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Conserved Enhancer and Silencer Elements Responsible for Differential *Adh* Transcription in *Drosophila* Cell Lines

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The distal promoter of *Adh* is differentially expressed in *Drosophila* tissue culture cell lines. After transfection with an exogenous *Adh* gene, there was a specific increase in distal alcohol dehydrogenase (ADH) transcripts in ADH-expressing (ADH^+) cells above the levels observed in transfected ADH-nonexpressing (ADH^-) cells. We used deletion mutations and a comparative transient-expression assay to identify the *cis*-acting elements responsible for enhanced *Adh* distal transcription in ADH^+ cells. DNA sequences controlling high levels of distal transcription were localized to a 15-base-pair (bp) region nearly 500 bp upstream of the distal RNA start site. In addition, a 61-bp negative *cis*-acting element was found upstream from and adjacent to the enhancer. When this silencer element was deleted, distal transcription increased only in the ADH^+ cell line. These distant upstream elements must interact with the promoter elements, the Adf-1-binding site and the TATA box, as they only influenced transcription when at least one of these two positive distal promoter elements was present. Internal deletions targeted to the Adf-1-binding site or the TATA box reduced transcription in both cell types but did not affect the transcription initiation site. Distal transcription in transfected ADH^- cells appears to be controlled primarily through these promoter elements and does not involve the upstream regulatory elements. Evolutionary conservation in distantly related *Drosophila* species suggests the importance of these upstream elements in correct developmental and tissue-specific expression of ADH.

The alcohol dehydrogenase gene (*Adh*) of *Drosophila melanogaster* is transcribed from two promoters, each of which has separate developmental and tissue-specific patterns of expression (6, 44; reviewed in references 11 and 51). Because differential gene expression in eucaryotes can be controlled at the level of transcription initiation by interactions between *cis*-acting elements and sequence-specific *trans*-acting factors (16, 33), we have begun to characterize the *cis*- and *trans*-acting elements responsible for differential ADH expression.

Our model system for studying *Adh* gene regulation has been a collection of *Drosophila* cell lines (48) that show large (ADH^+) or barely detectable (ADH^-) expression of the *Adh* gene (4). Analysis of the mRNA in these cell lines indicated differing levels of ADH mRNA specific to the distal promoter, whereas the proximal transcript appeared to be entirely absent (4). Detailed characterization of the *Adh* locus in these cell lines has shown that the gene is intact (4, 16a; also unpublished observations). We have hypothesized that the observed levels of distal transcripts reflect differential distal promoter transcriptional activity in different cell lines, which may in turn result from differences in *trans*-acting factors present in these cells.

To test this hypothesis in vivo and to define the DNA sequences responsible for the differential pattern of expression in *Drosophila* cell lines, we used a transient-expression assay to study transcription of exogenous *Adh* templates in these cell lines. In this study we compared ADH expression from a plasmid containing all *cis*-acting elements required for normal patterns of distal *Adh* expression in flies (39) with that of a series of 5′ and internal deletion mutations. We have identified a 15-base-pair (bp) *cis*-acting element located 480 bp upstream from the distal RNA start site which is responsible for enhanced distal transcription in ADH^+ cells. In addition we have detected a negative *cis*-acting element upstream and adjacent to the enhancer element (−584 to −523), which when deleted results in increased distal transcription in ADH^+ cells. Within these elements, which are conserved in distantly related species, are sequences with close similarity to the binding sites of known DNA-binding proteins.

Using transient-transfection analyses of internal linker-replacement deletion mutations targeted to the distal TATA box (−32 to −26) and the binding site of the distal-specific transcription factor Adf-1 (−86 to −46) (20), we have shown that both behave as promoter elements in this tissue culture system. The enhancer- and silencer elements defined in this study act through these promoter elements to specifically regulate the level of correct distal transcription. These enhancers and silencer elements, however, do not appear to affect proximal (larval) promoter transcription, even in the absence of a functional distal promoter.

MATERIALS AND METHODS

Cell culture. Cell lines S2 and 1006-2 were grown in M3 medium (47, 48) supplemented with 10% fetal calf serum (Armour) and 50 μg of gentamicin (GIBCO) per ml at 23°C and split every 3 to 5 days at approximately 2×10^6 cells per ml.

Construction of plasmids. The plasmids p5′Xba3.2 and p3′Xba3.2 were derived from pXba3.2 (a gift of J. Posakony), which contains a 3.2-kilobase (kb) *Xba*I fragment of an Adh^F allele in pUC13. p5′Xba3.2 and p3′Xba3.2 each contain a mutation at one of the *Xba*I sites, leaving one intact *Xba*I site at the 5′ and 3′ end of the gene, respectively. These plasmids were constructed by partially digesting the plasmid pXba3.2 with *Xba*I, filling in the 5′ overhang with T4 DNA polymerase as described before (30), and circularizing the plasmid DNA with T4 DNA ligase.

A series of 5′ deletion mutations were produced from the plasmid p5′Xba3.2. p5′Xba3.2 (5 μg) was restricted with *Xba*I at the one remaining *Xba*I site at the 5′ end of the gene

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(−662) and digested with 1 U of Bal 31 (Bal 31 slow; International Biotechnologies Inc.) for 5 to 15 min at 30°C. The exonucleolytic digestion was terminated with addition of EGTA (ethylene glycol tetraacetic acid) to 20 mM, and DNA was purified by phenol-chloroform-isoamyl alcohol (PCI) extraction and ethanol precipitation. The DNA was blunt-ended with T4 DNA polymerase and 0.25 mM each of the four dNTPs. Following purification of the DNA, *Xba*I linker DNA (pGAGATCTC; New England Biolabs) was ligated to the blunt-end DNA, and multiple linkers were removed with subsequent *Xba*I digestion. DNA was digested with *Eco*RI, which cuts in the multiple cloning site beyond the 3′ end of the *Adh* gene. The DNA was separated by electrophoresis on 1% low-melting-point agarose (Sea Plaque; FMC Corp.), and the DNA fragment bearing the *Adh* gene was isolated. This DNA fragment was ligated into the vector pUC13, which was restricted in the multiple cloning site with *Xba*I and *Eco*RI. The extent of deletion was determined by sequencing the plasmid DNA with modified T7 DNA polymerase (Sequenase; US Biochemical Corp.) (54) according to the conditions specified by the manufacturer. The names of these plasmids reflect the extent of the 5′ deletion.

A series of 3′ deletion mutations were made from p3′Xba3.2 by a strategy similar to that for the 5′ deletion mutations. Appropriate 3′ and 5′ deletion mutations were combined to make the internal linker-replacement deletion mutations.

Plasmid preparation. Single colonies of *Escherichia coli* DH5α containing the plasmid of interest were used to inoculate 500 ml of SOB (30) containing ampicillin (50 μg/ml) and grown overnight at 37°C. Cells were harvested, and plasmid DNA was prepared by alkaline lysis (30). Supercoiled plasmid DNAs were purified by banding twice on cesium chloride-ethidium bromide gradients and dialyzed against TE (10 mM Tris hydrochloride [Tris-HCl, pH 7.5], 1 mM EDTA). DNA concentrations were determined by absorbance at 260 nm. Agarose gel electrophoresis was used to determine the percent supercoiled DNA and to confirm the concentration. Only samples with greater than 90% supercoiled DNA were used for transfections.

Transfection of *Drosophila* tissue culture cells. *Drosophila* cells were plated in T75 flasks at approximately 2×10^6 cells per ml and allowed to grow at 23°C for an additional 24 h. Only cells that appeared healthy and had been growing at a steady rate were used for transfections.

Calcium phosphate precipitates (2 ml) were prepared by first mixing 20 μg of *Adh*-containing plasmid DNA and 1 μg of pPAcLacZ (15) with 60 μl of 2 M $CaCl_2$ in a volume of 0.98 ml. This solution was then added dropwise to a second solution containing 1 ml of HEPES-buffered saline (0.05 M HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 0.28 M NaCl, pH 7.1) and 20 μl of 0.07 M sodium phosphate, pH 7.1. Mixing was accomplished by delivering a gentle, continuous stream of air through a 50-μl microcapillary pipette. The precipitate was allowed to form for 15 min. One half of the precipitate (1 ml) was added dropwise to each tissue culture flask containing ADH^+ *Drosophila* cells, and the other half was added to ADH^- cells to control for variation in precipitate quality.

