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Redundant *cis*-acting elements control expression of the *Drosophila affinidisjuncta Adh* gene in the larval fat body

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ABSTRACT

The alcohol dehydrogenase (*Adh*) gene in the Hawaiian species of fruit fly, *Drosophila affinidisjuncta*, like the *Adh* genes from all *Drosophila* species analyzed, is expressed at high levels in the larval fat body via a larval-specific promoter. To identify the *cis*-acting elements involved in this highly conserved aspect of *Adh* gene expression, deleted *D.affinidisjuncta* genes were introduced into *D.melanogaster* by somatic transformation. Unlike previously described methods, this transformation system allows analysis of *Adh* gene expression specifically in the larval fat body. The arrangement of sequences influencing expression of the proximal promoter of this gene in the larval fat body differs markedly from that described for the *Adh* gene from the distant relative, *D.melanogaster*. Multiple redundant elements dispersed 5′ and 3′ to the gene, only some of which map to regions carrying evolutionarily conserved sequences, affect expression in the fat body. *D.affinidisjuncta* employs a novel mode of *Adh* gene regulation in which the proximal promoter is influenced by sequences having roles in expression of the distal promoter. This gene is also unique in that far upstream sequences can compensate for loss of sequences within 200 bp of the proximal RNA start site. Furthermore, expression is influenced in an unusual, context-dependent manner by a naturally-occurring 3′ duplication of the proximal promoter — a feature found only in Hawaiian species.

INTRODUCTION

Adh genes in *Drosophila* display complex temporal- and tissue-specific expression, with some aspects of this expression held in common by even distantly related species. Despite the estimated 40 to 60 million years since the divergence of *D.melanogaster* and *D.affinidisjuncta* (1,2,3), the *Adh* genes from these species retain certain aspects of their regulatory patterns as analyzed by germ-line transformation (4,5). Both genes are expressed at high levels in larval midgut, and in larval and adult fat bodies. Each gene contains two promoters (distal and proximal) that display temporal- and tissue-specific expression (6,7). The proximal transcript predominates in larvae increasing through the third-instar stage. The distal promoter becomes active during late third-instar and is responsible for the predominant transcript in adults. The importance of two promoters in the developmental expression of *Adh* genes is unknown but presumably critical as the arrangement is conserved in the distantly related *D.melanogaster* and *D.affinidisjuncta*.

Germ-line (8,9) and transient somatic transformation of *D.melanogaster* (10,11,12,13) have been used to identify regulatory elements on *Adh* genes from various species. Numerous studies indicate that different *Adh* genes are expressed as in the donor species when assayed by these methods (4,5,14,15,16,17,18,19). Several regulatory elements upstream of and influencing the distal promoters of various *Drosophila Adh* genes have been identified by P element transformation (16,20,21,22,23,24). Relative to the distal promoter, little is known about elements critical to activation of the proximal promoter, or larval-specific promoter, in any *Drosophila* species. As assayed by P element transformation, the *D.melanogaster* gene carries an *Adh* larval enhancer (ALE), located between 660 and 5000 bp upstream of the distal promoter (25). In *D.mulleri*, a putative enhancer element is located 3′ to the larval-specific gene, *Adh-1* (16). Other studies have addressed the importance of sequences immediately upstream of the larval-specific promoters. One such sequence, within 100 bp of the transcription start site, has been shown to contribute to full levels of expression in all cases tested (17,26,27,28). Other sequences in the immediate upstream region are needed for full expression of the larval-specific promoters of various *Drosophila Adh* genes, but the functions of these are unique to particular genes (12,26,27).

To identify potential regulatory sequences on the *D.affinidisjuncta Adh* gene, Rowan and Dickinson (29) compared the sequence of this gene to the *D.melanogaster* gene. In the 2.8 kb of 5′ flanking sequences compared, there is little overall similarity with the exception of several short regions of greater than 70% identity. Many studies indicate that the size of these regions, their degree of sequence conservation, and their organization are consistent with regulatory roles (30). There is also a remarkable degree of correlation between these conserved elements and the sites that footprint on the *D.melanogaster* gene (28,31).

These observations pose an obvious question regarding the distantly related species, *D.melanogaster* and *D.affinidisjuncta*.

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Do small clusters of similar sequences account for highly conserved aspects of *Adh* gene expression such as transcription from the proximal promoter in the larval fat body? To test if it is these conserved regions or other sequences that are involved in the regulation of the proximal promoter of the *D.affinidisjuncta* *Adh* gene in the larval fat body, we have developed a transient expression system allowing analysis of *Adh* gene expression specifically in this tissue. The site of DNA injection was chosen to optimize for larval fat body expression, and a non-*Adh* control plasmid was used to avoid the possibility of interplasmid competition for limiting *trans*-acting factors (32). Derivatives of the *D.affinidisjuncta* gene, carrying deletions in the upstream and downstream regions were introduced into *D.melanogaster*. In fact, multiple sequences with a high degree of redundancy and dispersed throughout the 5′ and 3′ flanking regions affect expression. While some of these correspond to conserved sequences others, including a 3′ duplication of the proximal promoter, are unique to the *D.affinidisjuncta* gene. Interestingly, the highly conserved sequences immediately upstream of the RNA initiation site are dispensable for expression in the presence of far upstream sequences.

MATERIALS AND METHODS

Plasmid constructions

The cloning and characterization of the *Adh* gene from the *D.affinidisjuncta* stock, S36G1, has been described previously (4,19,33,34). The full-length *Adh* fragment (Fig.1) is a 5.4 kb *BglII/XhoI* genomic fragment inserted into the vector, pBRXB2 to produce p11BXB2 (4). Other genes, constructed by standard methods (35) using available restriction enzyme cleavage sites, were inserted into pBRXB2 in the same orientation. These and other plasmids, described below, were purified by CsCl density gradient centrifugation prior to injection.

