The two small introns of the *Drosophila affinidisjuncta* Adh gene are required for normal transcription

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ABSTRACT

All *Drosophila* alcohol dehydrogenase (Adh) genes sequenced to date contain two small introns within the coding region. These are conserved in location and, to some extent, in sequence between the various species analyzed. To determine if these introns play a role in Adh gene expression, derivatives of the *Drosophila affinidisjuncta* Adh gene lacking one or both introns were constructed and analyzed by germline and transient transformation of *Drosophila melanogaster*. Removal of both introns lowered expression, whether measured by enzyme activity or by RNA levels. The decrease was seen in both germline transformed and transiently transformed larvae, with the effect being larger for germline transformants. Similar decreases (averaging 5-fold) were also seen at the embryonic and adult stages for germline transformants. Nuclear run-off transcription with nuclei from germline transformed embryos indicated that the reduction in RNA levels is due to decreased transcription. However, *LacZ* fusion constructs designed to test for the presence of a classical enhancer in the introns provided no evidence for such a mechanism. Removal of each intron individually resulted in more complex phenotypes. The introns have smaller, additive effects on expression in adults. In larvae, removal of the upstream intron significantly increases RNA levels but modestly decreases enzyme activity. Removal of the downstream intron lowers expression in both germline and transiently transformed larvae, but also increases position effects in germline transformants. Therefore, the small introns are clearly needed for optimal transcription of this Adh gene, but multiple mechanisms are involved.

INTRODUCTION

The Adh genes from many *Drosophila* species contain two promoters (1,2). Primary transcripts originating from the upstream (distal) promoter contain three introns: a large distal-specific intron (intron 1) in the non-translated leader and two smaller introns (introns 2 and 3) within the coding region. Transcripts arising from the downstream (proximal) promoter carry only the two small introns. The positions and approximate sizes of these two smaller introns are strongly conserved among the Adh genes from various species (3). Therefore, they could conceivably contribute to conserved aspects of Adh gene expression, such as transcription, in the larval and adult fat bodies. Though there is precedent for transcriptional regulatory elements within introns (4–8), no prior study has shown that the small introns (<100 bp) within the coding region of *Drosophila Adh* genes are needed for normal expression. Shen and co-workers addressed this for the *Drosophila melanogaster* Adh gene, by employing a transient transformation system that measured gene expression predominantly in the larval midgut (9). Their results suggest that the small introns of this gene are not essential for normal expression. On the other hand, phylogenetic analyses hint that there may be important roles for the small introns. Sullivan and colleagues (10) point out that rates of intron sequence divergence between some *Drosophila* species are less than expected relative to silent substitution rates. Furthermore, co-variational analysis suggests that a conserved stem–loop structure is formed by sequences within the second intron of the Adh genes from 10 diverse species of *Drosophila*, including *Drosophila affinidisjuncta* (11).

To determine if the small introns within the coding region of the *Daffinidisjuncta* gene are needed for normal transcription, genes lacking one or both of these small introns were constructed and tested by both germline and transient transformation. The gene lacking both introns is expressed at a significantly lower level than the unaltered gene. The decrease in expression was most pronounced in germline transformed embryos, larvae and adults, but was also seen in the fat body of transiently transformed larvae. Both introns contribute to expression, as deletions of each intron individually have distinct yet relatively modest effects. In germline transformed larvae, an unexpected increase in RNA levels resulted from deletion of intron 2, which contains the putative stem–loop structure. Deletion of the third intron caused increased susceptibility to chromosomal position effects in germline transformed larvae. The observed decrease in expression upon deletion of both introns is due to lowered transcription as shown by nuclear run-off transcription. However, gene fusions provided no evidence for the presence of a DNA enhancer in the introns. Taken together, these results suggest that the small introns are needed for normal transcription but that mechanisms other than a classical DNA enhancer are involved.

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To produce germline transformants, plasmids were injected into post-hatching flies. To assess enhancer function, a heat-shock promoter–LacZ fusion gene, SP73Lac1, was used as a host for both P element-mediated germline and transient transformation. Unless otherwise specified, transformants were harvested as embryos (aged 13–19 h), feeding third instar larvae (aged 7–8 days post-hatching), or as adults (aged 4–8 days post-eclosion).