After 48 h at 23°C, cells were harvested, and 2 to 3 ml was saved for protein extract after washing in 1 ml of ice-cold phosphate-buffered saline (M.A. Bioproducts). RNA was prepared from the remaining cells. Cell pellets were frequently frozen until protein or RNA could be isolated.

ADH enzyme assay. Cell pellets were thawed and diluted in 0.2 ml of phosphate-buffered saline containing 10% glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by sonication and centrifuged in a microfuge for 10 min. The clear supernatant was removed and kept on ice. Protein concentration was determined by Coomassie binding with the BioRad Laboratories protein determination kit, with bovine serum albumin as a standard. An amount of protein empirically determined to give quantitative results was used in the ADH enzyme assay: 10 to 50 μg for ADH^+ cells and 200 μg for ADH^- cells. Extract was added to 2.5 ml of a solution containing 100 mM Tris-HCl (pH 7.5), 1.25% 2-butanol, and 1.4 mg of NAD^+ per ml. The production of NADH was measured fluorometrically at time points between 1 and 15 min. ADH activity was determined as units of fluorescence per microgram of protein per minute.

β-Gal enzyme assay. From 10 to 25 μg of protein extract was diluted to 0.5 ml with 100 mM sodium phosphate (pH 7.0)–10 mM KCl–1 mM $MgSO_4$–50 mM β-mercaptoethanol. Then, 100 μl of 4 mg/ml *o*-nitrophenyl-β-D-galactoside (Sigma Chemical Co.) was added, the reactions were stopped after 15 to 30 min at 24°C by addition of 2 ml of 0.4 M sodium carbonate, and the absorbance at 420 nm was determined. β-Galactosidase (β-Gal) activity was defined as A_{420} units per microgram of protein per minute.

Isolation of RNA. Frozen cell pellets were thawed at room temperature and diluted in 0.3 ml of lysis buffer (15 mM Tris-HCl [pH 7.5], 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.3 M sucrose, 0.1 mM PMSF, 10 mM vanadyl-ribonucleoside complex [Bethesda Research Laboratories], and 0.5% Nonidet P-40) and were placed on ice for 10 min with periodic vortexing. Lysed cells were then layered onto a 0.3-ml, 1.7 M sucrose cushion containing lysis buffer (without PMSF, Nonidet P-40, or vanadyl-ribonucleoside complex) in a 1.5-ml microfuge tube. The interface was broken by stirring, and the samples were centrifuged for 15 min. The cytoplasmic supernatant above the cushion was removed and extracted with PCI until the vanadyl-ribonucleoside complex was removed (six to seven times), extracted twice with chloroform-isoamyl alcohol (24:1), and ethanol precipitated. RNA was dissolved in 0.2 ml of H_2O, and Tris-HCl (pH 7.5) was added to 50 mM and $MgCl_2$ was added to 10 mM. DNase I digestions containing 40 U of RNasin (Promega Biotech) and 5 U of RNase-free DNase I (RQ1; Promega Biotech) were done at 37°C for 1 h, followed by two extractions with PCI and two extractions with chloroform-isoamyl alcohol and ethanol precipitation. RNA was suspended in 0.2 ml of H_2O, and the concentration was determined by absorbance at 260 nm. A sample of RNA (2 μg) was analyzed by formaldehyde-agarose gel electrophoresis for evidence of degradation and to confirm the concentration.

RNase protection mapping. Riboprobe transcripts were prepared by using either T3 or SP6 RNA polymerase, depending on the particular probe. Reaction mixes included 1 μg of linearized DNA template, 50 μCi of [^{32}P]CTP (800 Ci/mmol; Amersham or New England Nuclear Corp.), 30 μM unlabeled CTP, 0.3 mM each ATP, GTP, and UTP, 40 U of RNasin (Promega Biotech), and 10 U of RNA polymerase in the buffer supplied by the manufacturer (Bethesda Research Laboratories). Reaction mixes were incubated at 37°C for 1 h. Template DNA was removed by digestion with 5 U of RNase-free DNase I (RQ1; Promega Biotech) at 37°C for 15 min. Reaction mixes were extracted once with PCI and once with chloroform, and 5 μg of yeast tRNA was added as a carrier prior to ethanol precipitation. The riboprobe was suspended in 100 μl of deionized formamide, and

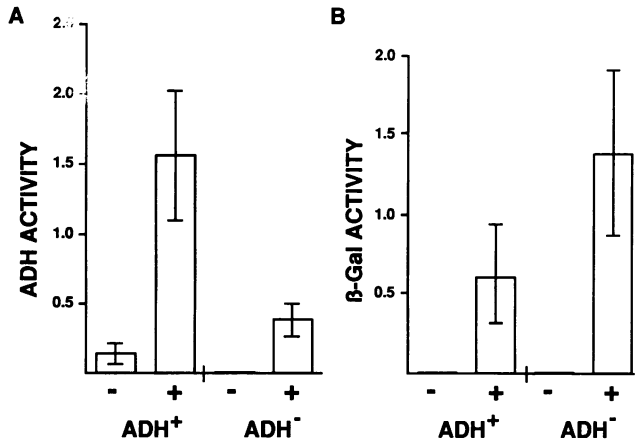


FIG. 1. ADH and β-Gal enzyme activities in transfected *Drosophila* cell lines. ADH$^+$ (1006-2) and ADH$^-$ (S2) cells were transfected with 10 μg of an *Adh*-containing plasmid (A) or 1 μg of a plasmid expressing β-Gal from the *Drosophila act5C* promoter (B). (A) ADH activity produced in two *Drosophila* tissue culture cell lines with (+) and without (−) transfection of the plasmid p5′Xba3.2, containing the entire *Adh* gene with 662 bp of 5′-flanking DNA. The data presented are the averages ± 1 standard deviation of 28 separate transfections with multiple preparations of DNA. (B) β-Gal activity produced in two *Drosophila* tissue culture cell lines before (−) and after (+) transfection of the plasmid pPAcLacZ (15). The data presented are the averages ± 1 standard deviation of 66 separate transfections.

1-μl samples were analyzed on an acrylamide-urea gel and counted in a scintillation counter to determine that the probe was both intact and of high specific activity.

RNA was precipitated and suspended in 24 μl of deionized formamide–6 μl of 5× hybridization buffer (0.2 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.7], 2 M NaCl, 5 mM EDTA), and 0.5 to 1 μl of riboprobe was added. Samples were covered with 20 μl of paraffin oil, heated to 95°C for 5 min, and then allowed to cool slowly (greater than 30 min) to 60°C, at which temperature they remained for 8 to 15 h. Samples were removed, immediately quenched in dry ice, and allowed to thaw at room temperature. A solution (0.3 ml) containing RNases A (40 μg/ml) and T_1 (3,000 U/ml) was added, and RNA was digested at room temperature (24°C). After 1 h, 5 μl of 10% sodium dodecyl sulfate and 50 μg of proteinase K were added, and samples were incubated at 37°C for 10 min, PCI extracted, and ethanol precipitated with 5 μg of yeast tRNA as carrier. Undigested RNA was resuspended in 5 μl of deionized formamide containing 10 mM Tris-HCl (pH 8.0), 1 mg of xylene cyanol per ml, and 1 mg of bromophenol blue per ml heated to 95°C for 5 min and then electrophoresed on a 10% acrylamide–8 M urea gel. The dried gel was exposed to Kodak X-ray film (XAR-5) with intensifying screens (Cronex Lightning Plus; Du Pont) at −80°C.

RESULTS

Differential ADH expression following transfection of *Drosophila* tissue culture cells. Before transfection, the ADH$^-$ (S2) cells had very little ADH activity compared with the ADH$^+$ (1006-2) cells (4) (Fig. 1A). After transfection of these ADH$^+$ and ADH$^-$ cell lines with the plasmid p5′Xba3.2, an Adh^F gene construct which contains all the distal *cis*-acting elements (39) and 662 bp of 5′-flanking DNA, ADH activities went up in both cell types; however, the ADH$^+$ cells continued to show higher levels of ADH activity than the ADH$^-$ cells (greater than fourfold). Analysis of the RNA from these transfected cell lines showed that although the ADH$^-$ cells transcribed both distal and proximal ADH RNAs from the exogenous templates (Fig. 2, lanes 3 and 4), the ADH$^+$ cells showed a much greater abundance of distal transcripts than proximal (Fig. 2, lanes 15 and 16). Therefore, most of the enhanced ADH expression in transfected ADH$^+$ cells is the result of enhanced distal transcription. It is possible that the ADH$^+$ cells contain *trans*-acting factors, specific to the distal promoter, which are different from those of the ADH$^-$ cells. The ADH$^+$ cells either possess additional positive *trans*-acting factors or lack negative factors which are present in the ADH$^-$ cells, or both.

