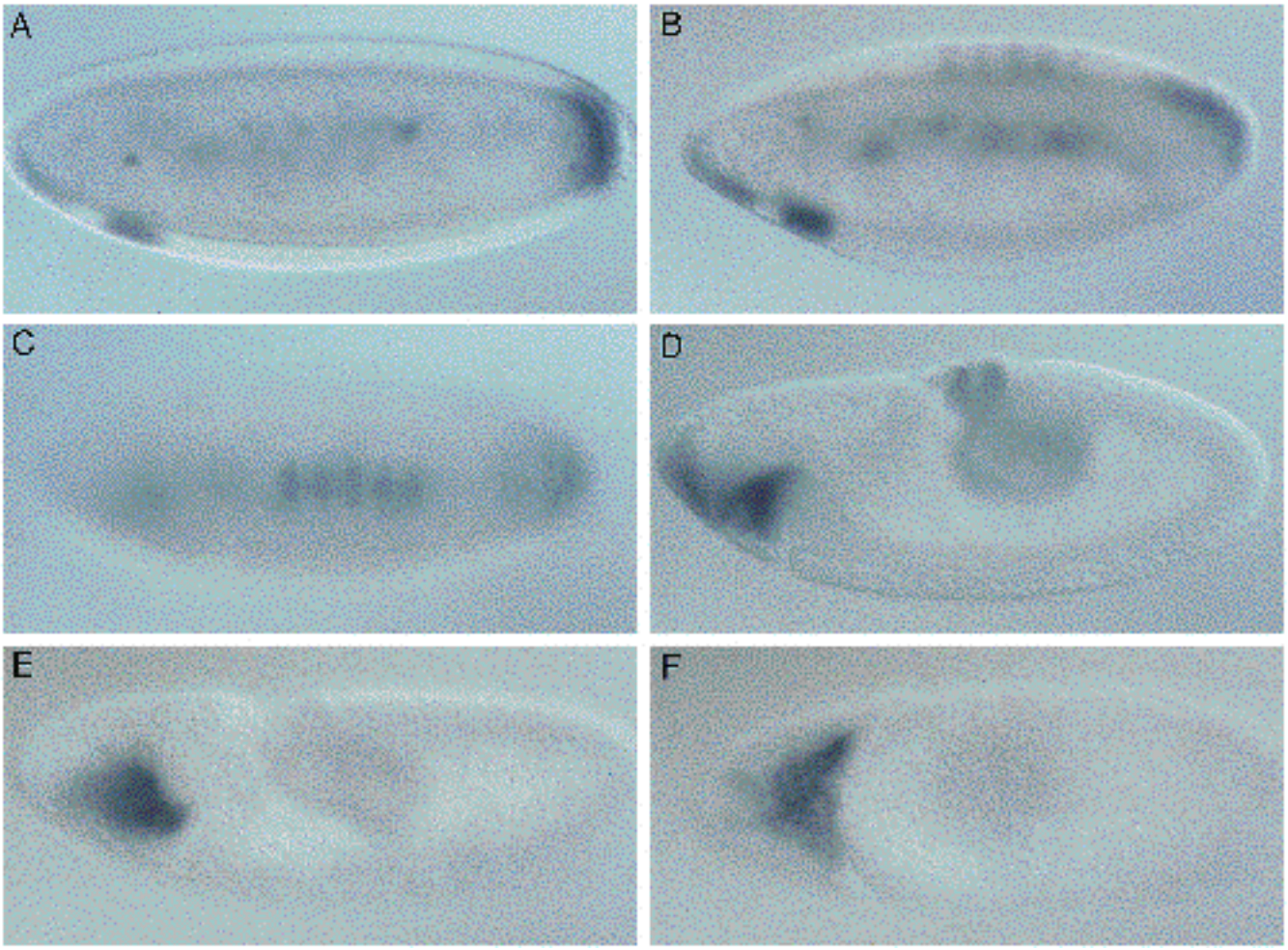


Fig. 5. Early in embryogenesis ABF is expressed in a variety of tissues. In situ hybridization to whole-mount embryos with digoxigenin-labeled probe (Tautz and Pfeifle, 1989) was used to determine the spatial distribution of ABF mRNA in embryos of various stages. The product of the alkaline phosphatase reaction indicates the presence of ABF transcripts. Embryos were staged according to Campos-Ortega and Hartenstein (1985). In all views, anterior is to the left. Lateral views, with dorsal at the top, are shown in A,B,D and E; a dorsal view is shown in C; a ventral view is shown in F. (A) ABF expression first appears just following cellularization (late stage 5) in the anterior and posterior midgut primordium, the vitellophages, and the primordium of the cephalic mesoderm. Note the unstained pole cells, which are the germ line precursors, at the posterior pole. (B,C) As gastrulation begins and the ventral furrow forms (stage 6), ABF transcripts are detected in the amnioserosa. (D) During germ band elongation, staining for ABF becomes weaker in the anterior and posterior midgut and amnioserosa, but continues at high levels in the cephalic mesoderm. (E,F) At the completion of germ band extension, ABF transcripts are seen only in the cephalic mesoderm.

