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Genetics

Cloning of *Drosophila* transcription factor Adf-1 reveals homology to Myb oncoproteins

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ABSTRACT **The *Drosophila* sequence-specific DNA binding protein, Adf-1, is capable of activating transcription of the alcohol dehydrogenase gene, *Adh*, and is implicated in the transcriptional control of other developmentally regulated genes. We have cloned the cDNA encoding Adf-1 by generating specific DNA probes deduced from partial amino acid sequence of the protein. Several cDNA clones encoding an extended open reading frame were isolated from a phage λ library. The complete amino acid sequence of Adf-1 deduced from the longest cDNA reveals structural similarities to the putative helix–turn–helix DNA binding motif of Myb and Myb-related proteins. DNA sequence analysis of genomic clones and Northern blot analysis of mRNA suggest that Adf-1 is a single-copy gene encoding a 1.9-kb transcript. Purified recombinant Adf-1 expressed in *Escherichia coli* binds specifically to Adf-1 recognition sites and activates transcription of a synthetic *Adh* promoter *in vitro* in a manner indistinguishable from the protein purified from *Drosophila*. Temporally staged *Drosophila* embryos immunochemically stained with affinity-purified anti-Adf-1 antibodies indicate that Adf-1 protein is not detectable in very early embryos and does not appear to be maternally inherited. During later stages of embryogenesis, Adf-1 appears to be expressed in the nucleus of most somatic cells in the embryo with possibly higher concentrations found in some tissues.**

Recent advances in the biochemical and genetic analysis of *Drosophila melanogaster* gene regulation have established that transcriptional initiation is a primary mechanism controlling patterns of gene expression during development and cellular differentiation. The cis regulatory DNA elements and trans-activating proteins of many *Drosophila* genes have subsequently been identified. One class of transcriptional regulators, the sequence-specific DNA binding factors, have been of particular interest because they are responsible in large measure for governing the temporally programmed and spatially restricted patterns of gene expression during *Drosophila* embryogenesis (1). For example, the *Drosophila* alcohol dehydrogenase gene, *Adh*, is transcribed in a tissue-specific and temporally regulated manner during the life cycle of the fly (2). The expression of *Adh* in *Drosophila* is directed in an unusual and complex manner by two tandemly arrayed promoters, distal and proximal, that are 700 base pairs (bp) apart. These two promoters are activated in different cell types and are subject to different temporal programs (3, 4). The cis-controlling DNA elements responsible for mediating temporal and tissue-specific expression of *Adh* have been mapped, both by introducing altered gene sequences into *Drosophila* by *P*-element-mediated transformation (5–8) and by transcription of mutant templates either *in vitro* or following transfection into *Drosophila* cells (9–11). A complex assortment of distinct promoter and enhancer elements was found to be required for regulated transcriptional initiation from the distal and proximal *Adh* promoters.

In vitro transcription and DNA binding studies using extracts derived from temporally staged *Drosophila* embryos or established tissue culture cell lines have identified multiple site-specific transcription factors that recognize and bind to the cis-controlling elements of the *Adh* promoter (9, 12). These studies have revealed that one of these transcription factors, Adf-1, binds to and activates transcription from a specific upstream recognition site in the distal *Adh* promoter. Recently, we succeeded in purifying Adf-1 to homogeneity and found that the purified protein also binds specifically to DNA sequences in the promoters of several other developmentally regulated *Drosophila* genes, including the Antennapedia (*Antp*) P_1 promoter and the dopa decarboxylase gene promoter (10). The next step toward achieving a more comprehensive biochemical and molecular characterization of Adf-1 required cloning the gene encoding Adf-1. Here, we report the isolation and sequence† of the gene encoding *Drosophila melanogaster* Adf-1. In addition, we have raised antibodies against this transcription factor and determined the temporal and spatial patterns of Adf-1 expression during different stages of *Drosophila* embryogenesis.

MATERIALS AND METHODS

HPLC Purification of Adf-1 and Peptide Sequencing. Adf-1 was affinity-purified from *Drosophila* embryos as described (10). An estimated 2.4 μg of affinity-purified Adf-1 was alkylated with 4-vinylpyridine and digested with trypsin, and the partial amino acid sequence of several tryptic peptides was determined—all as described (13).