Alternatively, the ADH$^+$ cells may be better able to take in the transfected DNA or possess greater amounts of general transcription factors, such as the TATA-binding protein or RNA polymerase II. To test this hypothesis, a plasmid, pPAcLacZ (15), which contains the *Drosophila* actin 5C (*act5C*) promoter and RNA start site as well as the *Adh* translation initiation site fused in frame to the *E. coli lacZ* gene, was transfected into ADH$^+$ (1006-2) and ADH$^-$ (S2) cell lines, and the cells were assayed for β-Gal activity. Both S2 (ADH$^-$) and 1006-2 (ADH$^+$) cells were able to take in this DNA and express β-Gal (Fig. 1B). The ADH$^-$ cell line consistently showed nearly twofold greater β-Gal activity than the ADH$^+$ cell line. Therefore, the differences in ADH expression in ADH$^+$ and ADH$^-$ cells cannot be the result of differential DNA uptake or differences in general transcriptional machinery. This β-Gal assay has proven to be a reliable indication of transfection efficiency, and therefore this plasmid has been used to standardize the results of all subsequent transfection analyses of *Adh* promoter deletions.

It is likely that this increase in ADH activity after transfection is the direct result of transcription from the exogenous *Adh* template and not through activation of the endogenous *Adh* gene. Transfections with a splicing-defective mutant template of *Adh* have been shown not to affect the level of transcripts from the endogenous template (5). Furthermore, in transfection studies in which the distal *Adh* regulatory sequence was placed upstream of the chloramphenicol acetyltransferase reporter gene (*cat*), we observed differential chloramphenicol acetyltransferase activity with no change in either endogenous ADH activity or RNA levels (J. McKeon and C. Benyajati, unpublished observations).

Analysis of 5′ deletion mutations following transfection into ADH$^+$ and ADH$^-$ cells. Plasmids containing 5′ deletion mutations of the *Adh* distal promoter and the actin-*lacZ* control plasmid (pPAcLacZ) were cotransfected into ADH$^+$ and ADH$^-$ cell lines, and the levels of ADH activity were compared with that of the plasmid p5′Xba3.2, which contains 662 bp of 5′-flanking DNA. Cells were harvested and assayed for ADH and β-Gal expression. After subtracting background ADH activity, which is the level seen with mock transfections with plasmids lacking *Adh*, the ADH data were corrected for transfection efficiency as determined by variations in β-Gal activity. Relative ADH activity is presented as units of ADH activity per unit of β-Gal activity (Fig. 3).

Transfected ADH$^-$ cells. There was little change in ADH activity in the ADH$^-$ cells with transfections of any of the 5′ deletions from −662 to −85 (Fig. 3). This was confirmed by RNase protection analysis of the RNA isolated from transfected cells (Fig. 2). The level of distal RNA as well as proximal RNA remained uniform with respect to the level of

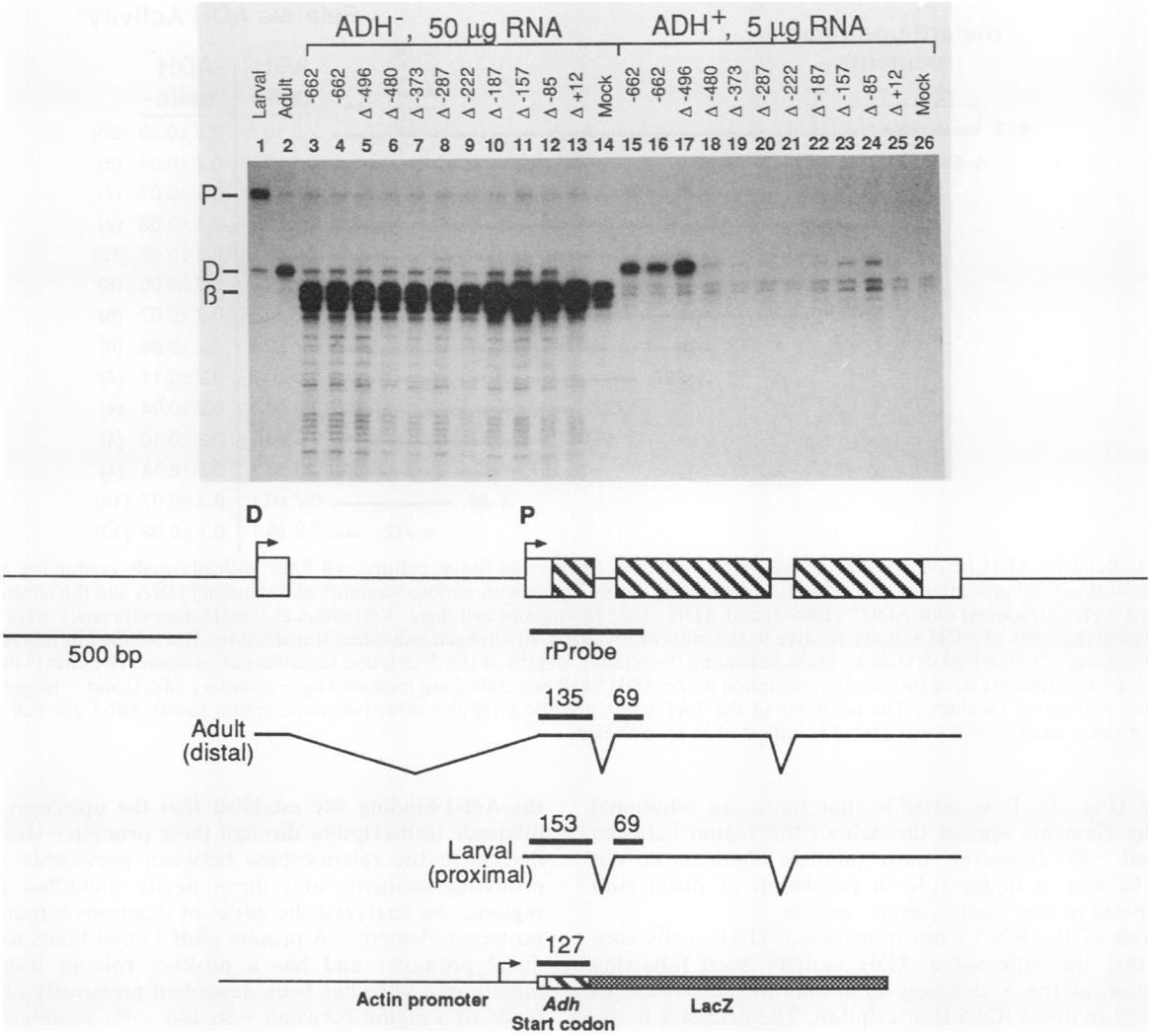


FIG. 2. RNase mapping analysis of RNA isolated from ADH$^+$ and ADH$^-$ cells transfected with plasmids containing 5′ deletion mutations of the *Adh* distal promoter. After transfection of ADH$^+$ and ADH$^-$ cells with plasmids containing 5′ deletion mutations of the distal promoter and the control plasmid pPAcLacZ, total RNA was analyzed by RNase mapping with a riboprobe designed to distinguish between proximal (P), distal (D), and RNA transcribed from the *act5C* promoter of the control plasmid (β). A diagram indicating the *Adh* gene, its transcripts, and the sizes of the protected riboprobe is shown. The multiple bands seen for the actin-*lacZ* mRNA are an artifact of the RNase mapping technique. The 69-bp protected fragment was run off this gel. The data presented in this figure are from a single set of transfections and are representative of multiple experiments. Lanes: 1 and 2, 1 μg of total RNA from third-instar larvae and adult flies, respectively; 3 to 14, 50 μg of total RNA from ADH$^-$ cells transfected with the plasmid p5′Xba3.2 (−662), the 5′ deletion mutations, or no *Adh*-containing plasmid (Mock); 15 to 26, 5 μg of total RNA from ADH$^+$ cells transfected with the plasmid p5′Xba3.2 (−662), the 5′ deletion mutations, or no *Adh*-containing plasmid (Mock).