During mammalian development, the four members of the GATA family are expressed in a variety of tissues (Orkin, 1992). GATA-1 was originally isolated as an erythroid cell nuclear protein that recognizes a sequence motif found in globin gene promoters and enhancers (Evans and Felsenfeld, 1989; Tsai et al., 1989). The cell-type specificity of this factor suggested an important developmental role and, indeed, GATA-1 is required for erythroid cell development (Pevny et al., 1991). GATA-2 is more ubiquitous in its expression (Yamamoto et al., 1990), whereas GATA-3 is expressed at high levels in the nervous system and in T cells (Ho et al., 1991; Joulin et al., 1991; Ko et al., 1991; Yamamoto et al., 1990). GATA-4, recently identified, is expressed in a limited set of tissues, which include tissues of endodermal and mesodermal origin (Arceci et al., 1993; C. Kelly and L. Zon, personal communication).

During *Drosophila* development, two GATA factors, dGATAa (Winick et al., 1993) and ABF (dGATAb), are expressed in a limited range of tissues, suggestive of a developmental role for these proteins. dGATAa is initially expressed just after cellularization in the dorsalmost ectodermal region of the embryo. As development continues, dGATAa transcripts are observed within the dorsal epidermis. ABF transcripts are initially observed in the anlagen of the anterior and posterior midgut and the cephalic mesoderm. Like GATA-4, ABF is found in both the endodermal and mesodermal germ layers. For a brief period during gastrulation, ABF is expressed in the amnioserosa, in a subset of the portion of the dorsal ectoderm in which