**MATERIALS AND METHODS**

**Drosophila stocks**

The *D. affinidisjuncta* stock S36G1 was used as the source of the genomic and cDNAs for cloning (12). All *D. melanogaster* stocks were maintained as previously described (13). The Adh null stock, *Adh* flies, was used as a host for both P element-mediated germline and transient transformation. Unless otherwise specified, transformants were harvested as embryos (aged 13–19 h), feeding third instar larvae (aged 7–8 days post-hatching) or as adults (aged 4–8 days post-eclosion).

**Plasmid constructs**

The cDNA clone pADH3A (14) and the 5.4 kb *D. affinidisjuncta* fragment (–2832/+2618), abbreviated Aff, were used to construct intron-deleted genes (Fig. 1). Sequence identity between the coding regions of the genomic and cDNA clones was confirmed by dideoxynucleotide sequencing (14; M. Brennan, unpublished data, GenBank accession no. for cDNA U63563). Other genes were constructed by standard methods (15) using available restriction enzyme cleavage sites. Unless otherwise stated, restriction endonucleases and other DNA modifying enzymes were obtained from New England Biolabs and used according to the supplier’s recommendations. To produce plasmids for germline transformation, genes were inserted into Carnegie 20 as described (16,17). A heat-shock promoter–LacZ fusion gene, SP73Lac1 (kindly provided by T. Abel), served as the reporter to assess enhancer function. Briefly, this construct consists of the *D. melanogaster* hsp70 promoter (–43 to +265) fused in-frame to the Escherichia coli LacZ gene inserted into the Smal site of the polylinker of the vector pSP73 (18). Fragments of the *D. affinidisjuncta* Adh gene tested for enhancer function were inserted in the sense orientation between the XbaI and XhoI sites of the polylinker immediately downstream of the LacZ gene. All plasmids were purified by CsCl density gradient centrifugation prior to injection.

**Germline and transient transformation**

To produce germline transformants, plasmids were injected into pre-blastoderm embryos and homozygous stocks were established from transformed progeny as described previously (13,17,19–21). Only transformed stocks carrying single intact transposons in autosomal locations (as determined by Southern analysis) were analyzed (22). Determination of alcohol dehydrogenase enzyme (ADH) specific activity for germline transformants was as described (23,24). RNA levels were determined by RNAse protection assays with the probes and methods detailed previously (25). Quantification of RNA levels was by scanning densitometry of autoradiograms or by phosphorimaging (Molecular Dynamics PhosphorImager) and, unless otherwise specified, all ADH values were corrected by normalization to the actin control (25).

Transient transformation of the larval fat body, including measurement of ADH activity and β-galactosidase activities, was as described previously (26). Unless otherwise specified, the vermillion–LacZ fusion gene (pTUF1.1 v-LacZ) served as an internal control for injection efficiency (27).

