Deletions at intervening sequence splice sites in the alcohol dehydrogenase gene of *Drosophila*

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ABSTRACT

Two formaldehyde-induced, homozygous viable ADH-negative mutants, *Adh^{fn4}* and *Adh^{fn6}*, possess no material that cross-reacts with antibody directed against ADH, no mature mRNA of wild-type size, and greatly reduced amounts of RNA that hybridizes with an *Adh* probe. We have cloned the genomic DNA sequences from these mutants in bacteriophage λ Charon 4 and subcloned the *Adh* region into plasmid vector pBR327. Restriction analyses revealed one small deletion in each of these mutants and DNA sequencing showed that the splice junctions of the 65-base pair (bp) intervening sequence (IVS) were altered. Both cloned mutant *Adh* genes, as well as the wild-type gene, are capable of promoting correct specific transcription initiation in HeLa cell nuclear extracts in vitro. We conclude that *Adh^{fn4}* and *Adh^{fn6}* are defective in RNA processing. Our results provide evidence for the importance of the splice junction sequences in normal ADH RNA processing and stabilization in *Drosophila*. We also speculate that splicing of ADH RNA proceeds in a nonrandom manner: mutations in one of the intervening sequences appear to cause accumulation of a large ADH RNA containing at least one other IVS.

INTRODUCTION

The regulation of gene expression in eucaryotic cells has been demonstrated at the level of transcription, nuclear RNA processing, and mRNA stability (1). Our strategy for studying the expression of the alcohol dehydrogenase gene (*Adh*) of *Drosophila melanogaster* has been to secure mutants at the *Adh* locus (2,3) and analyze them at the molecular level.

ADH is coded by a single-copy gene (4,5) that is regulated during development and displays tissue-specific expression (6). From DNA sequence analysis of a cloned genomic sequence (5), we find that there are 2 intervening sequences (IVS) of 65 and 70 bp interrupting the protein-coding region. In addition, our recent studies on mRNA sequencing of the ADH message from adult flies indicate that there is another IVS of 654 bp interrupting the genomic DNA sequence that encodes the 5' untranslated region of adult ADH RNA (C. Benyajati *et al.*, in preparation). All 3 IVS are flanked by the 5' GT and 3' AG dinucleotides characteristic of IVS boundaries in eucaryotic genes (7).

By using chemical selection procedures that allow isolation of flies that lack ADH enzyme activity (8,9), we have recovered a large number of Adh mutants after ethyl methanesulfonate and formaldehyde mutagenesis (2,3). Among these mutants we hoped to find some with lesions in the regulatory sites affecting gene expression. With the advent of recombinant DNA technology, we have started to characterize those mutants that appear most likely to fall into this class at the molecular level. The detailed analysis of two such mutants is presented here.

MATERIALS AND METHODS

Organisms

Adh^D is an ADH-positive strain, which is homozygous for the Adh^D allele. Adh^{fn23}, Adh^{fn24}, Adh^{fn6}, and Adh^{fn4} are homozygous viable, null activity derivatives of Adh^D generated by formaldehyde mutagenesis (3).

Construction of Drosophila bacteriophage λ libraries, identification of the λ plaques containing the Adh gene, and their subcloning

DNA of each of the 4 Adh⁻ strains and Adh^D was isolated from adult flies and partially digested with EcoRI. Fragments of 12 to 20 kilobases (kb) were purified by sucrose gradient centrifugation (10). They were then inserted into the bacteriophage Charon 4 (11), and the DNA was packaged in vitro (12). The λ phages containing the Drosophila Adh region were identified by plaque hybridization (13) using a ³²P-labeled nick-translated genomic Adh clone (in pBR322) (4) as probe. The entire Adh gene region was subcloned as a 4.8 kb EcoRI fragment into the EcoRI site of pBR327 (14). All the plasmid DNAs used in this study were cloned in the same orientation, which is shown in Fig. 4.

Restriction analysis of the cloned DNA

Plasmid DNA was isolated by the cleared lysate method (15) and was twice purified by CsCl-ethidium bromide equilibrium centrifugation. Restriction enzymes were from Bethesda Research Labs and New England Biolabs. Restriction fragments were fractionated on agarose gels using the 0.05 M Tris-borate, 1 mM EDTA buffer system. High-resolution gel electrophoresis was carried out on 0.5% agarose - 3.5% polyacrylamide gels and also on 6% polyacrylamide gels.

RNA

Total RNA was isolated from adult flies as described previously (16), except that the low-speed centrifugation step was omitted.

RNA blot hybridization

RNA samples were denatured and fractionated on 1.5% agarose gel containing formaldehyde (17) as described by Goldberg (4). The RNA was trans-

ferred to nitrocellulose paper and hybridized with ^{32}P -labeled nick-translated pADH cDNA (16) as described by Wahl *et al.* (18). The *E. coli* 16S (1541 bases, ref. 19) and 23S (2904 bases, ref. 20), the mouse 18S and 28S (about 1800 and 4000 bases, ref. 21) ribosomal RNAs were used as unlabeled RNA size markers on the same gel.