The targeted deletion, Δ51, was made by site-directed mutagenesis. A fragment from *NdeI* (blunt-ended) at −203 to *EcoRI* at +18, was inserted into pBluescriptKS+ (Stratagene) that had been digested with *SmaI* and *EcoRI*. To create the 51 bp deletion, an oligonucleotide (5′-GACCACGAGAAACTTA-GC-3′) containing wild-type flanking sequences and the internal deletion was used for site-directed mutagenesis by the method of Kunkel *et al.* (36). The mutation was verified by dideoxynucleotide sequencing (37). Then, intact 3′ ends (from +18 to +2618, Fig. 1) were inserted into the plasmids carrying wild-type and deleted sequences from −203 to +18.

Somatic transformation

Plasmid mixtures consisting of *Adh* genes with the control plasmid, vermilion-*lacZ* (pTUF1.1 *v-lacZ*, gift of L. Searles) (38) were injected into preblastoderm embryos of the *D.melanogaster* strain Adh^{fn6},*cn*;ry^{506} (39). This *Adh* allele produces a low level of incompletely processed *Adh* RNA and no detectable alcohol dehydrogenase protein (ADH) (39). The vermilion-*lacZ* fusion shows fat body-specific expression in larvae (38). Unless otherwise specified, *Adh* plasmids and pTUF1.1 *v-lacZ* were mixed at equimolar concentrations for injection (20 nM). Embryos were collected, injected, and reared as described (10) except that injections were at 18°C at the injection position specified in the text. Larvae were collected, transferred to vials containing Formula 4−24 instant Drosophila medium (Carolina Biological), placed in a humid chamber, and reared at 27°C for 5−7 days. Feeding third-instar larvae were hand-dissected in ice-cold Tris-buffered saline (129 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 20 mM Tris−HCl pH 7.5) to remove midguts. Dissections were performed to lower endogenous β-galactosidase activity, most of which is in the midgut. Ten dissected larvae were pooled and homogenized in 400 μl ice-cold lysis buffer (15 mM $MgSO_4$, 4 mM EGTA, 10 mM β-mercaptoethanol, 1% Triton X-100 (v/v), 20 mM HEPES, pH 7.3). Insoluble material was removed by centrifugation (12,000×*g* for 2 min at 4°C).

Enzyme activity measurement

Determination of ADH activity was done as previously described (40) using 2-propanol as the substrate. Assay mixtures were preincubated in absence of substrate at room temperature for 20 minutes to deplete pools of endogenous substrates that could be used for NAD reduction.

To measure β-galactosidase activity, extracts from dissected larvae (100 μl) were mixed with 200 μl of assay buffer (1 mM $MgSO_4$, 100 mM β-mercaptoethanol, and 100 mM $NaPO_4$ pH 8.0). The sample was divided in half. One aliquot was mixed with 50 μl of assay buffer containing 4 mg/ml of substrate, o-nitrophenyl β-D-galactopyranoside, and the other aliquot was mixed with 50 μl of assay buffer lacking substrate. Following a 2 hour incubation at 37°C, the reactions were stopped by placing the samples on ice. Insoluble material was pelleted by centrifugation (12,000×*g* for 2 min). β-galactosidase activity was determined by reading absorbance of the plus-substrate samples at 420 nm and subtracting the corresponding no-substrate control values. To correct for residual endogenous β-galactosidase activity, extracts from Adh^{fn6} *cn; ry^{506}* larvae (injection stock) were similarly assayed, and this background value was subtracted from the value for injected larvae.

Histochemical localization of ADH was performed on dissected tissue stained at 37°C for fifty minutes. In all cases, control tissues were incubated in the absence of substrate. The fixing and staining procedure was described previously (4).

RNase protection assays

Total nucleic acids were prepared by homogenizing whole larvae in RNA extraction buffer followed by phenol/chloroform extraction as described (19). RNA levels were determined by RNase protection assays performed as described (41) with the detailed modifications specified by Fang and Brennan (15). The template used for production of the *D.affinidisjuncta Adh* probe and measurement of *Adh* RNA by densitometric scanning of X-ray film was also as described (15).

RESULTS

Fat body expression in somatic transformants is optimized by ventral injection

Adh gene expression in larval *D.affinidisjuncta* and *D.melanogaster* is primarily in the fat body. However, injection of plasmids carrying *Adh* genes into the posterior pole of embryos results in mostly midgut expression for somatically transformed larvae (10,11,12, and below). It is likely that the expression patterns observed in somatic transformation are determined by both the tissue-specific regulation of the injected gene and by the particular cells into which the injected DNA diffuses. As we are interested in analyzing proximal promoter expression in the larval fat body, we tested the effect of injection position on expression patterns in somatic transformants carrying the *D.affinidisjuncta Adh* gene (Fig. 1).