Isolation and Analysis of Genomic and cDNA Clones. Oligodeoxynucleotide probes designed according to considerations described previously (14, 15) using *Drosophila* codon preferences (16) were prepared from the sequences of peptides 4 and 5 (see Fig. 1). The sequence of probe 1 (peptide 4) was 5′-GTACTTGTTGTGCTGGGCCAGCAGCTGCATGTCGGTCAGGTA-3′ and the sequence of probe 2 (peptide 5) was 5′-CTGGTCCTCGGCIGAIACGGCCTGIGAIACGTTGTTCTGGAAGAT-3′ (I represents inosine). These probes were used to screen a *Drosophila* genomic library in λFIX (Stratagene). Two stringent washes were with 0.30 M NaCl/0.03 M Tris, pH 7.5/5 mM EDTA/0.5% SDS for 30 min at 58°C (probe 1) or 54°C (probe 2).

To isolate Adf-1 cDNA clones, a high-specific-activity single-stranded DNA probe was prepared from a subclone of genomic clone 1. This probe was used to screen a phage λgt11 cDNA library prepared from mRNA isolated from 9- to 12-hr-old *Drosophila* embryos (17). The three largest clones obtained (numbers 4, 19S, and 22) were cloned in both

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Abbreviation: Adh, alcohol dehydrogenase.

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†The sequence reported in this paper has been deposited in the GenBank data base under the name DROADF1A (accession no. M37787).

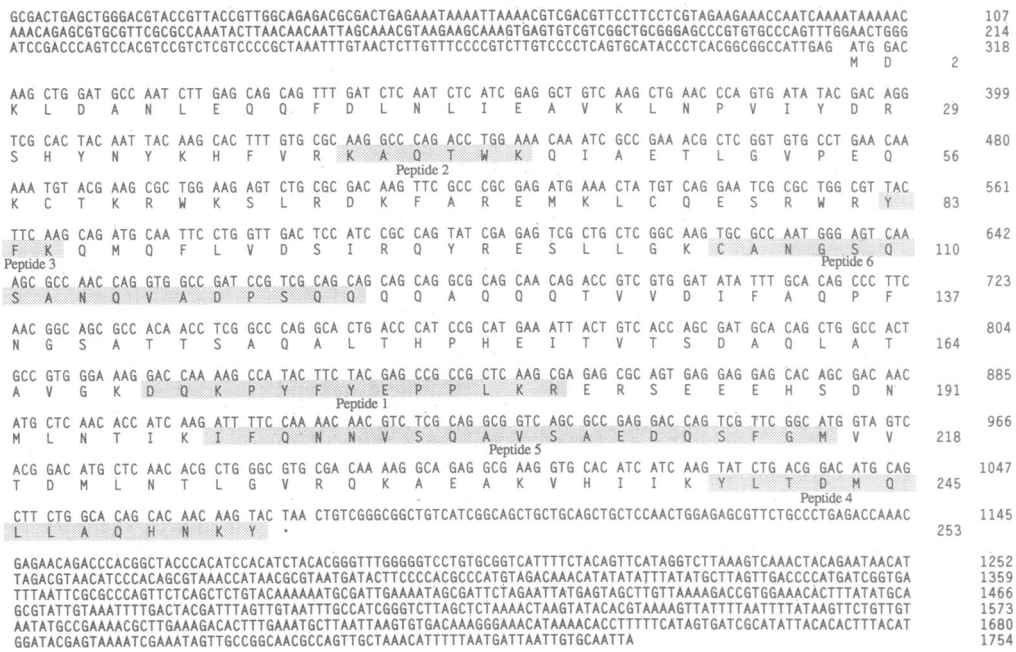

FIG. 1. cDNA and amino acid sequence of Adf-1. The complete insert of phage λ cDNA clone 19-S was sequenced on both strands, and the consensus sequence is shown. The deduced Adf-1 peptide sequence is shown below the DNA sequence. Adf-1 tryptic peptides are indicated by shading.

orientations into the *Eco*RI site of pBluescript SK(+) (Stratagene), and the DNA sequences of the ends of these clones were determined by using Sequenase (United States Biochemical). The complete sequence of clone 19S was determined from a 5′ and a 3′ series of nested deletions prepared with exonuclease III (18).