**Nuclear run-off transcription**

Nuclear run-off transcription reactions were carried out by a modification of previously described methods (28,29). Embryos (aged 13–19 h) were collected on nylon mesh and rinsed with chilled water (10–15°C). They were dechorionated for 90 s in a 2-fold dilution of commercial bleach at room temperature and rinsed quickly in chilled wash solution buffer (0.7% w/v NaCl, 0.04% v/v Triton X–100) using 10 mg/ml embryos. They were then rinsed in chilled water (10–15°C), blotted dry and weighed. Unless otherwise specified, all subsequent steps were performed at 0–4°C. Typical yields were 50–150 mg embryos from four half-pint bottles of adult flies. Volumes, where specified, are those used for 100 mg embryos. Embryos were homogenized in a 7 ml Dounce homogenizer (Wheaton Scientific) containing 1 ml ice-cold buffer I (15 mM HEPES (K+), pH 7.6, 10 mM KCl, 0.1 mM MgCl2, 0.1 mM EDTA, 0.5 mM EGTA, 350 mM sucrose, 1 mM dithiothreitol, 1 mM sodium bisulfite, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride] using the B pestle. Homogenates were filtered through one layer of Miracloth (Calbiochem) and the debris retained by the Miracloth was rinsed with an additional 0.2 ml buffer I. The nuclei were transferred to a 15 ml Corex tube and centrifuged at 42°C for 60 min followed by two extractions with an equal volume of commercial bleach at room temperature and then resuspended in 0.1 ml freshly prepared reaction buffer containing 1 mM each of ATP, CTP and GTP by gentle pipetting. The reaction mixture was transferred to a 1.5 ml Eppendorf centrifuge tube and incubated in a Sorvall SS-34 rotor at 9200 r.p.m. (10 000 g) for 15 min. The supernatant was carefully decanted from the loose pellet of nuclei and the nuclei were suspended, avoiding both the hard yellow yolk pellet and lipid deposits, in 1 ml chilled reaction buffer (5 mM Tris–HCl, pH 8, 5 mM MgCl2, 0.3 M KCl). The nuclei were transferred to a 15 ml Corex tube and dispersed by gentle pipetting through a 1–200 µl pipette tip. They were centrifuged in a Sorvall SS-34 rotor at 9200 r.p.m. for 15 min and then resuspended in 0.1 ml freshly prepared reaction buffer containing 1 mM each of ATP, CTP and GTP by gentle pipetting. The reaction mixture was transferred to a 1.5 ml Eppendorf centrifuge tube, 5 µl [α–32P]UTP (ICN, 760 Ci/mmol, 10 µCi/ml) was added and the tube was agitated at 25°C for 30 min. To prepare RNA, 150 µl HS buffer (0.5 M NaCl, 50 mM MgCl2, 2 mM CaCl2, 10 mM Tris–HCl, pH 7.4) containing 0.12 U/µl RNase-free DNase I (Stratagene) were added to the reaction mixture followed by incubation at 30°C for 5 min. Digestion was stopped by adding 50 µl SDS/Triton buffer (5% SDS, 0.5 M Tris–HCl, pH 7.4, 0.125 M EDTA, 2.5 µl proteinase K (20 µg/ml) and 10 µl yeast tRNA (10 mg/ml). This was incubated at 42°C for 60 min followed by two extractions with an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous phase was precipitated overnight at −80°C with 2 volumes of ethanol and 0.5 volumes of 3 M sodium acetate (pH 5.2) and resuspended in 50 µl DEPC water.
The reaction was quenched with 375 µl 1 M NaOH were added and the mixture was placed on ice for 10 min. The reaction was then 375 µl 1 M HEPES (free acid) in a 15 ml Corex tube followed by precipitation with sodium acetate (0.33 M) and 2.5 vol cold ethanol. Nucleic acids were recovered by centrifugation for 30 min at 10 000 r.p.m. in a HB-4 rotor and dissolved in 400 µl TES (10 mM Tris–HCl, pH 7.4, 10 mM EDTA, 0.2% SDS). RNA was precipitated with 0.075 vol 4 M NaCl and 2.5 vol ethanol, recovered by centrifugation, dried under vacuum and dissolved in 250 µl TES. Incorporation of 32P was determined by scintillation counting; typical incorporation was 4–8 x 106 c.p.m./ml. Samples were then precipitated with 0.5 vol 7.5 M ammonium acetate and 2.5 vol cold ethanol. RNA was dissolved in 250 µl TES. An additional 250 µl TES solution containing 0.6 M NaCl (1× TES/NaCl) was then added. The total volume of solution was hybridized to DNA immobilized on filter strips with gentle agitation for 36 h at 65°C.

Plasmids were linearized and bound to nitrocellulose as described (29). Three plasmids were used as controls: pUC19, actin 5C and rp49. The actin 5C plasmid, a gift of S.Tobin, is a 3.7 kb EcorI–HindIII genomic fragment carrying exons 1 and 2 of the actin 5C gene (30) inserted into pGEM-1 (Promega). The rp49 (ribosomal protein-49) plasmid, HR0.6, has been described elsewhere (31). The cDNA-containing Adh plasmid used was pADH3A (14). Membranes were blocked with 1× TES/NaCl containing 10× Denhardt’s by gentle agitation for 3 h at 65°C, followed by a similar incubation with prehybridization buffer [1× TES/NaCl solution, 10× Denhardt’s, 50 µg/ml sonicated, single-stranded calf thymus DNA, 10 µg/ml poly(A) (Boehringer Mannheim) and 0.1% w/v sodium pyrophosphate]. Following hybridization, membranes were washed with 2× SSC ([1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0] for 1 h at 65°C and then incubated in 2× SSC containing RNase A (10 µg/ml) at 37°C for 30 min. Membranes were washed with 2× SSC at 37°C for 1 h, followed by washes in 1× and 0.5x SSC for 10 min each at 65°C. Autoradiograms were prepared and signal strength was determined by scanning densitometry.