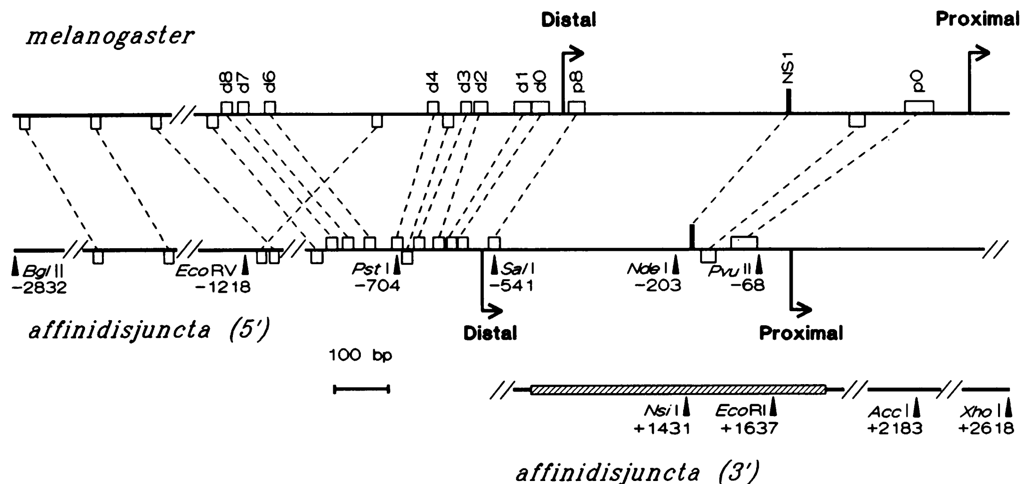

Figure 1. Comparison of *D.melanogaster* and *D.affinidisjuncta Adh* sequences. The top map represents the *D.melanogaster* gene on which labeled boxes above the line denote regions protected from DNase digestion by nuclear extracts from *D.melanogaster* embryos (31). The bottom map represents the full-length *D.affinidisjuncta* gene used for somatic transformation. Arrows labeled D and P designate the distal and proximal promoters. Open boxes above the line show the position of sequences that are at least 70% identical to portions of sequences footprinting on the *D.melanogaster* gene. Open boxes below the lines represent sequences conserved between *D.melanogaster* and *D.affinidisjuncta* that have not been shown to footprint. The vertical bar represents an element, NS1, required for full expression of the *D.melanogaster* proximal promoter (12,13). The hatched bar represents a 525 bp duplication that is shown aligned with the corresponding 5′ sequences. Restriction endonuclease cleavage sites used for construction of deleted genes are also shown. Position numbering is relative to the proximal transcription start site.

In the method described by Martin and coworkers (10), a plasmid carrying the *D.melanogaster Adh* gene was injected into the posterior pole of embryos. Strong ADH activity was observed in larval midgut, Malpighian tubules, and moderate activity was observed in larval fat body. Similarly, in the present study, posterior injection of the *D.affinidisjuncta Adh* gene (Fig. 1) results in moderate ADH activity in larval middle midgut (15/16 larvae) and Malpighian tubules (14/15), and weak activity in larval fat body (3/16) (Table 1.).

We tested three additional injection locations (the anterior pole, the dorsal midline, and the ventral midline). Third-instar larvae were dissected to determine the histochemical distribution of ADH as summarized in Table 1. Anteriorly injected embryos give rise to larvae that exhibit strong activity exclusively in the anterior midgut (15/15 larvae). Dorsal injection results in very weak staining of the fat body (6/17) and hypodermis (11/17). Consistent with the fate map of the *D.melanogaster* blastoderm (42), larvae derived from ventrally injected embryos display intense ADH activity limited exclusively to the fat body (16/16 larvae). The relatively large amount of fat body-specific activity makes determination of ADH activity by spectrophotometry possible. Consequently all subsequent analyses employed ventral injections.

To determine if *Adh* activity is directly proportional to the concentration of the injected DNA, *Adh* plasmid concentrations from 10 ng/μl to 200 ng/μl were tested (Fig 2). The ratio of ADH to β-galactosidase activity increased linearly with a correlation coefficient of 0.99. This confirms the usefulness of this assay system in measuring relative gene expression over this 20-fold range. For all subsequent experiments *Adh* plasmid concentrations of 20nM (corresponding to 100 ng/μl of the parental plasmid) were used.

Multiple 5′ elements affect expression of the proximal promoter

As a starting construction we used a 5.4 kb genomic fragment from *D.affinidisjuncta* (Fig. 1). This carries 2832 bp of sequence upstream of the proximal promoter (−2274 relative to the distal promoter). Previous experiments have shown that germ-line transformants carrying this fragment express the *Adh* gene in larval fat body at levels comparable to *D.affinidisjuncta* (4). Thus genomic sequences downstream of −2832 are sufficient for normal expression in the larval fat body. To identify the *cis*-acting sequences upstream of the proximal start site of transcription that affect expression, blocks of conserved sequences were deleted from the *D.affinidisjuncta* gene, and the resultant constructions were tested for expression.

Removal of 1.6 kb of the *D.affinidisjuncta* gene, corresponding in position to portions of the *Adh* larval enhancer (ALE) in *D.melanogaster*, results not in a decrease, but rather in a small, but significant, increase in expression (*cf.* Aff and −1218, Fig. 3, $P<0.02$). Within the region removed in the −1218 construction are two small sequences held in common between *D.melanogaster* and *D.affinidisjuncta* (Fig. 1). These were identified by us in computer-aided comparison of the *D.melanogaster* (43) and *D.affinidisjuncta* (29) genes. The two sequences, 14 bp and 13 bp in length, are 100% conserved and are separated by nearly identical spacing on the two genes.

Table 1. Histochemical measurement of ADH in larval tissues[a,b]

Injection Position	Fat Body	Midgut	Tissue: Malpighian Tubules	Hypodermis
Posterior	++	+++[c]	+++	−
Anterior	−	++++[d]	−	−
Dorsal	+	−	−	+
Ventral	++++	−	−	−

[a] The gene shown in Fig. 1 was injected.
[b] (++++) Strong, (+++) moderate, (++) weak, (+) very weak, (−) undetectable.
[c] Middle and posterior midgut.
[d] Anterior midgut.