Expression of Adf-1 in *Escherichia coli*. As described previously for two other *Drosophila* transcription factors (13), the coding portion of Adf-1 cDNA clone 19S was placed in the T7 RNA polymerase expression vector pET-3a (19), and the resulting plasmid, pET-3a/Adf-1, was transformed into *E. coli* strain BL21 (20). Adf-1 expression was induced, and bacterial protein extracts were prepared as described (13). Adf-1 was purified by passing the bacterial extract directly over an Adf-1 affinity resin prepared and run as described (10). DNase I footprinting reactions and *in vitro* transcription reactions with this purified recombinant Adf-1 were performed as described (10).

Antibody Studies. Rabbits were immunized by subcutaneous injection of 100 μg of recombinant Adf-1 suspended in 500 μl of phosphate-buffered saline (PBS) and 500 μl of Freund's complete adjuvant. Rabbits were given three boosters of 100 μg of Adf-1 in incomplete adjuvant at 3-week intervals. Antisera used for the experiments shown were obtained 1 or 2 weeks after administration of the third booster. To affinity-purify Adf-1 antibodies, 100 μg of affinity-purified, recombinant Adf-1 was coupled to 600 μl of Affi-Gel 10 (Bio-Rad) according to the manufacturer's recommendations. Adf-1 antiserum was diluted 3-fold with PBS, applied to the affinity resin, and washed with PBS. Bound antibodies were eluted with 100 mM glycine (pH 2.3) and neutralized immediately after elution with 1/10th volume of 2 M Tris (pH 8.0). Immunohistochemical staining of whole *Drosophila* embryos was performed as described (21).

RESULTS AND DISCUSSION

Isolation and Sequencing of Adf-1 Genomic and cDNA Clones. To clone the gene and cDNA coding for Adf-1, we first determined the amino acid sequences of several Adf-1 tryptic peptides. The amino acid sequences of tryptic peptides 4 and 5 (see Fig. 1) were chosen to generate the synthetic DNA probes used to screen a *Drosophila* genomic library. Our initial screen identified two phage λ clones that hybridized strongly to both Adf-1 probes. Partial DNA sequence analysis confirmed that both of the genomic clones isolated contained the coding sequences for the two peptides that had been used to generate the probes as well as the coding sequence for peptide 1.

To isolate Adf-1 cDNA clones, a DNA probe was prepared from a fragment of genomic clone 1 and used to screen a λgt11 cDNA library. We isolated eight cDNA clones that, by restriction analysis, all appeared to be derived from the same message (data not shown). Sequence analysis of the ends of the three longest cDNA clones confirmed that they were derived from the same transcript.

A Northern blot of embryo mRNA hybridized with a probe made from the genomic clone reveals a single Adf-1 message of ≈1.9 kilobases (kb) (data not shown), and the lengths of seven of the eight cDNA clones isolated are in the range of 1.5–1.8 kb. Therefore, it seemed likely that the largest of these clones, at ≈1.8 kb, was a nearly complete copy of the Adf-1 mRNA. The complete DNA sequence of this clone, 19S, was determined and is shown in Fig. 1. This cDNA includes a long open reading frame that, when translated, contains the sequences of all of the Adf-1 tryptic peptides that had been determined by peptide sequencing, thus providing strong evidence that we have indeed cloned the cDNA for Adf-1. Between peptide 2, the most N-terminal of these peptides, and the first in-frame upstream stop codon (nucleotides 256–258) there is only one methionine codon, which we have designated as the initiation codon of the Adf-1 protein. The polypeptide shown has a calculated molecular weight of 29.2 kDa, which is in close accord with Adf-1's apparent molecular mass of 34 kDa based on SDS/PAGE (see Fig. 3).