DNA sequencing

DNA sequence analyses were performed according to Maxam and Gilbert (22) using restriction fragments that had been labeled at the 5' termini by T4 polynucleotide kinase (PL Biochemicals). The strategy for determining the sequence of the *Adh* genes was as described previously (5).

Preparation of HeLa cell-free extract containing endogenous RNA polymerase II

The extracts were made according to Weil *et al.* (23) with a slight modification of Talkington *et al.* (24). The modification involved freezing of the extract between the homogenization and centrifugation steps. This resulted in an S-100 preparation highly enriched with RNA polymerase II and eliminated the requirement for exogenous RNA polymerase II. The protein concentrations of the extracts were approximately 12-17 mg/ml. The extracts were stored in aliquots at -80°C .

In vitro transcription and analysis of RNA products

RNA was synthesized in S-100 extracts of HeLa cell according to Talkington *et al.* (24). Plasmid DNAs, each containing the cloned *Adh* wild-type and mutant 4.8-kb *EcoRI* fragments, were cleaved with *PvuII* and *EcoRI* to generate DNA fragments of 3.27, 2.5, and 2.3 kb in length which were used as templates in 50- μl *in vitro* transcription reaction mixtures with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ as label. Each reaction mixture consisted of 2 μg of restricted plasmid DNA, 7.5 mM MgCl_2 , 10 mM Hepes, pH 7.9, 10% glycerol, 0.1 mM EDTA, 0.25 mM dithiothreitol, 50 mM KCl, 4 mM creatine phosphate, 60 μM uridine $[\alpha\text{-}^{32}\text{P}]$ triphosphate ($[\alpha\text{-}^{32}\text{P}]\text{UTP}$ at 400 Ci/mmol was purchased from Amersham), 600 μM of each rNTPs (PL Biochemicals), and 25 μl of the S-100 extract in the presence or absence of 1 $\mu\text{g}/\text{ml}$ α -amanitin. The reactions were incubated at 30°C for 45 minutes and terminated by the addition of 200 μl of 66 mM sodium acetate, pH 5.5, 0.3% sodium dodecyl sulfate, and 2 μg yeast tRNA. The resultant transcripts were extracted twice with phenol and chloroform (1:1), once with chloroform and precipitated with ethanol. The samples were reprecipitated with ethanol once again to further remove the unincorporated rNTPs. Each dried pellet was resuspended in 10 μl of 90% deionized formamide, 0.05% xylene cyanol, bromophenol blue, heated at 90°C for 3 minutes and electrophoresed on 8M urea-4% polyacrylamide gel in

0.05 M Tris-borate, 1 mM EDTA buffer, pH 8.3. The fractionated transcripts were visualized by autoradiography (Kodak XAR-5 film). Fragments of ϕ x174 DNA cleaved with HaeIII and end-labeled, were used as size markers on the same gel.

RESULTS

Four formaldehyde-induced ADH⁻ mutant strains (Adh^{fn23}, Adh^{fn24}, Adh^{fn6}, Adh^{fn4}, all derivatives of Adh^D) have been classified as cross-reacting material positive (CRM⁺, Adh^{fn23}) and cross-reacting material negative (CRM⁻, Adh^{fn24}, Adh^{fn6} and Adh^{fn4}) by using goat anti-ADH antibody (unpublished results). They were next examined for the presence of ADH RNA by RNA blot hybridization (Fig. 1). Total RNA prepared from wild-type and Adh mutant adult flies was electrophoresed on a denaturing formaldehyde agarose gel, transferred to nitrocellulose, and probed with ³²P-labeled pADH cDNA (16). These 'Northern' blots revealed the following: the Adh^D strain possesses an ADH mRNA of about 1150 bases (the same as other wild-type strains), which can be translated *in vitro* into ADH monomeric protein and which accounts for 1-2% of the total translational activity in the adult fly (16). The Adh^{fn23} strain has an ADH-like RNA species of about 1100 bases, just slightly shorter than that of the wild-type; the Adh^{fn24} strain has an RNA species of about the same size as Adh^D. In contrast, both the Adh^{fn6} and Adh^{fn4} strains show very low amounts of an ADH-like RNA that is about 850 bases longer than normal and no detectable mature wild-type size RNA. Quantitative solution hybridization studies indicate that the amounts of RNA hybridizing to a cloned Adh-genomic probe (5) in Adh^{fn23} and Adh^{fn24} adult flies are approximately 90% and 50% of Adh^D, respectively, whereas both Adh^{fn6} and Adh^{fn4} adult flies possess only 5-10% of the wild-type RNA levels (unpublished results).

The Adh gene organization of Adh^D and these 4 Adh-null derivatives were compared by Southern blot hybridization of genomic DNA cleaved with several restriction endonucleases. We were unable to detect any changes in restriction sites or sizes of DNA fragments in these mutants by this analysis (data not shown). The restriction maps obtained for the 5 strains are identical to those reported previously (4,5).