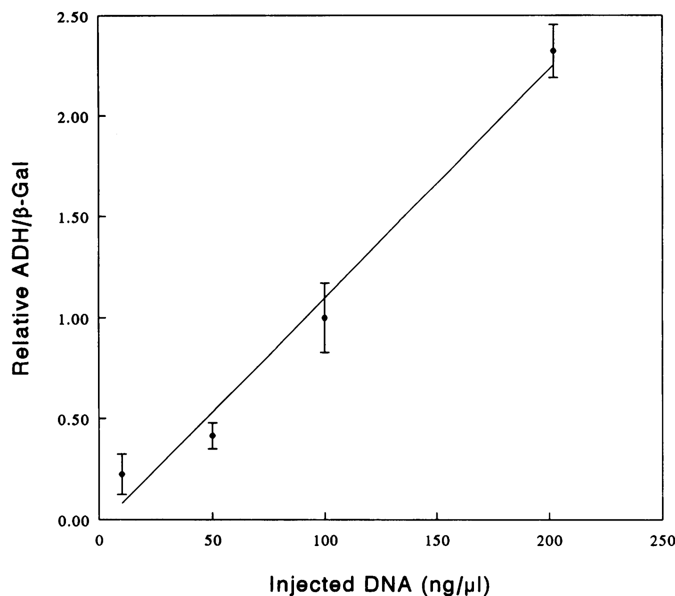

Figure 2. ADH/β-galactosidase activity following somatic transformation with different concentrations of injected *Adh* DNA. A plasmid carrying the *D.affinidisjuncta Adh* fragment shown in Fig. 1 was coinjected at various concentrations with the control plasmid pTUF1.1 *v-lacZ*. Concentration of the control plasmid was held constant at 20 nM (206 ng/μl). ADH and β-galactosidase (β-Gal) activities were measured, and ratios of the two were determined. The ratios are expressed relative to the value determined for injection of 100 ng/μl of the *Adh* plasmid. Three to four samples at each concentration were assayed and the results averaged. Error bars show standard deviations. The line represents a least squares fit of the data.

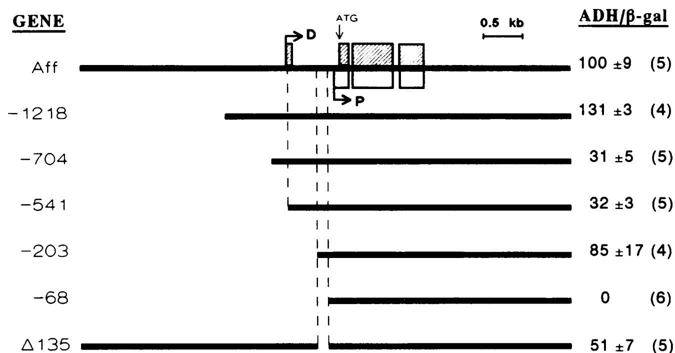

Figure 3. Relative expression of 5′ deletions. Plasmid mixtures containing the control pTUF1.1 *v-lacZ* and *D.affinidisjuncta Adh* genes were introduced into *D.melanogaster*. Transformation efficiency is controlled for by expressing ADH activity relative to β-galactosidase activity. The average ADH/β-gal ratio is shown ± SEM, with the number of samples assayed given in parentheses. Maps depict relative lengths of the genes. The full-length *D.affinidisjuncta* 5.4 kb fragment (−2832/+2618), abbreviated Aff, is shown at the top followed by the altered genes. On the 5.4 kb fragment, exons corresponding to transcription from the distal promoter (D) are shown above the line, and those from the proximal promoter (P) are shown below the line. Genes carrying 5′ deletions are named according to their 5′-most nucleotide relative to the start site of proximal transcription. The designation, Δ135, indicates the extent of the deletion in bp.

Despite this similar organization, these sequences are not essential for high levels of expression in the larval fat body.

Deletion to −704 results in a 4-fold drop in expression (*cf.* −1218 and −704, Fig. 3, $P<0.001$). This loss of activity suggests that one or more positive control elements influencing

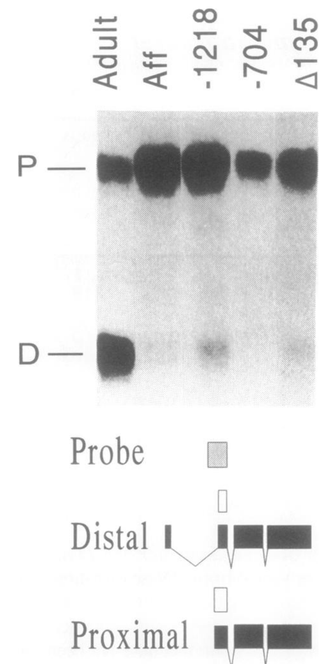

Figure 4. Confirmation of proximal transcripts in somatic transformants. Control RNA (Adult), from germ-line transformed adults carrying the parental *D.affinidisjuncta Adh* fragment, is included to indicate the positions of the protected fragments resulting from the proximal (P) and distal (D) transcripts. Fragments correspond to exon 2 (120 bp) of the distal transcript and exon 1 (152 bp) of the proximal transcript. Labels above the gel lanes designate gene constructions as described in Fig. 3.

the proximal promoter in the larval fat body lie within the 500 bp removed by the −704 deletion.

Further deletion, to −541, removes the highly conserved region from −146 upstream to +17 downstream of the distal RNA initiation site (Fig. 1). This deletion results in no significant change in expression levels (*cf.* −704 and −541, Fig. 3). This construction was also analyzed histochemically to determine if truncation of the gene altered the expression pattern. Injection of −541 results exclusively in fat body staining as for the unaltered gene. However the staining for −541 is relatively weak (data not shown).

The construction, −203, which is missing an additional 338 bp, shows nearly a 3-fold increase in activity to a level not significantly different from the unaltered gene (Fig. 3, *cf.* Aff vs. −203, $0.2<P<0.5$). This suggests that there may be at least one negative regulatory element for the proximal promoter between −541 and −203.

The region of the *D.affinidisjuncta* gene between −203 and −68 contains sequences homologous to those required for full expression of the larval-specific genes in several other *Drosophila* species. In all cases examined, elements within about 100 bp of the larval-specific promoter are critical to expression of the various genes. In *D.melanogaster* a 50 bp sequence from −115 to −66, referred to as NS6, is an enhancer of proximal transcription and is required for correct tissue-specific expression (11). The NS6 sequence contains most of P0 (−97 to −60), a region of the gene footprinted by embryonic nuclear extracts (28,31). Related sequences with documented or suggested roles in transcription of the larval-specific promoters have been identified near the transcription start sites in *D. mulleri* and

A.