β-Gal RNA (Fig. 2, lanes 3 to 12). All distal ADH expression in this ADH$^-$ cell line appeared to be regulated by promoter elements between −85 and +12, because only when this region was removed was there any effect on distal transcription (compare Fig. 2, lanes 12 and 13). The 5′ deletion Δ+12 lacks all sequences flanking the distal RNA start site, the start site itself, and the first 12 nucleotides of the RNA coding region. The residual ADH activity seen following transfection with this deletion (Fig. 3) must be due to ADH protein translated from proximal message, since analysis of the RNA (Fig. 2, lane 13) revealed the absence of distal mRNA in the ADH$^-$ cells transfected with deletion Δ+12 and the presence of proximal RNA.

Transfected ADH$^+$ cells. In the ADH$^+$ cell line 1006-2, the 5′ deletion mutant templates exhibited considerable differences in their levels of expression (Fig. 2 and 3), suggesting the presence of several *cis*-acting elements with roles in the regulation of distal RNA transcription. The various *cis*-acting elements are diagrammed in Fig. 3. First, deletion to −584 resulted in a twofold reduction in ADH activity, suggesting the presence of a *cis* element with a positive influence on transcription. Second, deletions to −540, −523, and −496 all resulted in a four- to sixfold increase in ADH activity over deletion to −584, suggesting the removal of a *cis*-acting element with a negative effect on transcription. Third, deletions to −481 and to −480 resulted in a fivefold reduction in the level of expression seen with the deletion to −496 and a three- to fourfold reduction from that seen with p5′Xba3.2, suggesting the presence of a strong positive *cis*-acting element.

Transfected templates containing deletions between −480 and −85 showed a fluctuation in ADH activity in the ADH$^+$

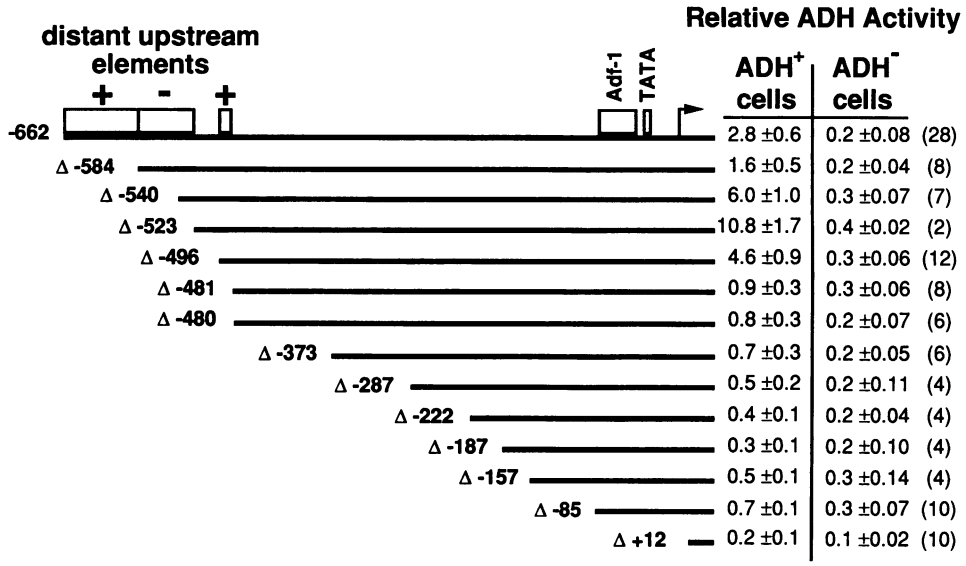


FIG. 3. Relative ADH activity following transfection of two *Drosophila* tissue culture cell lines with plasmids containing 5′ deletion mutations of the distal promoter of *Adh*. Plasmids containing the *Adh* gene with various amounts of 5′-flanking DNA and the control plasmid pPAcLacZ were introduced into ADH$^+$ (1006-2) and ADH$^-$ (S2) *Drosophila* cell lines. Variations in transfection efficiency were corrected by expressing the units of ADH activity relative to the units of β-Gal activity in each individual transfection. The average of this ADH/β-Gal ratio is presented ±1 standard deviation. Maps indicating the relative lengths of the 5′ deletion mutations are shown. The effects that various distant upstream elements have on distal transcription in the ADH$^+$ cell line 1006-2 are indicated by + (positive effect) and − (negative effect) above their respective locations. The positions of the TATA box and the promoter-selective transcription factor Adf-1 are indicated. The number of times each plasmid was tested is indicated in parentheses.

cell line (Fig. 3). It is possible that there are additional *cis*-acting elements spread throughout the region between −480 and −85. However, these possible elements do not appear to play a major role in regulation of distal *Adh* transcription in this tissue culture system.

Analysis of the RNA from transfected ADH$^+$ cells confirmed that the differential ADH activity seen following transfection of the 5′ deletion mutations was the result of differences in distal RNA transcription. The decrease in the level of distal transcripts associated with the deletion of 16 bp between −496 and −480 was quite striking (compare band D in Fig. 2, lanes 17 and 18). Because deletion of this small region resulted in such a dramatic reduction in distal *Adh* transcription only in the ADH$^+$ cells, this region must be an important *cis* element responsible for the enhanced distal *Adh* transcription in the ADH$^+$ cells.

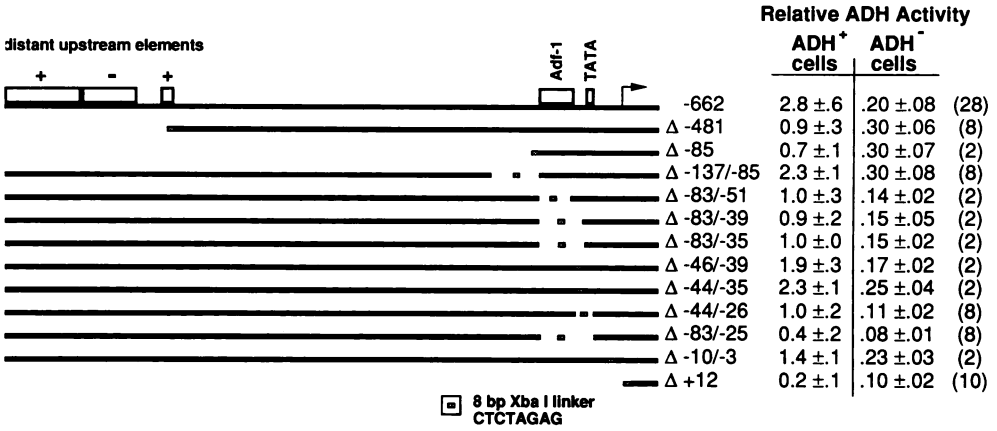
To summarize, these results demonstrate the presence of at least two distant *cis*-acting elements which, when removed, affected expression of the exogenous *Adh* template in the ADH$^+$ cells but not in the ADH$^-$ cells. One of these elements (−584 to −523) had a negative effect on distal *Adh* transcription, because when it was removed ADH expression increased more than fivefold. Another element has been delimited to a 15-bp region between −496 and −481. This region shows enhancer activity in that it stimulated transcription in a cell type-specific manner, and at a distance of 480 bp from the distal RNA start site.

Further evidence that these distant elements have properties associated with classical enhancers and silencers (46) comes from the ability to change the spacing of these elements to the promoter. The linker-replacement deletion mutation Δ−135/−85 had little effect on distal transcription in the ADH$^+$ cell line (Fig. 4 and 5). This deletion changes the spacing by 43 bp, suggesting that these distant elements will operate over a range of distances.