The cytological locus of the *Adf-1* gene was determined by hybridizing a biotinylated *Adf-1* genomic probe to salivary gland polytene chromosome squashes and visualizing the annealed probe by biotin-specific histochemical staining (22). The *Adf-1* clone hybridized to a single cytological locus,

42C2-7 (data not shown). No previously identified *Drosophila* genes have been mapped to this interval (23).

Comparison of the Adf-1 sequence with available sequences in the GenBank and National Biomedical Research Foundation–Protein Identification Resource databases revealed potentially interesting similarities between Adf-1 and other proteins. First, the peptide sequence between amino acid residues 44 and 63 has some homology to a bacterial helix–turn–helix DNA-binding domain. Although this homology appears somewhat weak, when it is analyzed by a statistical method designed to evaluate the similarity of any protein sequence to the phage λ Cro type helix–turn–helix domain (24), we find that this segment of Adf-1 is rated as more similar to the Cro-type binding domain than are the homeodomains of the products of *Ubx, en,* and *ftz*. These *Drosophila* homeodomains form a helix–turn–helix-type structure (25, 26) but show almost no similarity to the potential helix–turn–helix domain of Adf-1. Second, it has been proposed that the DNA-binding domains of the Myb oncoproteins and other related proteins also adopt a helix–turn–helix tertiary structure (27). In contrast to the homeodomain, the proposed Myb helix–turn–helix domain shows significant similarities to the potential helix–turn–helix domain of Adf-1 (Fig. 2). Although a gap must be inserted between Adf-1 residues Leu-52 and Gly-53 to optimize the alignment of Adf-1 with the Myb sequences, this same gap must be introduced into both the bacterial helix–turn–helix consensus and the homeodomain consensus to align them with Myb sequences (27). It is interesting to note that the best alignment was observed between Adf-1 and REB1, a Myb-related yeast transcription factor involved in transcription of rRNA (30). Although the amount of amino acid identity between Adf-1 and the prokaryotic helix–turn–helix proteins and the Myb family of proteins is somewhat limited, it occurs primarily at highly conserved positions that have been identified as important for maintaining a helix–turn–helix structure by virtue of hydrophobic interactions with the protein core (25).

Another noteworthy feature of the Adf-1 protein sequence is the large number of glutamine residues in the protein (11.1%) and the presence of a polyglutamine run interrupted by a single alanine residue from amino acid 120 through amino acid 127. Glutamine-rich protein segments have been shown to direct transcriptional activation by the human transcription factors Sp1 and Oct-2 (31, 32). Mutational analysis will be required to test whether the glutamine-rich sequence of Adf-1 serves the same function.

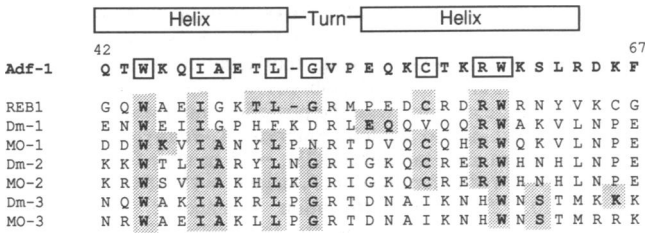

FIG. 2. Comparison of Adf-1 with portions of the DNA-binding repeats of Myb proteins and the Myb-related region of yeast transcription factor REB-1. The Adf-1 sequence from amino acid residue 42 to residue 67 is shown aligned with parts of the repeats of *Drosophila* c-Myb (Dm) (28) and mouse c-Myb (MO) (29) and to yeast REB1 (30). Residues identical to the corresponding Adf-1 residue are indicated by shading. Boxed residues in the Adf-1 sequence indicate amino acid identity with the majority of the sequences shown. Dashes indicate gaps inserted into the sequences to optimize alignment. The numbers 1, 2, or 3 after the Myb protein abbreviations indicate first, second, or third Myb repeat, respectively. The helix–turn–helix motif shown is that previously proposed for Myb proteins (27).