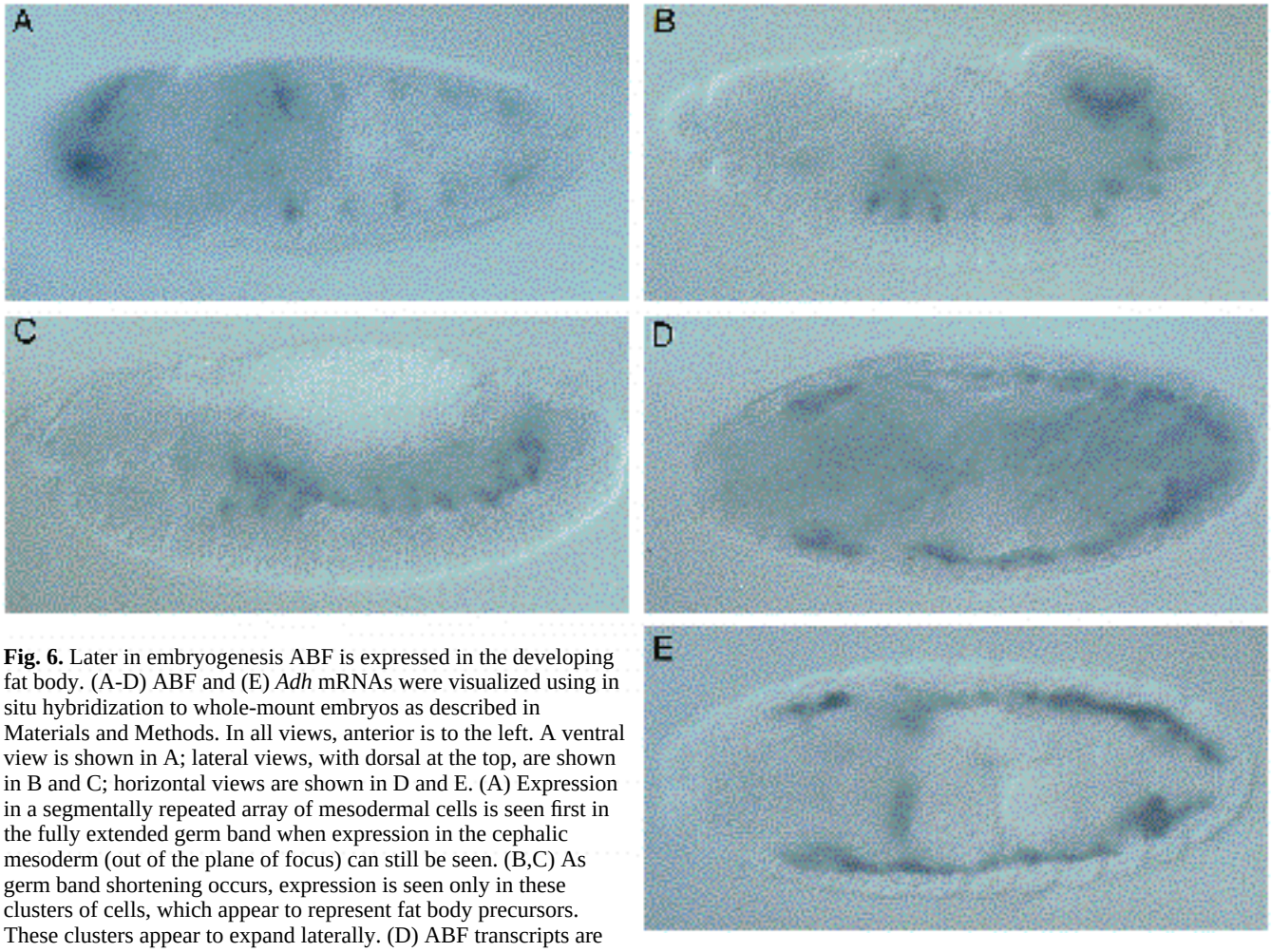


Fig. 6. Later in embryogenesis ABF is expressed in the developing fat body. (A-D) ABF and (E) *Adh* mRNAs were visualized using in situ hybridization to whole-mount embryos as described in Materials and Methods. In all views, anterior is to the left. A ventral view is shown in A; lateral views, with dorsal at the top, are shown in B and C; horizontal views are shown in D and E. (A) Expression in a segmentally repeated array of mesodermal cells is seen first in the fully extended germ band when expression in the cephalic mesoderm (out of the plane of focus) can still be seen. (B,C) As germ band shortening occurs, expression is seen only in these clusters of cells, which appear to represent fat body precursors. These clusters appear to expand laterally. (D) ABF transcripts are present in the mature fat body of a stage 15 embryo. (E) At this same stage, *Adh* transcripts are observed exclusively in the fat body.

dGATAa is expressed. Thus the expression patterns of ABF and dGATAa partially overlap. ABF is more restricted in its lateral extent, however, which is consistent with the later brief localization of ABF transcripts to the amnioserosa. During germ band retraction, ABF expression becomes restricted to the fat body, a tissue of mesodermal origin which expresses *Adh* at high levels.

The role of GATA sites in fat body-specific expression

Our studies of ABF have demonstrated that this protein binds to the sequence $^{T}/_{A}$GATA present within box A of the *D. mulleri Adh-1* gene and within the proximal promoter of the *D. melanogaster Adh* gene. Mutagenesis experiments using either P-element transformation (Corbin and Maniatis, 1990; Fischer and Maniatis, 1988) or somatic transformation (Shen et al., 1991) have shown that the region of these promoters containing this sequence is required for expression in vivo. Further, DNase I footprinting studies have revealed that this region within the proximal promoter is recognized by a factor found in embryonic (Moses et al., 1990) and K_c cell nuclear extracts (Heberlein et al., 1985). Together, these data suggest that the ABF-binding site is recognized by a positive regulatory factor. Our results are consistent with the possibility that ABF is this transcriptional activator: ABF binds to these positive regulatory elements, functions as a transcriptional activator and is expressed in the embryonic fat body.