Statistical methods

Four to five different germline transformed lines were analyzed for each construct. Enzyme activity and RNase protection values for germline transformed larvae and adults were obtained from three independent preparations. The mean value for the transformed line was then treated as a single determination such that n for each gene analyzed by germline transformation was equal to the number of independent lines. For transient transformation, five samples, each consisting of 10 dissected larvae, were analyzed for each construct. Mean values for different genes were compared by the two sample Student’s t-test for two-tailed hypotheses (32).

RESULTS

Removal of the small introns lowers expression in adults

To determine if the two small introns are critical to expression of the Adh gene in adults, enzyme activities and RNA levels of adult germline transformants carrying the same constructs were analyzed. Loss of both introns results in a 4- to 14-fold (mean 7-fold) decrease in enzyme activity and a 3- to 10-fold (mean 4-fold) drop in total Adh RNA levels. The latter can be partitioned into a 3- to 13-fold (mean 5-fold) drop in distal transcript levels and a 2- to 6-fold (mean 3-fold) drop in proximal transcript levels (Fig. 3).

As in larvae, deletion of only the second intron causes a less profound but measurable difference. Enzyme activities and RNA levels decrease ~2-fold (Fig. 3). Unlike the case for larvae, however, the two measures of expression correlate well in adults, not only for this deletion but for the other intron deletions as well. Pairwise comparisons of mean enzyme activity and total Adh RNA levels for each of the three intron-deleted genes in adults show no statistically significant differences between the two measures of gene expression. The fact that RNA and activity values correlate well in adults but not in larvae may relate to the presence of the large distal intron uniquely present in adult primary transcripts.

Transformants carrying the construct lacking only the third intron show a 2.9-fold decrease in enzyme activity and a 2.4-fold decrease in RNA levels (Fig. 3). Thus in adults the introns appear to act additively to affect expression, as the decrease in expression for ΔIn is about the same as that for the combined decrease in expression for the two single deletions. In both larvae and adults, the most profound impact on expression is seen upon deletion of both introns. This suggests that both introns contribute to expression, with the third intron having a slightly greater effect.
Deletion of both small introns lowers transcription but does not remove a DNA enhancer

Deletion of the two small introns lowers Adh RNA levels 3- to 5-fold in both larvae and adults. This drop in steady-state levels of RNA is due either to decreased transcription or to enhanced degradation of transcripts from the altered genes. The former possibility would be expected if the introns contain elements that contribute to transcription by any of several possible mechanisms, including one or more DNA or RNA enhancers or altered chromatin structure. However, it is conceivable that loss of the two small introns, along with the intrinsic splicing signals, affects the ability of these altered transcripts to be properly processed (capping, proper 3'-end generation and polyadenylation). This, in turn, may hinder any of a number of processes that could affect RNA stability, such as export from the nucleus, subcellular localization or ribosome targeting (33).

In order to differentiate between effects on transcription and stability, transcription rates for the intron-deleted gene (ΔInts) must be compared with that of the intron-containing gene by nuclear run-off transcription. The previously described experiments involved larval and adult flies. However, nuclear run-off experiments in Drosophila are more conveniently accomplished using embryos, because the methods for obtaining nuclei from embryos are relatively well established.

To determine the developmental profile of Adh transcription in D. melanogaster embryos carrying the unaltered D. affinidisjuncta Adh gene, RNA was isolated from staged, transformed embryos.
Figure 4. Temporal pattern of *D. affinis* *Adh* RNA accumulation during embryogenesis. RNA levels were determined by RNase protection analysis of *Adh* RNA isolated from embryos 0–20 h of age. Each gel lane represents the total nucleic acids from 100 mg embryos. The probe fragment protected by the proximal transcript is labeled P. The probe fragment protected by the distal transcript is labeled D. The two fragments protected by the actin 5C probe are labeled A. The graph at the bottom shows the densitometric scanning values for the proximal and distal transcripts. As is the case for all of our RNase protection analyses, the signal strengths are corrected for the lengths of the protected products. However, because the levels of the actin transcripts vary with developmental stage, the *Adh* RNA values are expressed per mg of embryos rather than being normalized with reference to the actin values.

Figure 5. Deletion of introns lowers RNA levels in embryos. RNA levels were determined by RNase protection analysis of *Adh* RNA isolated from embryos (13–19 h of age) carrying either the unaltered gene (Aff) or the gene lacking both small introns (ΔInts). Protected fragments are labeled as in Figure 4.