DNA Binding and Transcription Properties of Recombinant Adf-1. To produce large amounts of Adf-1 for biochemical studies, we expressed the *Adf-1* gene in *E. coli* using a bacteriophage T7 RNA polymerase expression system (20). Upon induction with isopropyl β-D-thiogalactoside, a new polypeptide of ≈34 kDa appeared in crude lysates of cells carrying the *Adf-1* expression plasmid but not in control lysates from cells bearing the vector alone. Crude extracts from the induced bacteria were passed directly over an Adf-1 DNA-binding affinity column and were eluted with a high-salt buffer, yielding a single polypeptide with a mobility on SDS/PAGE identical to that of *Drosophila* Adf-1 (Fig. 3*A*).

The purified recombinant Adf-1 was tested for the characteristic Adf-1 DNA-binding activity in a DNase I footprint assay and was found to bind to the *Adh* distal promoter Adf-1 site with a pattern identical to that of the native *Drosophila* protein (Fig. 3*B*). Since recombinant Adf-1 appeared to bind to its recognition element with the same specificity as the authentic *Drosophila* protein, we next tested its ability to activate transcription in an Adf-1 site-dependent manner. Using an Adf-1-depleted extract described (10), *in vitro* transcription reactions were carried out with DNA templates containing one or three copies of an Adf-1 binding site. Addition of recombinant Adf-1 stimulated *in vitro* transcription to approximately the same extent (5- to 8-fold) as was previously observed with *Drosophila* Adf-1 (Fig. 4). These results establish that the cDNA clone we isolated codes for an *Adf-1* product that is active for both DNA binding and promoter selective transcription. The demonstration that purified recombinant Adf-1 has the same biochemical properties as the purified *Drosophila* protein confirms the finding that the 34-kDa Adf-1 protein is the essential component required for Adf-1 site-dependent transcriptional activation.

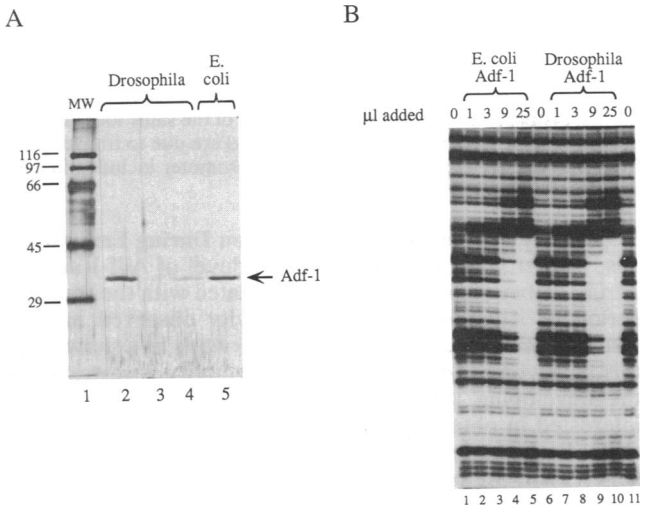

FIG. 3. Expression of Adf-1 in *E. coli*. (*A*) SDS/8% polyacrylamide gel comparing purified *E. coli*-expressed Adf-1 with Adf-1 purified from *Drosophila* embryos. Lanes: 1, molecular weight standards, 100 ng of protein per band; 2, 5 μl of affinity-purified embryo Adf-1 estimated at 2 DNase I footprint units/μl; 3 and 4, 1 and 5 μl of a fraction of affinity-purified embryo Adf-1 of undetermined activity; 5, 1 μl of affinity-purified *E. coli*-expressed Adf-1 estimated at 20 DNase I footprint units/μl. (*B*) DNase I footprints of purified *E. coli* expressed Adf-1 and purified *Drosophila* embryo Adf-1. Protein samples shown in lanes 2 and 5 of *A* were diluted by factors of 50 and 500, respectively (to estimated concentrations of 0.32 and 0.14 μg/ml, respectively), and assayed for Adf-1-characteristic footprinting on the *Adh* distal promoter. Lanes: 1, 6, and 11, DNase I digestion pattern in the absence of added Adf-1; 2–5, DNase I footprint due to increasing amounts of *E. coli*-expressed Adf-1, as indicated at the top; 7–10, DNase I footprint due to increasing amounts of embryo Adf-1, as indicated at the top.