Linker-replacement deletions targeted to the TATA box and the Adf-1-binding site establish that the upstream elements influence transcription through these promoter elements. To determine the relationships between previously identified promoter elements and these newly identified upstream regions, we analyzed the effect of deletions targeted to the promoter elements. A protein (Adf-1) that binds to the *Adh* distal promoter and has a positive role in transcription initiation in vitro has been described previously (20). Adf-1 binds to a region between −86 and −46, as determined by DNase I footprinting (20), just upstream of the TATA box (−32 to −26) (see Fig. 7A). Adf-1 has been suggested to be responsible for transcriptional activation of the distal promoter in early embryos, as its presence in nuclear extracts of developmentally staged embryos is correlated with ADH expression (21).

It was of interest to determine the role of this region in the differential ADH expression patterns of cell lines. Specific linker-replacement deletions targeted to the Adf-1-binding site (−86 to −46) and to the TATA box (−32 to −26) were tested in the comparative transient-transfection assay. The results of these experiments are presented in Fig. 4 and 5.

Transfected ADH$^-$ cells. In the ADH$^-$ cell line, deletion of either the Adf-1-binding site (Δ−83/−51, Δ−83/−39, and Δ−83/−35) or the TATA box (Δ−44/−26) reduced ADH activity to nearly the same level seen when the entire distal promoter, including the distal RNA start site, was removed (Δ+12) (Fig. 4). Analysis of the RNA (Fig. 5) showed that although the proximal RNA level was not affected, the distal message was greatly reduced in cells transfected with the Adf-1-binding site deletions (Fig. 5, lanes 6, 7, and 10; Fig. 6, lane 11) and TATA box deletion (Fig. 5, lane 11; Fig. 6, lane 12). Deletion of either of these two regions did not affect correct RNA start sites (Fig. 6, lanes 9 to 12). Deletion of both the Adf-1-binding site and the TATA box (Δ−83/−25) further reduced ADH activity and distal transcription in the ADH$^-$ cell line (Fig. 4; Fig. 5, lane 12).



	Relative ADH Activity ADH⁺ cells	ADH⁻ cells	
-662	2.8 ±.6	.20 ±.08	(28)
Δ -481	0.9 ±.3	.30 ±.06	(8)
Δ -85	0.7 ±.1	.30 ±.07	(2)
Δ -137/-85	2.3 ±.1	.30 ±.08	(8)
Δ -83/-51	1.0 ±.3	.14 ±.02	(2)
Δ -83/-39	0.9 ±.2	.15 ±.05	(2)
Δ -83/-35	1.0 ±.0	.15 ±.02	(2)
Δ -46/-39	1.9 ±.3	.17 ±.02	(2)
Δ -44/-35	2.3 ±.1	.25 ±.04	(2)
Δ -44/-26	1.0 ±.2	.11 ±.02	(8)
Δ -83/-25	0.4 ±.2	.08 ±.01	(8)
Δ -10/-3	1.4 ±.1	.23 ±.03	(2)
Δ +12	0.2 ±.1	.10 ±.02	(10)

FIG. 4. Relative ADH activity following transfection of two *Drosophila* tissue culture cell lines with plasmids containing 5′ deletion and linker-replacement deletion mutations of the distal promoter of *Adh*. Plasmids containing the *Adh* gene with various mutations in the flanking DNA were introduced into ADH⁺ and ADH⁻ *Drosophila* cell lines. Variations in transfection efficiency were corrected by expressing the units of ADH activity relative to the units of β-Gal activity in each individual transfection. The average of this ADH/β-Gal ratio is presented with error of ±1 standard deviation. The number of times each plasmid was tested is indicated in parentheses. The data for −662 and the 5′ deletion mutations are the same as presented in Fig. 3 and are presented here for comparison. Other symbols are the same as described in the legend to Fig. 3.

Transfected ADH⁺ cells. The effect of removing the entire promoter region from −83 to −25 was nearly the same in the ADH⁺ cells as in the ADH⁻ cells, that is, nearly complete elimination of ADH activity and distal RNA (Fig. 4; Fig. 5, compare lane 24 and lane 26). Deletion of either the Adf-1-binding site (Δ−83/−51, Δ−83/−39, and Δ−83/−35) or the TATA box (Δ−44/−26) resulted in only a threefold reduction of ADH activity compared with deletion to −662 (Fig. 4). RNase mapping supported this observation (Fig. 5).

It is interesting that complete removal of the distal TATA box (Δ−44/−26) had no observed effect on correct initiation of distal transcription in either cell line (Fig. 6, lanes 6 and 12). This is not consistent with the presumed role of the TATA box in establishing the correct start site (reviewed in references 16, 24, and 31).

The strong effect of the deletions within the region between −83 and −25 confirms its importance on distal transcription. Even in ADH⁺ cells these deletions were dominant over the presence of distant positive upstream elements defined in this study. However, this region (−83 to −25) is unlikely to play a major role in differential ADH activity in these two cell lines. It is likely that the Adf-1-binding site and the TATA box are promoter elements with functions in establishing correct transcription, and it is the actions of distant elements, such as the enhancerlike element at −496 to −481, which, by stimulating transcription through these

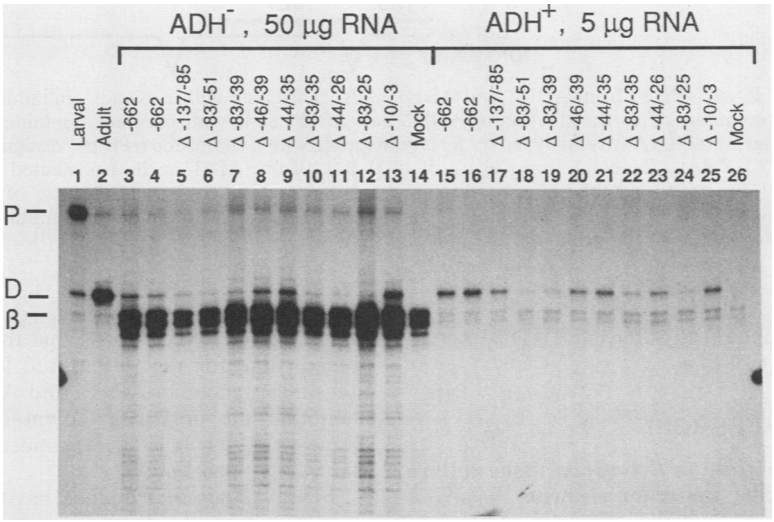


FIG. 5. RNase mapping analysis of RNA isolated from ADH⁺ and ADH⁻ cells transfected with plasmids containing linker-replacement deletion mutations of the *Adh* distal promoter. After transfection of ADH⁺ and ADH⁻ cells with plasmids containing linker-replacement deletion mutations of the distal promoter and the control plasmid pPAcLacZ, total RNA was analyzed by RNase mapping. Lanes: 1 and 2, 1 μg of total RNA from third-instar larvae and adult flies, respectively; 3 to 14, 50 μg of total RNA from ADH⁻ cells transfected with the plasmid p5′Xba3.2 (−662), the linker-replacement deletion mutations, and no *Adh*-containing plasmid (Mock); 15 to 26, 5 μg of total RNA from ADH⁺ cells transfected with the plasmid p5′Xba3.2 (−662), the linker-replacement deletion mutations, and no *Adh*-containing plasmid (Mock). P, Proximal RNA; D, distal RNA; β, β-Gal mRNA.