In addition to being required for expression in all tissues, box A has the interesting property of expanding the tissue specificity of box B, a fat body-specific enhancer (Fischer and Maniatis, 1988). Our studies have shown that ABF is expressed in the fat body and thus it may play a role in regulating expression from box A in this tissue. The factor that mediates expression in the gut and Malpighian tubules remains an open question. One possibility is that ABF is expressed in these tissues during larval development and future studies will explore the larval expression pattern of ABF using appropriate antibodies. Alternatively, other GATA family members may regulate expression in these tissues. In this context, it is interesting to note that on certain promoters, mammalian GATA-1 functions synergistically with other factors (Zon et al., 1991) in a way analogous to the interaction observed between box A and box B. Lastly, box A, as defined by linker scanning mutagenesis experiments (Fischer and Maniatis, 1988) extends beyond the GATA site. Therefore, proteins recognizing additional

sequences within this element may also mediate the action of box A in a variety of tissues.

A large number of *Adh* genes have been cloned from a variety of *Drosophila* species and all of these genes contain the sequence $^{T}/_{A}$GATAA within the larval promoter around position −70. In addition to *Adh*, six major polypeptides, P1, P6, LSP-1α, LSP-1β, LSP-1γ and LSP-2, are synthesized in the larval fat body (Delaney et al., 1987; Maschat et al., 1990; Rat et al., 1991). The regions required for the proper expression of some of these genes in vivo have been mapped (Delaney et al., 1987; Maschat et al., 1991), and in all cases these elements include a sequence that matches the GATA consensus found in the larval *Adh* promoters. In this way, the GATA regulatory element may be a common motif involved in mediating the expression of genes within the larval fat body.

ABF is the earliest known marker of fat body differentiation

The larval fat body, which is fully formed late in embryogenesis, has been described from morphological studies of wild-type embryos (Rizki, 1978) and enhancer trap lines (Hartenstein and Jan, 1992) as a loose sheet of cells located between the gut and the somatic musculature. Because of the lack of a suitable marker, the early development of the fat body has remained obscure. Staining for a variety of genes, including *Adh* (Lockett and Ashburner, 1989), and analysis of a variety of enhancer trap lines (Hartenstein and Jan, 1992) have revealed only the pattern of the mature tissue. By comparing the morphology of the larval fat body in wild-type and homeotic bithorax mutant larvae, Rizki and Rizki (1978) concluded that the larval fat body has a segmental origin whose organization is dependent upon the activity of genes within the bithorax complex.

ABF transcripts are present in the developing and fully differentiated fat body. The latter pattern is very similar to that of *Adh* in the later embryo. However, we cannot determine directly whether ABF, or indeed *Adh*, are expressed in all cells of the fat body. ABF expression within the germ band mesoderm is first observed during germ band retraction in segmentally repeated clusters of cells. These clusters appear to lie deeper within the mesoderm than do the unfused myogenic cells which express *nautilus* (Michelson et al., 1990), consistent with the anatomical location of the mature fat body. During shortening, the ABF-expressing clusters expand and coalesce, thus forming the continuous sheet of cells which is the mature fat body. The isolation and characterization of mutations in the ABF locus will be required to investigate the role that ABF plays in *Adh* expression and in fat body development. Our analysis of the expression pattern of ABF during development thus provides the most detailed description of the genesis of the fat body available, and this gene will provide a useful reagent to analyze the role of homeotic genes in the development of the mesoderm. Furthermore, the expression pattern of ABF, along with its identification as a member of the GATA family of transcriptional regulatory proteins, points to an important role for ABF in the organogenesis of a variety of tissues including the midgut and the fat body.

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