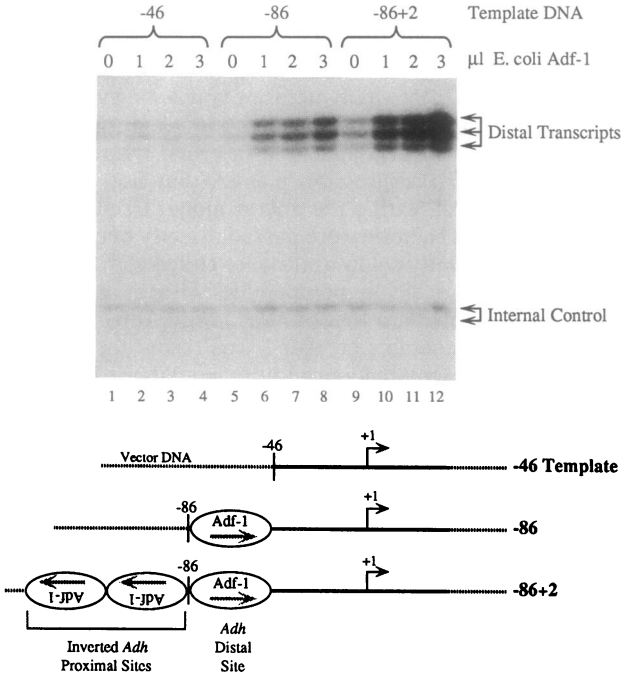

FIG. 4. *In vitro* transcriptional activation by *E. coli*-expressed Adf-1. Twenty-five-microliter transcription reaction mixtures contained 50 ng (33 fmol) of the indicated distal promoter template DNA, 200 ng of pADHP.Δ5′-55 internal control DNA, 9.5 μl of 3× Adf-1-depleted embryo nuclear extract (20 mg of protein per ml), and purified *E. coli*-expressed Adf-1 as indicated. The concentration of the Adf-1 used was estimated to be 7 μg/ml. Ovals in template diagrams indicate sequences protected by Adf-1 in DNase I footprint reactions. Lanes: 1–4, bands marked "Distal Transcripts" are due to transcription from the −46 distal promoter template in the presence of 0, 1, 2, and 3 μl of Adf-1 as indicated at the top of the lanes; 5–8, transcription from the −86 template in the presence of the same four amounts of Adf-1; 9–12, transcription from the −86+2 template (which contains two inserted Adf-1 binding sites in addition to the normal distal promoter site) in the presence of the same four amounts of Adf-1. Internal control bands in lanes 1–9 are due to transcription from the pADHP.Δ5′-55 internal control promoter included in each reaction.

Antibody Studies of Adf-1 Expression During Embryogenesis. Earlier work suggested that the level of Adf-1 activity during embryogenesis might be correlated with the transient period of *Adh* distal promoter activity observed midway through embryogenesis (12). Further work has shown that this embryonic *Adh* distal promoter activity is restricted to the developing larval fat body (3). Therefore, we were interested in obtaining antibodies directed against Adf-1 to study both the levels of Adf-1 during *Drosophila* embryogenesis and the embryonic tissues in which it is expressed.

Rabbits were immunized with SDS/PAGE-purified recombinant Adf-1, and antibodies of high titer and specificity were obtained (data not shown). To further increase the specificity of these antibodies, they were affinity-purified by passage over a resin of purified Adf-1 coupled to agarose beads. The affinity-purified anti-Adf-1 antibodies were shown by Western blotting to still bind specifically to Adf-1 when present in a crude nuclear extract from *Drosophila* K_c cells (data not shown).