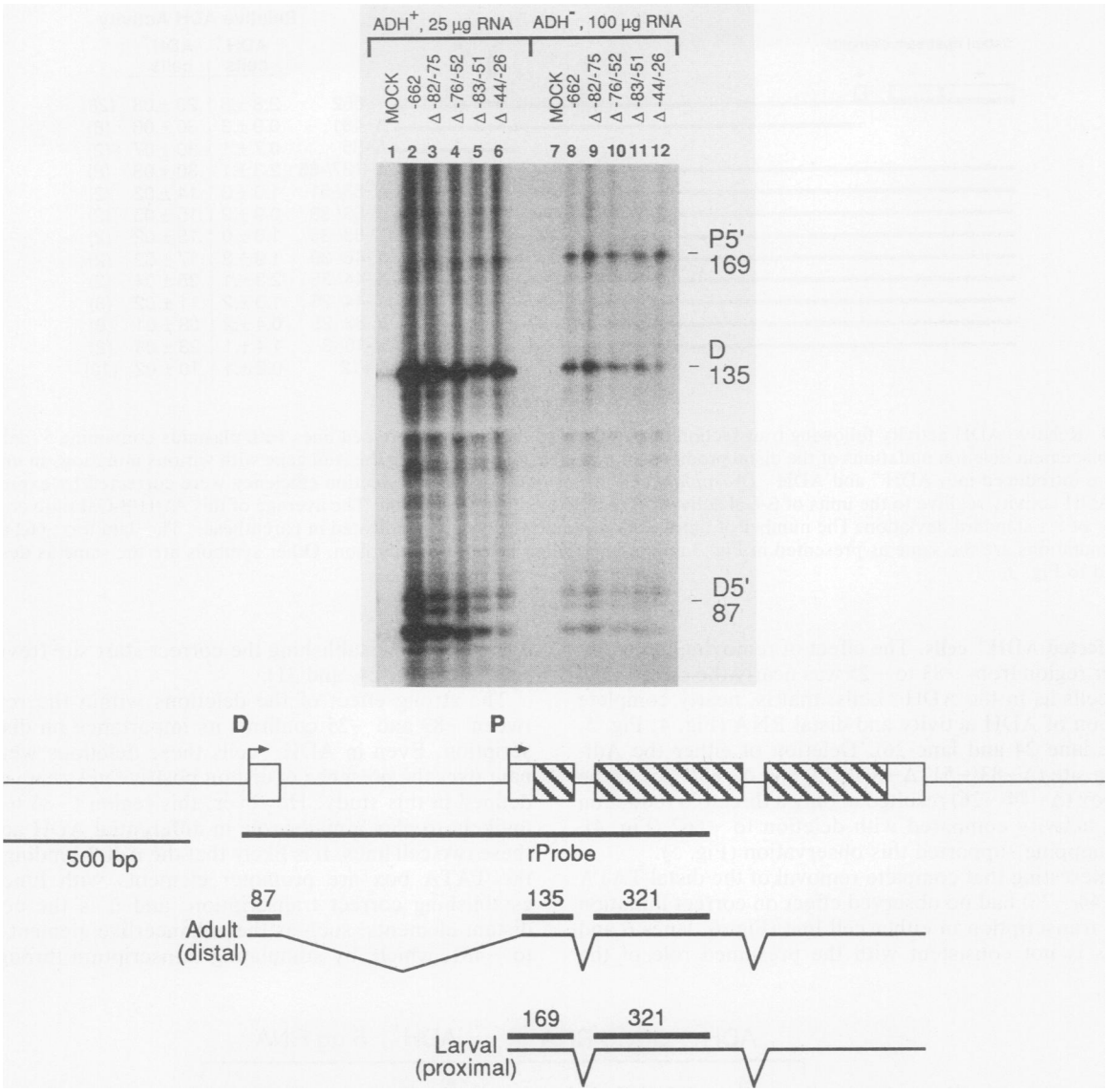


FIG. 6. 5′-End analysis of RNA isolated from ADH$^+$ and ADH$^-$ cells transfected with plasmids containing linker-replacement deletion mutations of the *Adh* distal promoter. After transfection of ADH$^+$ and ADH$^-$ cells with plasmids containing linker-replacement deletion mutations of the distal promoter, total RNA was analyzed by RNase mapping with a riboprobe (rProbe) designed to map the start sites of the proximal (P5′) and distal (D5′) RNAs. Lanes: 1 to 6, 25 μg of total RNA from ADH$^+$ cells transfected with no plasmid (Mock), the "wild-type" plasmid p5′Xba3.2 (−662), and the linker-replacement deletion mutations; 7 to 12, 100 μg of total RNA from ADH$^-$ cells transfected with no plasmid (Mock), the plasmid p5′Xba3.2 (−662), and the linker-replacement deletion mutations. A diagram indicating the *Adh* gene, its transcripts, and the sizes of the protected riboprobe is shown.

promoter elements, bring about the differential ADH expression observed in different cell lines.

DISCUSSION

Differential distal transcription in *Drosophila* tissue culture cells is the result of specific upstream elements. Using a transient-expression assay to test the effect of 5′ deletion mutations, we have identified at least two elements upstream of the distal promoter which are responsible for differential *Adh* distal transcription in *Drosophila* tissue culture cells. One element, −496 to −481 (relative to the distal RNA start site), has a positive effect on distal transcription. The other element, −584 to −524, has a negative effect on distal transcription. We propose that these upstream elements are responsible for the differential levels of ADH distal transcripts observed in ADH$^+$ and ADH$^-$ *Drosophila* cell lines and that the positive element (−496 to −481) plays the largest single role in the enhanced expression that is seen in the ADH$^+$ cell line.

Several lines of evidence have suggested that sequences upstream from the distal promoter elements are functional in the regulation of distal transcription. Corbin and Maniatis (12) described the region between −590 and −128, with respect to the distal RNA start site, as the *Adh* adult (distal) enhancer. Deletion of this large region reduced distal transcription by 10- to 20-fold in P-element-mediated transformation studies. Also, Cartwright (10) found DNase I-hyper-

sensitive sites upstream from the distal promoter in fat body cells of late third-instar larvae, suggesting an open chromatin configuration in the upstream region. The appearance of these hypersensitive sites correlates with the switch from proximal transcription to distal transcription. These hypersensitive sites may be caused by chromatin structural changes brought about by protein-DNA interactions at the upstream regulatory elements defined in this study.

The upstream enhancer and silencer elements interact with distal promoter elements to influence transcription. Transfection studies with the linker-replacement deletion mutations suggest that the Adf-1-binding site and the TATA box are positive promoter elements. Removal of this region greatly reduces distal transcription in both cell lines. 5′ deletion analysis suggests that the Adf-1 and TATA box region (the promoter region) is all that is required for distal transcription in ADH^- cells. An additional positive enhancer element which functions only in the ADH^+ cells stimulates distal transcription only in the presence of the distal promoter (Fig. 4, compare −662 with Δ−83/−25; Fig. 5, compare band D in lanes 15 and 16 with band D in lane 24). Having one or the other of the two promoter elements present is sufficient for the enhancer element to stimulate distal transcription (Fig. 4, compare −662 with Δ−83/−51, Δ−83/−39, Δ−83/−35, and Δ−44/−26; Fig. 5, compare band D in lanes 15 and 16 with band D in lanes 18, 19, 22, and 23). This is why deletion of either the TATA box or the Adf-1 site did not reduce distal transcription as completely in the ADH^+ cell line as in the ADH^- cell line, where the enhancer is not functional.

The fact that we can remove the TATA box without influencing the position of the correct distal start site raises questions as to the generally accepted role of the TATA box for positioning the start site (as discussed in references 16, 24, 29, and 31). The distal promoter of *Adh* must contain features other than the TATA box that correctly position the RNA initiation site. It is possible that Adf-1 can replace some of the functions of TATA-binding factor, such as determining the RNA start site and assembling the active transcription complex (23, 56). Alternatively, there may be additional proteins which are responsible for assembling the transcription complex and selecting the correct initiation site.

The distal promoter of *Adh* also contains binding sites for two additional sequence-specific DNA-binding proteins, Adf-2 at −7 to +21 (C. Benyajati, A. Ewel, M. Chovav, J. McKeon, J. Jackson, R. Kapoor, and E. Juan, unpublished data) and the GAGA factor at −42 to −37 (7) (Fig. 7A). Adf-2 appears to play a role in repression of *Adh* distal transcription in ADH^- cells as assayed by transient cotransfection competition experiments (C. Benyajati, A. Ewel, M. Chovav, J. McKeon, J. Jackson, R. Kapoor, and E. Juan, unpublished data), whereas the GAGA factor stimulates transcription of the *Drosophila Ubx* promoter in vitro (7). The same or a closely related protein, the CT-binding protein (19), is believed to maintain certain genes, including *Drosophila his3*, *hsp26*, and *hsp70*, in a state poised for transcriptional activation. Linker-replacement deletions which specifically remove the GAGA site (Δ−46/39 and Δ−44/35) did not significantly affect distal transcription in either the ADH^+ or ADH^- cell line (Fig. 4; Fig. 5, lanes 8, 9, 20, and 21). Therefore, it is unlikely that the GAGA factor plays a major role in the regulation of distal *Adh* transcription in these cell lines.