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D. melanogaster     -105 CATAGCcgA GATCGCGTAACGGTA  GATAATGAAAAGCTCTACGTAAccgaag -54
D. affinidisjuncta  -103 cagtgcTGATGATCGCC AACATTACTGATAAACAGCTGCG  AGG AAAtACTC -53
D. affinidisjuncta +1529 --------------a-- ---g-g-gc----------a---  --t ------g- +1579
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B.

GENE	ADH/β-gal
−203	100 ±14 (6)
Δ51	5 ± 2 (5)

Figure 5. The P0 homolog is required for expression. **A.** The P0 region of *D.melanogaster* (−97/−60) aligned with the P0 homolog and the 3′ P0 duplication in *D.affinidisjuncta*. Gaps in the sequence have been introduced to maximize matching. For the 5′ sequences, upper case letters denote nucleotides protected by embryonic nuclear extracts (31; J. Hu and M. Brennan unpublished data). The GATAA motif identified by Sullivan and coworkers (17,26, see text) is shown in bold type. The 9 and 16 bp elements identified by Rowan and Dickinson (29, see text) are underlined. In the 3′ duplication, nucleotides differing from the 5′ P0 homolog in *D.affinidisjuncta* are shown in lower case, and dashes denote identical nucleotides. **B.** Relative expression of 5′ deletions. Genes designations and data presentation are as described in the legend to Fig. 3. These two genes were constructed and analyzed using pBluescript as the vector.

GENE	ADH/β-gal
Aff	100 ±9 (5)
+2183	77 ±3 (5)
+1637	53 ±7 (4)
+1431	108 ±16 (5)
−203	85 ±17 (4)
−203/+1431	14 ±1 (5)
−203/+1637	45 ±3 (5)

Figure 6. Relative expression of 3′ deletions. Data are displayed as described in the legend to Fig. 3. Genes carrying 3′ deletions are named according to their 3′-most nucleotide relative to the start site of proximal transcription. Those genes with 5′ deletions have, in addition, their 5′-most nucleotide indicated. Open bars on the gene maps depict duplicated sequences.

D. mojavensis (26,27). Indeed, for *D. mojavensis*, sequences downstream of −70 are sufficient to allow full levels of expression in germ-line transformants (26).

To test for the presence of regulatory elements within the corresponding region of the *D.affinidisjuncta* gene, we deleted sequences upstream of −68. Truncation to −68 abolishes expression (Fig. 3). Given this, it appears that the region from −203 to −68 is required for function of the proximal promoter of the *D.affinidisjuncta* gene in the larval fat body. To test if the sequences between −203 and −68 are required in the presence of upstream sequences, we constructed a gene (Δ135) lacking only this region. As Fig. 3 shows, this construction retains function, being about 50% as active as the intact gene.

This result led us to consider the possibility that our somatic transformation system may result in aberrant promoter utilization by the injected genes. Conceivably, the unexpected activity level of Δ135 could be the result of transcription from the distal promoter in the larval fat body where normally the proximal promoter is active (15). Expression of the distal promoter could also account for the influence of sequences between −1218 and −704 on expression in the larval fat body (above), since these sequences have established roles in distal transcription.

To determine if there are high amounts of distal transcript produced in our assay system, we used RNase protection to measure the relative levels of the two transcripts (Fig. 4). As can be seen, transcripts from the proximal promoter were the predominant form for all genes analyzed. Distal transcripts were found for Aff, for the 5′ deletion, −1218, and for Δ135. However the levels were less than 5% of the total. No distal transcript was detected for the 5′ deletion, −704, which retains only 146 bp of sequence upstream of the distal transcription start site. Consistent with these results, proximal transcripts also predominate in germ-line transformed larvae carrying these various constructions (our unpublished data). The fact that −203, which entirely lacks the distal promoter, is fully functional (Fig. 3) also lends support to the idea that the bulk of measured ADH activity is due to protein encoded by proximal transcripts.

To address the possibility that the activity of the Δ135 construction was due to inappropriate expression in tissues other than the fat body, we analyzed the histochemical localization of ADH in larvae transformed with Δ135. Ventral injections of Δ135 result in histochemical staining that is localized exclusively to the fat body (data not shown).

Therefore, unexpectedly high levels of expression for Δ135 are not due to transcription from the distal promoter or to aberrant tissue-specific expression but rather are the result of proximal transcription in the larval fat body. This indicates that sequences upstream of −203 compensate for loss of sequences between −203 and −68.

This compensation by upstream sequences required that further tests for function of sequences between −203 and −68 be done with a gene truncated to −203. Specifically, we wanted to test the hypothesis that sequences homologous to P0 in *D.melanogaster* (Fig. 1) play a role in expression of the *D.affinidisjuncta* gene. The P0 homolog includes two sequences (−99/−84 and −81/−73, Fig. 5A), the smaller of which contains a GATAA motif that is conserved immediately upstream of the larval-specific promoters in many *Drosophila* species (17). The P0 homolog shows 63% identity with the *D.melanogaster* sequence and has recently been shown to be protected from DNase digestion by embryonic nuclear extracts (J. Hu and M. Brennan, unpublished data). Deletion of 51 bp (from −103 to −53) containing the P0 homolog results in a 20-fold drop in expression (Fig. 5B). Thus, for a *D.affinidisjuncta* gene lacking upstream sequences, the P0 homolog is required for normal expression of the proximal promoter in the larval fat body.