To examine the spatial pattern of Adf-1 expression during embryogenesis, we immunostained a collection of whole *Drosophila* embryos from 0 to 14 hr old. Fig. 5*A* shows the pattern of Adf-1 expression over the time course of embryogenesis from the cellular blastoderm stage, ≈2.5 hr after fertilization (embryo 1), to the stage just prior to dorsal closure, ≈11 hr after fertilization (embryo 7). Adf-1 appears to be distributed in most cells of these embryos with the possible exception of the amnioserosa (unstained, dorsally located region in embryos 5, 6, and 7). However, since Adf-1

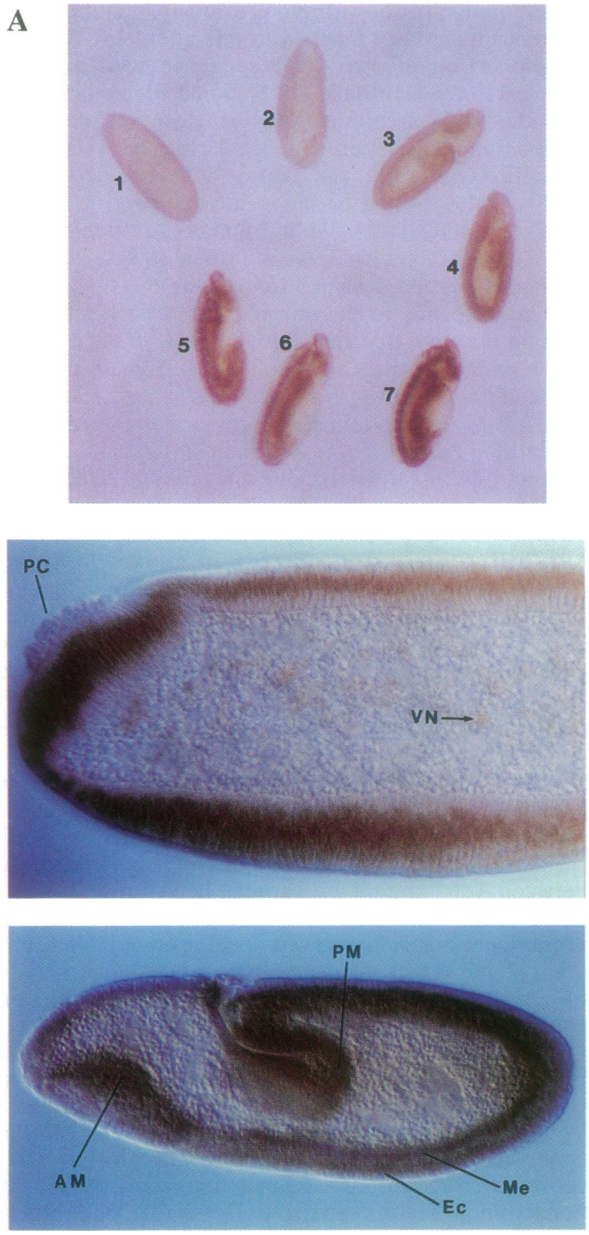

FIG. 5. Immunohistochemical staining of Adf-1 during embryogenesis. Embryos (0–14 hr old) were dechorionated, fixed, devitellinized, and then incubated with a 1:4000 dilution of affinity-purified anti-Adf-1 antibodies followed by 3,3′-diaminobenzidine staining. (*A*) Time course of Adf-1 staining. Individual embryos from representative stages are shown. All embryos (with the possible exception of embryo 1) are oriented with the posterior end toward the bottom and the ventral side to the left. Embryos: 1, embryo at stage 5 of Campos–Ortega and Hartenstein (33), ≈2.5 hr after fertilization; 2, stage 6–7, ≈3 hr after fertilization; 3, stage 9, ≈4 hr after fertilization; 4, stage 10, ≈5 hr after fertilization; 5, stage 12, ≈8.5 hr after fertilization; 6, stage 13, ≈10 hr after fertilization; 7, stage 14, ≈11 hr after fertilization. (*B*) Optical section through gastrulating embryo (stage 6), ≈3 hr after fertilization. The embryo is oriented with the posterior to the left and ventral surface to the bottom. Nuclear localization of Adf-1 is most clearly seen along the ventral side. VN, possible vitellophage nucleus staining positively for Adf-1; PC, pole cells unstained for Adf-1. (*C*) Optical section through a germ band-extended embryo (stage 9), ≈5 hr after fertilization. Posterior end is to the right, and the ventral surface is to the bottom. Me, mesoderm; Ec, ectoderm; AM, anterior midgut primordium; PM, posterior midgut primordium.

is localized in the nucleus (as shown below), this lack of staining may not reflect an absence of Adf-1 in the amnioserosa but rather the relative paucity of nuclei in this thin monolayer. The increase in stain intensity observed with embryos of increasing age may be due to an increase in the intranuclear concentration of Adf-1, to cellular proliferation or to a combination of these two factors. However, the intranuclear concentration of Adf-1 was observed to definitely increase over the time between the syncytial blastoderm stage and gastrulation (data not shown).