It is a common feature of enhancers to stimulate transcription through heterologous promoters (46). In our experiments we never observed any effect of the distal enhancer-silencer region on proximal transcription even in the absence of a functional distal promoter. It has been shown that a productive interaction between an enhancer and promoter requires specific protein-protein interactions (55). The proximal promoter contains a TATA box with a slightly different sequence and presumably additional proximal-specific promoter elements (20). Therefore, it is also possible that there are proximal promoter-specific DNA-protein and protein-protein interactions which prevent distal-specific enhancers and silencers from enhancing or repressing proximal transcription. In P-element-mediated transformation studies, the *Adh* larval enhancer functions with the proximal promoter but fails to stimulate transcription from the distal promoter (13). The enhancer- and silencer elements defined in this study may indeed be specific to the distal promoter. Further experiments are required to study these apparent specific interactions between promoters and enhancers.

Sequence similarities to the binding sites of known DNA-binding proteins are found in the distal enhancer-silencer region. Within the 15-bp *cis* element (−496 to −481) believed to be responsible for much of the enhanced ADH expression in the ADH^+ cell line is a sequence motif which is similar to that of the AP-1 enhancer-binding protein (1, 28) (Fig. 7B). The AP-1 enhancer-binding protein, first detected in HeLa cells as a binding activity that footprints sequences in the simian virus 40 (SV40) enhancer (28), has both positive and negative roles in regulation of SV40 gene expression (32). This same sequence motif is responsible for 12-*O*-tetradecanoylphorbol-13-acetate (TPA) inducibility of various genes (1). The AP-1-binding protein is composed of two polypeptides, the product of the c-*jun* and c-*fos* proto-oncogenes (43 and references therein). The Jun and Fos proteins have since been found to be members of larger families of proteins. The cyclic AMP-responsive element-binding protein (34) appears to be a member of this family of DNA-binding/transcriptional activator proteins (22; reviewed in reference 25). The Jun- and Fos-like proteins interact with one another through an α-helix motif known as a leucine zipper and bind the DNA through a basic region (27).

AP-1-binding activity has been detected in organisms as diverse as *Saccharomyces cerevisiae* and *Drosophila melanogaster* (yAP-1 [35], dAP-1 [36]). The *Drosophila* AP-1 protein is also composed of a Jun-like and a Fos-like protein (37). There is in vitro evidence that dAP-1 can stimulate transcription from a heterologous promoter containing multiple copies of an AP-1 site (37); however, no *Drosophila* gene has yet been shown to be regulated by the *Drosophila* AP-1 protein.

Within the negative element (−583 to −523) (Fig. 7A) is the sequence ATGCAAATTA, a perfect match to the consensus binding site of the octamer-binding factors (49, 50) (Fig. 7B). Octamer-binding proteins have been detected in such diverse species as *Psammechinus miliaris* (3) and humans (52 and references therein; reviewed in reference 33). The octamer motif has been found to be functional in tissue-specific and non-tissue-specific promoters and enhancers (52 and references therein). Multiple octamer-binding proteins have been found; some are present only in specific tissues, which can explain the different patterns of expression by the octamer motif (3, 18, 26, 40, 53). In addition to transcription activation (reviewed in reference 45), the octamer motif has recently been reported to be responsible for repression of immunoglobulin gene transcription in T cells (57). The presence, in *D. melanogaster*, of

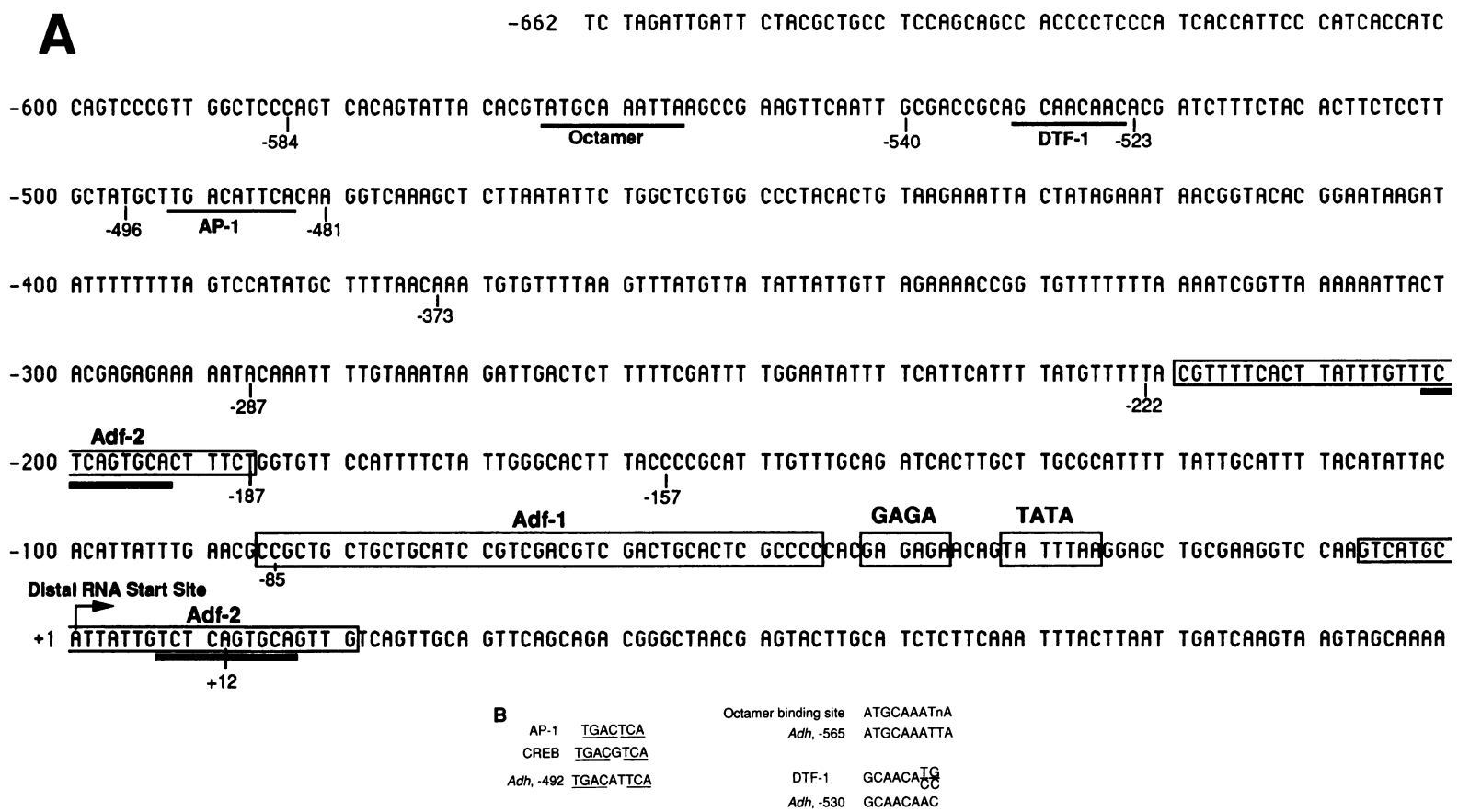


FIG. 7. Sequence of the DNA flanking and distal RNA start site with positions of 5′ deletion mutations and detail of sequence homology to known protein-binding sites. (A) Sequence of the region between −662 and +100 with respect to the distal RNA start site. The positions of the 5′ ends of the 5′ deletion mutations used in this study are indicated beneath the sequence. The binding sites of the known promoter-binding proteins (Adf-1, Adf-2, and GAGA) as well as the TATA box are indicated by boxes. The 10-bp direct repeats found in the Adf-2-binding sites are indicated by underlining. (B) The consensus binding sites of AP-1, cyclic AMP-responsive element-binding protein (CREB), DTF-1, and the octamer-binding proteins are presented along with the sequence of the distal *Adh cis*-acting elements which are similar to these binding sites.

octamer-binding activity has been noted (37), although no function has yet been described.

The possible binding site of another known DNA-binding protein has also been observed in the silencerlike region (Fig. 7). A sequence similar to the *Drosophila* transcription factor 1 (DTF-1) consensus (GCAACAT/CG/C) (38) is located at −530 (Fig. 7A and B). DTF-1 binds to sequences upstream of the *Antennapedia* P2 promoter and stimulates transcription from this promoter in vitro (38).