Elements 3′ of the coding region affect expression

The above results indicate that multiple upstream elements have quantitative effects on expression from the proximal promoter of the *D.affinidisjuncta Adh* gene. Previous results have demonstrated that sequences 3′ to the transcribed region also influence *Adh* gene expression in larval fat body as assayed by P element transformation (44). As mentioned above, 525 bp of DNA 3′ to the coding region is a direct, although imperfect, duplication of sequences just upstream of the start site for translation (hatched bar, Fig. 1) (29). Most of the distal-specific intron (95%) and the 60 bp region corresponding to the proximal mRNA leader sequences are duplicated. To further study the

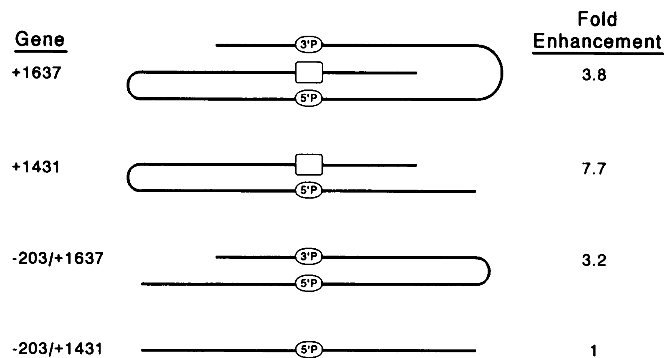

Figure 7. Model for context-dependent influence of 3′ promoter duplication. In the +1637 construction, activator proteins bound upstream of −203 (box) interact with both 5′ (5′P) and 3′ (3′P) promoter sequences resulting in relatively inefficient transcription from the authentic promoter. When the 3′ duplication is removed, (+1431) upstream sequences can interact exclusively with the 5′ promoter resulting in strong expression. In the absence of sequences upstream of −203, the 3′ duplication itself acts as an enhancer of the 5′ promoter (−203/+1637). Thus deletion of the 3′ duplication results in a decrease in expression (−203/+1431). For purposes of illustration, a mechanism involving looping out of intervening DNA is shown, but the data are equally consistent with binding of identical proteins by the three sequence elements with ultimate transfer of the proteins to the authentic promoter.

effects that sequences 3′ to the coding region have on expression of the proximal promoter, the constructions shown in Fig. 6 were analyzed.

Deletions of about 500 bp (+2183) and 1000 bp (+1637) from the 3′ end of the *D.affinidisjuncta Adh* gene have modest effects on ADH activity (Fig. 6). Within these regions there are no known homologies to the *D.melanogaster* gene. Results from the +1637 construction agree closely with those obtained previously in P element transformants in which a 50% reduction in expression was observed in the larval fat body (15). Removal of the next 200 bp causes a 2-fold increase in expression, resulting in activity levels comparable to those of Aff (*cf.* +1431 and +1637, Fig. 6). This construction, +1431, is missing about 50% of the 3′ duplication including sequences homologous to the 10 bp core of NS1 and to P0 (see Fig. 1 and Fig. 5A for comparisons).

Analysis of 3′ deletions of the −203 construction provides further evidence of redundancy

As discussed above, removing 2600 bp of upstream sequences does not significantly change expression (*cf.* −203 and Aff $0.2<P<0.5$, Fig. 6). Similarly, deletion of 1200 bp of downstream sequences (which removes about half of the downstream duplication) does not alter expression (*cf.* +1431 and Aff, Fig. 6, $P>0.5$).

Based on these results we wanted to test the hypothesis that sequences between −202 and +1431 are sufficient for normal expression levels. Interestingly, a gene carrying only these sequences shows an 8-fold drop in activity (*cf.* −203/+1431 and Aff, Fig. 6, $P<0.001$). Apparently in the presence of the upstream sequences, the 1200 bp segment at the 3′ end of the gene is dispensable (*cf.* +1431 and Aff, Fig. 6). However, this region contains elements capable of fully compensating for the loss of the upstream sequences (*cf.* −203 and Aff, Fig. 6).

To determine if the 3′ duplication is involved in this compensation for loss of upstream sequences, we constructed a gene (−203/+1637) that, in effect, restores the promoter-like segment of the 3′ duplication. As can be seen in Fig. 6, expression levels of this construction are similar to those of +1637 ($P>0.5$). These results highlight an interesting aspect of the regulation of this *Adh* gene. Removal of half of the 3′ duplication results in a 2-fold increase in expression in one case (*cf.* +1637 and +1431). However, deletion of the same fragment from −203/+1637 results in a 3-fold decrease in expression (*cf.* −203/+1637 and −203/+1431, Fig. 6). Stated differently, +1637 and −203/+1637 do not differ significantly in expression ($0.2<P<0.5$), but removal of the same 200 bp fragment from these two constructions has opposite effects: a 2-fold increase in one case, and a 3-fold decrease in the other.

For the above results to have bearing on the biologically relevant expression in the larval fat body, it is important to confirm that the observed ADH activity derives from this tissue. To determine if deleting 5′ and 3′ sequences alters the tissue specificity of *Adh* gene expression in our assay system, we analyzed the histochemical distribution of ADH in transformants carrying the shortest genes expressed at detectable levels. Both the −203/+1637 and −203/+1431 constructions show weak staining limited to the fat body (data not shown).

DISCUSSION

We have used a modified somatic transformation procedure to analyze the sequences contributing to expression of the proximal promoter of the *D.affinidisjuncta Adh* gene specifically in the larval fat body. This procedure circumvents the slowness and chromosomal position effects inherent in germ-line transformation (19), while allowing analysis of a tissue-specific expression phenotype that is highly conserved in evolution.

In conjunction with previous studies, our results indicate that major aspects of gene regulation can be evolutionarily conserved even though the specific sequences contributing to this regulation vary. Even the removal of several conserved sequences at one time generally had only modest effects on gene expression. As discussed below, our finding contrasts with those for the *D.melanogaster* gene. This is the only other *Drosophila Adh* gene that has been analyzed in detail and that shares a similar two-promoter structure.

Elements which enhance larval expression of *Adh* are arranged differently in *D.melanogaster* and *D.affinidisjuncta*. The *Adh* larval enhancer (ALE) in *D.melanogaster* comprises dispersed elements located between −5000 and −660 relative to the distal promoter (25). In contrast, for the *D.affinidisjuncta* gene, deletion of sequences upstream of −1218 (corresponding in position to the ALE) results in a small increase in expression. Further, sequences in both the 5′ and 3′ flanking regions of the *D.affinidisjuncta* gene have positive effects on expression, suggesting that the larval 'enhancer' in this species is dispersed throughout the flanking regions.