Figure 6. Deletion of both small introns lowers the transcription rate. Transcription rates for the unaltered gene (Aff) and the gene lacking both small introns (ΔInts) were compared by nuclear run-off transcription. Plasmids bound to the filter are indicated at left.

and analyzed by RNase protection. The signals were scanned densitometrically and the results are shown in Figure 4. Low levels of both distal and proximal transcripts are first detected at ∼1 h following egg laying. These remain relatively unchanged to ∼7 h, whereupon distal transcripts increase, followed by a similar increase in proximal transcripts at ∼10 h. Levels of distal RNA peak at ∼10 h, whereas levels of proximal RNA continue to increase until ∼17 h and then plateau. This profile is remarkably similar to that determined by others for the *D. melanogaster* gene (34,35).

To determine if removal of the two small introns affects expression of the *D. affinis* *Adh* gene in embryos, RNA levels were analyzed for 13–19 h old embryos from transformed stocks carrying Aff and ΔInts (Fig. 5). Depending on the particular stocks compared, levels of *Adh* RNA are 2- to 5-fold lower in embryos carrying ΔInts than in those carrying Aff.

Transcription rates for transformed genes were determined via nuclear run-off analysis. Nuclei were prepared from 13–19 h old *D. melanogaster* embryos carrying either the unaltered or intron-deleted gene. Two stocks carrying Aff and two stocks carrying ΔInts were used for this analysis. These stocks were chosen because they showed RNA levels in larvae and adults which were closest to the mean values for stocks carrying the respective gene. Radiolabeled RNA prepared by nuclear run-off transcription was hybridized to filters carrying four DNA probes: pUC19, as a vector control; actin 5C and rp49, as constitutively expressed positive controls; and pADH3A, a plasmid carrying a *D. affinis* *Adh* cDNA (Fig. 6). Densitometric analysis of the autoradiograms, followed by normalization of the *Adh* values to those from the control plasmids, indicated that the two transformed lines carrying Aff show 3- to 10-fold higher transcription rates than seen for the two lines carrying the intron-deleted gene. This difference is similar in magnitude to the differences in expression observed between the two genes in both larval and adult enzyme activities and in embryonic, larval and adult RNA levels. The fact that transcription rates, RNA levels and enzyme...
activities for these two genes are all within the same range suggests that the deletion of both introns affects expression primarily by decreasing the rate of transcription.

Since the introns influence transcription, we next wanted to determine if they contain a classical enhancer, capable of increasing transcription of a heterologous promoter. To address this, a fragment of the *D. affinidisjuncta* gene containing both of the small introns was inserted into SP73Lac1 (Material and Methods). This *hsp70* promoter–*LacZ* fusion gene was designed to test sequences for enhancer function and is known to respond to *D. melanogaster* *Adh* enhancer sequences in the larval fat body (18). A *Nrd–SmaI* fragment (+127 to +756 relative to the proximal transcription initiation site; 3) containing both small introns was excised from *Aff*. This fragment, +Ints, was inserted into the test vector to generate SP73Lac1/+Ints. The inserted fragment carries small portions of adjacent exonic sequences (25 bp of exon 2 and 44 bp of exon 4) as well as all of exon 3. Similarly, as a control, the plasmid SP73Lac1/Alnts was constructed using the *Adh* fragment from the cDNA-containing plasmid pADH3A (14). Therefore, this plasmid carries identical exonic *Adh* sequences but lacks the two small introns.

These two plasmids, in addition to SP73Lac1 as a vector-only control, were tested by transient transformation. Initial tests of varying concentrations of the *LacZ* reporter plasmid indicated that a 4-fold molar excess (relative to the *Adh* internal control plasmid) generated a β-galactosidase value within the linear range of the assay (data not shown). The intron-containing fragment had no effect on expression of the β-galactosidase reporter gene, as the value obtained for SP73Lac1/+Ints is not significantly different from the value for either of the two control plasmids (Fig. 7, *P* > 0.5 for all pair-wise comparisons).

**DISCUSSION**

**The small introns influence expression at the transcriptional level**

Prior to this study, no regulatory role had been assigned to the small introns of any *Adh* gene in *Drosophila*. The present study shows that the small introns of the *D. affinidisjuncta* gene individually have modest but significant effects on expression and that removing both introns lowers expression an average of 5- to 10-fold in embryos, larvae and adults. Expression from both the distal and proximal promoters is affected by the presence of both introns. Nuclear run-off transcription demonstrated that the two small introns clearly influence transcription. However, multiple mechanisms are likely to be involved.