Interestingly, a recent study suggested that these sequences are indeed protein-binding sites in vitro. Moses et al. (35) compared DNase I footprints on the *Adh* promoters of two closely related *Drosophila* species by using partially fractionated embryonic nuclear extracts. They showed that several blocks of highly conserved sequences are sites of protein binding. Three of these footprints are within the enhancer-silencer region identified in this report. They are positioned over the octamer-like (−576 to −551), DTF-1-like (−538 to −516), and AP-1-like (−491 to −473) elements (35). However, it is not known whether these known transcription factors interact with these sequences. Future experiments are needed to determine what proteins bind to this enhancer-silencer region and are responsible for the differential expression observed in this study. We propose that whatever transcription factors are interacting with these upstream elements, they must be either absent in the ADH$^-$ cells or, if present, in a nonfunctional state.

It has recently been reported that the *Adh* larval enhancer (−660 to −5000) contains several repeats of two sequence motifs which are also present in the *Adh* adult enhancer (14), namely the octamer-like and DTF-1-like elements. Although no evidence of function or protein binding has been ascribed to these larval (proximal) enhancer sequences, it is curious that they are also present within the 61-bp distal-specific silencer element identified in this study.

The distal enhancer and silencer elements are conserved in distantly related *Drosophila* species. It has been shown that the *Adh* genes of distantly related *Drosophila* species are expressed in their correct temporal and tissue-specific patterns when introduced into *D. melanogaster* by P-element-mediated germ line transformation (*D. mulleri*, of the repleta group of *Drosophila* [17], *D. affinidisjuncta*, of the Hawaiian picture-winged *Drosophila* [8]). This suggests that the factors controlling the regulation of *Adh* are conserved in different species.

The *Adh* gene of *D. affinidisjuncta* is organized much like that of *D. melanogaster*, with one gene and two promoters (9, 41). This gene can be expressed in correct temporal and tissue-specific patterns in *D. melanogaster* despite much divergence between the sequences upstream of the distal promoters in these two distantly related species (8). However, the upstream region in *D. affinidisjuncta* contains small blocks of sequences that are highly homologous to se-

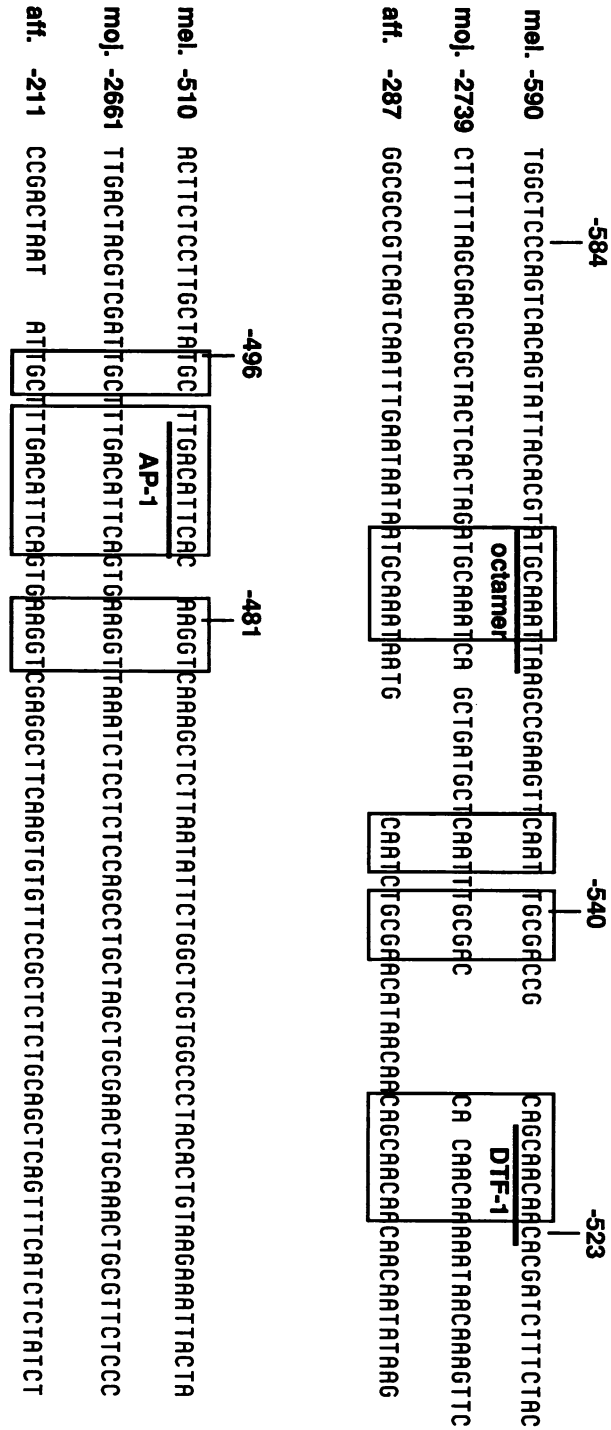


FIG. 8. Conservation of upstream sequences in *D. melanogaster*, *D. mojavensis*, and *D. affinidisjuncta*. The enhancer-silencer region defined in this study is compared with upstream sequences from *D. mojavensis* (moj.) (2) and *D. affinidisjuncta* (aff.) (42). Nucleotides within this region which are identical in all three distantly related species are indicated by boxes. The nucleotide numbers shown for *D. melanogaster* (mel.) and *D. affinidisjuncta* (42) correspond to the distance from the distal RNA start site. The nucleotide number shown for *D. mojavensis* is the distance from the initiation codon of the *Adh-2* gene (2). The endpoints of deletion mutations used to define the *cis*-acting elements are indicated with homology to the sites of known DNA-binding proteins are indicated.

quences upstream of the distal promoter in *D. melanogaster* (42), which includes the enhancer and the silencer regions identified in this study (Fig. 8).

Sequence far upstream (>2 kb) of the *Adh-2* (distal-like) gene in *D. mojavensis*, shown to act as an enhancer of *Adh-2* transcription, is highly conserved among members of the repleta group of *Drosophila* (D. Sullivan, P. Atkinson, C. Bayer, and M. Menotti-Raymond, in press). Within this sequence is a small region which is homologous to sequence upstream of the distal promoter in *D. melanogaster* and *D. affinidisjuncta* (Fig. 8). This region corresponds to the *D. melanogaster* sequence between ~−600 and ~−475, which includes the enhancer and the silencer elements identified in this study (Fig. 7 and 8). Outside of the enhancer and silencer elements there is little sequence conservation between these distantly related species (2, 42; Sullivan et al., in press).

Interestingly, the AP-1-like motif (TGACATTCA) is present and unchanged in *D. affinidisjuncta* (42), *D. mojavensis* (2), *D. mulleri*, *D. hydei*, and *D. melanogaster* (Sullivan et al., in press) (Fig. 8). The octamer motif found in *D. melanogaster* is also conserved in *D. affinidisjuncta* (42), *D. mojavensis* (2), *D. hydei* (M. Menotti-Raymond and D. Sullivan, unpublished data), and possibly other members of the repleta group as well. The spacing between these two motifs in the above species is similar to that in *D. melanogaster* (Fig. 8); 60 to 70 bp separate these two motifs. The conservation of these upstream elements in such distantly related species and the fact that they have been shown to be protein-binding sites (35) suggest that they are important for regulating ADH expression in the organism and may represent binding sites of important regulatory proteins.

Positive and negative *cis*-acting elements may together determine the level of distal *Adh* transcription. It appears that distal RNA transcription is controlled by activation and repression. The level of distal expression observed in the ADH^+ cell line may be controlled by both a positive element and a negative element which are in close proximity to each other. It is suggested by our results that this negative element is involved in modulating the level of distal transcription in cells where the enhancer element is active. However, we do not know whether the silencer element can function independently of the enhancer. Interestingly, the organization, with the silencer element upstream and adjacent to the enhancer element, as well as the relative spacing of these elements has been conserved throughout evolution (Fig. 8) (42; Sullivan et al., in press).

Detailed analysis of the upstream elements should begin to identify the factors that interact with them. P-element-mediated transformations with mutations targeted to the specific protein-binding sites will begin to address the functions of these *cis*- and *trans*-acting elements in the normally complex developmental and tissue-specific patterns of ADH expression.

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