Examination at higher magnification of Adf-1-stained embryos from the gastrulation [stage 6 of Campos–Ortega and Hartenstein (33)] and early germ-band extended (stage 9) stages confirms the observation that Adf-1 is distributed among almost all embryonic cells (Fig. 5 *B* and *C*). The nuclear localization of Adf-1 is most apparent along the ventral side of the optical sagittal section of the gastrulating embryo (Fig. 5*B*). The weak staining scattered throughout the yolk in this figure (indicated by an arrow) is probably due to Adf-1 in the vitellophage nuclei. The only nuclei that do not appear to contain appreciable Adf-1 at this stage are those in the pole cells, the germ-line primordia. Fig. 5*C* shows the pattern of Adf-1 staining after the formation of the three germ layers: ectoderm, mesoderm, and endoderm. Again, Adf-1 is present in all three cell types in comparable amounts. The striking double stripe pattern of Adf-1 staining most clearly seen along the ventral side is due to the regular arrangement of the nuclei of the ectoderm on the exterior and the nuclei of the mesoderm on the interior of the embryo. The anterior and posterior midgut primordia, indicated by arrows, are endodermal cells and also stain strongly for Adf-1.

The *Adh* distal promoter is active in embryos from 10 to 15 hr after fertilization, and this activity is restricted to the developing larval fat body (3). In contrast, the antibody data shows that Adf-1 is expressed at apparently constant levels from around 4 or 5 hr after fertilization onward, and this expression is distributed throughout the developing embryo. Therefore, it is unlikely that Adf-1 is the only factor required for the pattern of *Adh* distal transcription observed during embryogenesis. This conclusion is supported by the fact that *Adh* genes lacking the distal promoter Adf-1 site are nevertheless transcribed according to the correct temporal program during embryogenesis (8). However, these mutant *Adh* genes lacking the Adf-1 site are expressed at a much lower level than wild-type, demonstrating that Adf-1 plays an important role in regulating the level of *Adh* transcription.

We cannot at present explain the difference between the quantitative changes in Adf-1 footprinting activity during embryogenesis observed previously (12) and the more constant levels of Adf-1 protein observed over the same time period. This difference could reflect the presence of an activity in early and late embryo extracts that modulates the binding of Adf-1 to DNA. Alternatively, there could be a temporally programmed change in the affinity of Adf-1 for its binding site, controlled by some means such as protein modification.

Since Adf-1 binds to and may regulate transcription from the dopa decarboxylase gene promoter and the Antennapedia gene *P*1 promoter as well as the *Adh* distal promoter, it is not surprising that Adf-1 is found in many cells of the developing embryo. After all, these three genes have very different spatial and temporal patterns of embryonic expression. It remains to be determined just how widespread Adf-1-regulated promoters are in the *Drosophila* genome and what role, if any, Adf-1 plays in the orchestration of developmental gene expression. Perhaps Adf-1 acts in a manner analogous to the mammalian factor, Sp1, which is found to be essential for directing transcription of many genes, including cell-type specific genes, but is not itself cell-type specific. Instead, Sp1 appears to be a ubiquitous transcription factor that can act in conjunction with other tissue-specific enhancer proteins to specify unique transcription programs.

The best test of the role of Adf-1 in developmentally regulated gene expression would be to assess the transcriptional effect of mutations in the *Adf-1* gene. Such mutants, in conjunction with the Adf-1 clones, antibodies, and purified protein already available, and the powerful genetic and biochemical techniques available to *Drosophila* biologists, would make Adf-1 a useful model for addressing important questions regarding eukaryotic transcriptional regulation.

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