The effect on transcription is probably not due to the presence of a classical enhancer in one or both small introns. The reporter construct that we used to test for the presence of a DNA enhancer is known to be responsive to *D. melanogaster* *Adh* enhancers (18). However, we cannot rule out the possibility that the small introns contain an enhancer that fails to activate the *hsp70* promoter. Furthermore, although we have retained relative positioning and orientation within the intron-containing fragment tested, it is possible that a sub-element of a large enhancer lies outside the tested region (36).

One possible mechanism by which lack of intervening sequences could interfere with transcription of this gene involves torsional stress. Intronic sequences may serve in part to relieve such forces inherent in the transcription process (37). The relatively greater influence of the introns when the genes are assayed by incorporation into the chromosomes rather than by transient transfection is consistent with a systematic difference in torsional stresses between the chromosomal and plasmid environments. Other possible mechanisms are discussed below in the context of removing each of the small introns individually.

**Deletion of the second intron alters the RNA/activity ratios in larvae**

The RNA/activity ratios (relative RNA levels compared with relative enzyme activities) for the individual transformed stocks carrying the unaltered gene, *Aff*, show little deviation from 1. In contrast, RNA levels in larvae for stocks carrying ΔI(2) exceed enzyme activities consistently by at least 2-fold. This may reflect ordered splicing of the primary transcript. Evidence from several systems indicates that splicing of introns proceeds in an ordered fashion such that the presence of one intron facilitates removal of another (38–40). If splicing of the *D. affinidisjuncta Adh* primary
transcript also proceeds in an ordered fashion, deletion of the second intron may affect removal of the downstream third intron. Consistent with this possibility, the second intron contains 5′ and 3′ splice sites that more precisely match the consensus for small introns in Drosophila (3,41). Thus, the splicing machinery may interact less efficiently with primary transcripts lacking intron 2, which could lead to their accumulation in the nucleus or the export of incompletely processed RNAs to the cytoplasm (33,40). Given that the RNase protection assay measures only the 5′-termini of the Adh transcripts and we isolated total cellular RNA, this may account for inflated RNA levels for ΔI(2) larval transformants accompanied by low to normal levels of enzyme activity. Alternatively, if the ΔI(2) transcripts are transported normally but not translated efficiently, perhaps due to poor polyadenylation or capping (33,42–44), this could explain the altered relationship between RNA levels and enzyme activities, although it would not account for the inflated RNA levels.

A possible explanation for the relative importance of the second intron in larvae is suggested by the work of Stephan and Kirby (11), which provides evidence for a stem–loop that is conserved across 10 species of Drosophila, including D.affinidisjuncta. In the D.affinidisjuncta gene, the potential stem–loop is positioned toward the 5′-end of the 81 nt intron, with 13 nt preceding it. Such RNA secondary structures have been shown to be involved in splicing (45–47).

Results for a naturally occurring sequence variant of the D.melanogaster gene may parallel these findings. Laurie and Stam (8) demonstrated that deletion of sequences, containing a potential hairpin structure (48), within the large-distal-specific intron of the Fast allele of Adh in D.melanogaster, causes a 15–20% higher protein level in ADHfast adults compared with ADHslow adults. However, they found no statistically significant difference between levels of RNA for the two genes, suggesting that secondary structure in the primary transcript affects translational efficiency.

Secondary structure within primary transcripts can influence transcription as well (49). In HIV-1 transcripts, a stem–loop referred to as TAR interacts with a viral protein, Tat, to affect transcription. The manner in which this occurs has yet to be resolved. The ability of the TAR–Tat complex to activate the transcription has yet to be resolved. The mechanism by which intron 3 buffers against position effects in larvae is unclear. Interestingly, studies of linkage disequilibrium suggest the existence of a stem–loop structure in the corresponding intron of the D.pseudoobscura gene (55). So it is possible that an RNA enhancer is involved. Alternatively, by analogy with the D.melanogaster gene, nucleosomial positioning is likely to be important for transcription (56,57) and this could be affected by the presence of the third intron.

In summary, the two small introns of the D.affinidisjuncta Adh gene clearly affect its transcriptional efficiency. Therefore, such small introns may be more important in gene expression than generally assumed. The introns do not contain a classical DNA enhancer, at least for larvae. They may, however, contain one or more RNA enhancers. Additionally, in larvae removal of the second intron likely results in altered RNA processing or nuclear export, while removal of the third intron causes increased sensitivity to chromosomal position effects.

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