Another unexpected finding for the *D.affinidisjuncta* gene is that sequences upstream of the distal promoter and having roles in expression of the distal promoter also influence expression of the proximal promoter in the larval fat body. For example, previous work has shown that sequences upstream of −1218 exert a negative influence on the distal promoter of the *D.affinidisjuncta* gene (45). For a gene having the *D.affinidisjuncta Adh* distal promoter fused to a β-galactosidase reporter gene, deletion of upstream sequences between −1989 and −1218 (relative to the

start site of proximal transcription) results in a 67% increase in expression in *D.melanogaster* S2 cells (45). If this system is measuring the same negative element(s) we detect upstream of −1218, then the element can be further localized between −1989 and −1218. The two conserved sequences at −1963 and −1817 are within this region. One or both may exert a negative effect on expression from both promoters of the *D.affinidisjuncta* gene.

Similarly, sequences between −1218 and −704 affect both promoters. This region contains 7 stretches of sequence, 10 to 23 bp in length, that have an average identity of 81% with sequences in the *D.melanogaster* gene (Fig. 1) (29). A number of the latter are components of the *D.melanogaster* adult *Adh* (distal-specific) enhancer (20,23,46,47). Four of the conserved blocks of sequence correspond to footprinting sequences (d8 through d4) in *D.melanogaster* (31). Based on previous studies, a FTZ-F1 binding site (in the d6 homolog, Fig. 1) and an Adf-1/GAGA binding site (in the d4 homolog, Fig. 1) are possible candidates for positive regulatory sequences in this region of the *D.affinidisjuncta* gene. The d6 homolog, contains a perfect match to the consensus binding site for FTZ-F1, a transcriptional activator of the distal promoter of the *D.melanogaster* gene (21). The d4 homolog contains a positive control element that may interact with *Adh* distal factor (Adf-1) or (more probably) the GAGA factor to activate transcription of the distal promoter of the *D.affinidisjuncta* gene in S2 cells (45). The other potential binding site for a known transcriptional activator, BBF-2 (46,47), is not likely to function in transcription of the *D.affinidisjuncta* gene as it is poorly conserved in this gene. Other sequence homologies falling between −1218 and −704 are either predicted to bind negative regulatory proteins (20,24,48,49) or have no demonstrated function.

The proximal promoter of the *D.affinidisjuncta* gene is similar to other Drosophila larval-specific *Adh* promoters in that sequences within 100 bp upstream of the transcriptional start site are important for normal expression. Sequences homologous to P0 clearly contribute to the function of the proximal promoter of the *D.affinidisjuncta* gene (Fig. 5). In *D.melanogaster*, P0 is not essential for promoter function but is required for correct tissue-specific expression (11, 12). Upstream of the P0 homolog in *D.affinidisjuncta* we have identified an additional small element (between −172 and −163, AGAGCCGGCA) that is an 8 of 10 bp match to a region essential for transcription of the proximal promoter of the *D.melanogaster* gene (12, NS1 Fig. 1). This sequence appears to be needed for the enhancer function of the NS1 region of the *D.melanogaster* gene. While we have not directly tested its function as an enhancer for the *D.affinidisjuncta* gene, the observed sequence conservation and proximity to the P0 homolog suggest a similar role in *D.affinidisjuncta*.

A striking feature of the *D.affinidisjuncta* gene is the high degree of redundancy in positive regulatory sequences. Elements 3′ to the transcribed region can compensate for far upstream sequences and visa versa. Also, sequences upstream of −203 can compensate for the loss of sequences between −203 and −68. Studies on *Adh* genes from other species have indicated that the sequences immediately upstream of the larval promoters are needed for expression, but none have described compensation by naturally-occurring sequences elsewhere on the same gene (11,12,26,27,28). It is tempting to speculate, however, that this phenomenon could explain the apparent discrepancy regarding the need for Box B sequences in the expression of the *D. mulleri* and *D. mojavensis Adh-1* genes (26,27). Mutation of this upstream sequence lowers expression of the *D. mulleri* gene, but deletion of homologous sequences from a *D. mojavensis* gene (carrying additional 3′ sequences) has no effect.

The influence of the 3′ promoter duplication, a feature unique to the genes from the Hawaiian species, is context-dependent. In the presence of sequences upstream of −203, the 3′ promoter duplication is somewhat detrimental. As Figure 7 illustrates, elements present in +1637 but missing in +1431 may bind transcription factors, thus lowering expression from the proximal promoter due to the formation of inappropriate or abortive initiation complexes at the 3′ end. A similar phenomenon has been observed for constructions in which two *Adh* gene promoters on a single plasmid apparently share or compete for binding of one or more limiting components (50). Binding of proteins to the 3′ promoter-like sequences could nonetheless stimulate expression for genes that lack sequences upstream of −203. In this case, the 3′ sequences may bind factors that could then be utilized, even if somewhat inefficiently, by the proximal promoter (*cf.* −203/+1637 and −203/+1431, Fig. 7). Thus, the 3′ duplication may have enhancer activity that is dependent on gene context. Again, a parallel situation is seen for plasmids that carry two *Adh* genes. The two genes may provide complementary functions that allow expression from the promoters of both genes while at the same time lowering the level of expression from a given promoter (50).

Previous studies employing chimeric *Adh* genes suggest that multiple *cis*-acting sequences functioning in the fat body have accumulated in *Adh* genes from various *Drosophila* species (15,18,44). However, the present study is the first to provide direct support for this hypothesis. Taken together, the above results indicate that there are many sequences, of redundant function, contributing to the expression of the proximal promoter of the *D.affinidisjuncta* gene in the larval fat body.

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REFERENCES

1. Beverley, S.M., and A.C. Wilson. (1982) *J. Mol. Evol.* **18**, 251−264.
2. Beverley, S.M., and A.C. Wilson. (1985) *Proc.Natl. Acad. Sci. USA* **82**, 4753−4757.
3. Throckmorton, L.H. (1975) In R.C. King (ed.), Handbook of Genetics. Plenum Press, New York, pp. 421−469.
4. Brennan, M.D., and W.J. Dickinson. (1988) *Dev. Biol.* **125**, 64−74.
5. Goldberg, D.A., J.W. Posakony, and T. Maniatis. (1983) *Cell* **34**, 59−73.
6. Benyajati, C., N. Spoerel, H. Haymeryle, and M. Ashburner. (1983) *Cell* **33**, 125−133.
7. Rowan, R.G., M.D. Brennan, and W.J. Dickinson. (1986) *Genetics* **114**, 405−533.
8. Rubin, G.M., and A.C. Spradling. (1982) *Science* **218**, 348−353.
9. Spradling, A.C., and G.M. Rubin. (1982) *Science* **218**, 341−347.
10. Martin, P., A. Martin, A. Osmani, and W. Sofer. (1986) *Dev. Biol.* **117**, 574−580.
11. Shen, N.L.L., E.C. Hotaling, G. Subrahmanyam, P.F. Martin, and W. Sofer. (1991) *Genetics* **129**, 763−771.
12. Shen, N.L.L., G. Subrahmanyam, W. Clark, P. Martin, and W. Sofer. (1989) *Devel. Genet.* **10**, 210−219.
13. Sofer, W., and P.F. Martin. (1987) *Annu. Rev. Genet.* **21**, 203−225.

14. Brennan, M.D., C.-Y. Wu, and A.J. Berry. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6866–6869.
15. Fang, X.-M., and M.D. Brennan. (1992) *Genetics* **131**, 333–343.
16. Fischer, J.A., and T. Maniatis. (1986) *EMBO J.* **5**, 1275–1289.
17. Sullivan, D.T., P.W. Atkinson, and W.T. Starmer. (1990) In M.K. Hecht, B. Wallace, and R.J. MacIntyre (ed.), Evolutionary Biology. Plenum Press, New York, Vol. 24, pp. 107–147.
18. Wu, C.-Y., and M.D. Brennan. (1993) *Mol. Gen. Genetics* **240**, 58–64.
19. Wu, C.-Y., J. Mote, and M.D. Brennan. (1990) *Genetics* **125**, 599–610.
20. Ayer, S., and C. Benyajati. (1990) *Mol. Cell. Biol.* **10**, 3512–3523.
21. Ayer, S. and C. Benyajati. (1992) *Mol. Cell. Biol.* **12**, 661–673.
22. Corbin, V., and T. Maniatis. (1989a) *Nature* **337**, 279–282.
23. Corbin, V., and T. Maniatis. (1989b) *Gen. Dev.* **3**, 2191–2201.
24. Falb, D., and T. Maniatis. (1992a) *Mol. Cell. Biol.* **12**, 4093–4103.
25. Corbin, V., and T. Maniatis. (1990) *Genetics* **124**, 637–646.
26. Bayer, C.C., S.W. Curtiss, J.A. Weaver, and D.T. Sullivan. (1992) *Genetics* **131**, 143–153.
27. Fischer, J.A., and T. Maniatis. (1988) *Cell* **53**, 451–461.
28. Heberlein, U., B. England, and R. Tjian. (1985) *Cell* **41**, 965–977.
29. Rowan, R.G., and W.J. Dickinson. (1988) *J. Mol. Evol.* **28**, 43–54.
30. Faisst, S., and S. Meyer. (1992) Nucleic Acids Res. **20**:3–26.
31. Moses, K., U. Heberlein, and M. Ashburner. (1990) *Mol. Cell. Biol.* **10**, 539–548.
32. Livant, D.L., A.E. Cutting, R.J. Britten, and E.H. Davidson. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7607–7611.
33. Brennan, M.D., R.G. Rowan, L. Rabinow, and W.J. Dickinson. (1984) *J. Mol. Appl. Genet.* **2**, 436–446.
34. Dickinson, W. J. (1980) *J. Mol. Evol.* **16**, 73–94.
35. Sambrook, J., E.F. Fritsch, and T. Maniatis. (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y.
36. Kunkel, T.A., J.D. Roberts, and R.A. Zakour. (1987) *Methods Enzymol.* **154**, 367.
37. Sanger, F., S. Niklen, and A.R. Coulson. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
38. Fridell, Y.C., and L.L. Searles. (1992) *Mol. Cell. Biol.* **12**, 4571–4577.
39. Benyajati, C., A.R. Place, N. Wang, E. Pentz, and W. Sofer. (1982) *Nucl. Acids. Res.* **10**, 7261–7272.
40. Sofer, W., and H. Ursprung. (1968) *J. Biol. Chem.* **243**, 3110–3115.
41. Zinn, K., D. DiMaio, and T. Maniatis. (1983) *Cell* **34**, 865–879.
42. Technau, G.M. (1987) *Development* **100**, 1–12.
43. Savakis, C., and M. Ashburner. (unpublished sequence data). EMBL accession number #M14802.
44. Fang, X.-M., C.-Y. Wu, and M.D. Brennan. (1991) *J.Mol.Evol.* **32**, 220–226.
45. Ahmed, S.U., C. Sackerson, and W.J. Dickinson. (unpublished).
46. Abel, T., R. Bhatt, and T. Maniatis. (1992) *Genes Dev.* **6**, 466–480.
47. Falb, D., and T. Maniatis. (1992b) *Genes Dev.* **6**, 454–465.
48. Benyajati, C., A. Ewel, J. McKeon, and E. Juan. (1992) *Nucleic Acids Res.* **20**, 4481–4489.
49. Yu, H., B. Porton, L. Shen, and L.A. Eckhardt. (1989) *Cell* **58**, 441–448.
50. Friedman, R., E. Hotaling, L. Borack, and W. Sofer. (unpublished).