We next constructed bacteriophage λ Charon 4 libraries containing genomic Drosophila DNA from each fly strain. The λ phages containing the Drosophila Adh genes were identified by plaque hybridization. Because the entire Drosophila Adh gene has been previously located on a 4.8-kb EcoRI restriction fragment (4,5), we isolated and subcloned the Adh gene regions

Figure 1. Autoradiogram of *Drosophila* adult total RNA hybridizing to pADH-cDNA. RNA samples (about 5 μ g per lane) were denatured and fractionated on 1.5% agarose gels containing formaldehyde, transferred to nitrocellulose paper, and hybridized with 32 P-labeled nick-translated pADH cDNA. Two different preparations of RNA from each strain are shown. The *E. coli* 16S and 23S, and the mouse 18S and 28S ribosomal RNAs (see Materials and Methods) were used as unlabeled RNA size markers on the same gel.

from *Adh*^D and its four *Adh*-null derivatives as a 4.8-kb *Eco*RI fragment into the *Eco*RI site of pBR327 (see Fig. 4). High-resolution restriction maps of the *Adh* gene inserts were then generated using frequent-cutting restriction enzymes that recognize 4-bp sequences. An analysis with the restriction endonuclease *Hha*I is shown here. Ethidium bromide-stained DNA fragments from the 5 cloned *Adh* genes and pBR327 are shown in Fig. 2. We discovered one small deletion (ranging from ~10-40 bp) in each of the 4 *Adh* mutants. The deletions in the *Adh*^{fn4}, *Adh*^{fn6}, and *Adh*^{fn24} strains were located on a 270-bp *Hha*I fragment, which was identified by secondary restriction cleavage to be in the 5' region (or the N-terminal) of the *Adh* gene. The *Adh*^{fn23}

Figure 2. Restriction analysis of the cloned Adh mutants and wild-type. 1 μ g of each of the plasmid DNA was digested with restriction nuclease HhaI, fractionated on 0.5% agarose - 3.5% polyacrylamide gel, and visualized by ethidium bromide staining. pBR327 DNA was included to identify the HhaI fragments that originated from the vector.

strain, on the other hand, contained a deletion in a 650 bp HhaI fragment located in the 3' region (or the C-terminal) of the Adh gene. No other changes in restriction sites or sizes of DNA fragment were detected by this analysis. The positions and sizes of the deleted DNA were also confirmed by cleavages with several other restriction enzymes. We proceeded to sequence the DNA surrounding the deleted regions. The remainder of this report describes the characterization of the cloned genes from Adh^D, Adh^{fn4}, and Adh^{fn6} by DNA sequence analysis and *in vitro* transcription studies. The analyses of Adh^{fn23} and Adh^{fn24} will be reported in detail elsewhere.

The deletions of Adh^{fn4} and Adh^{fn6} are contained entirely within the 65-bp IVS (see Fig. 4). Figure 3 shows the 65-bp IVS of Adh^D and the corresponding regions in Adh^{fn4} and Adh^{fn6}. The protein-coding regions flanking this IVS are identical in Adh^D and the two Adh mutants. Adh^{fn4} has a 17-bp deletion and 1-bp substitution. One breakpoint is between the conserved dinucleotide AG of the 3' splice site (7) and is accompanied by an AG → GG transition. Adh^{fn6} has a 6-bp deletion and a 5-bp substitution. One breakpoint is between the conserved dinucleotide GT of the 5' splice site (7) and is accompanied by a GT → GC transition. These deletions and nucleotide rearrangements in both mutants drastically change the nucleotide context of the eucaryotic consensus splice junction sequences (7).

To further characterize the cloned Adh genes from Adh^D, Adh^{fn4}, and Adh^{fn6} at the functional level, we carried out transcription studies *in vitro* with these cloned genes as templates using HeLa cell-free extracts (23). Convincing evidence has been accumulated (24-30) that the HeLa whole cell extract system of Manley *et al.* (30) and the HeLa cytoplasmic extract system of Weil *et al.* (23) permit the accurate initiation of transcription by RNA polymerase II on cloned eucaryotic DNA templates from various species. We first identified a functional promoter for RNA polymerase II on the cloned Adh^D 4.8-kb EcoRI fragment by transcribing truncated DNA templates and analyzing the specific runoff RNA transcripts on denaturing gels (C. Benyajati *et al.*, in preparation). This promoter is on the 387-bp SalI-HpaI fragment of the Drosophila 4.8-kb EcoRI fragment; the *in vitro* transcript is specifically initiated approximately 60 bp from the SalI site towards the HpaI site

Figure 3. The DNA sequence of the 65-bp intervening sequence of the Drosophila wild-type and mutant Adh genes. Vertical lines show where the splicing events must occur to give mature mRNA. The number of nucleotides deleted in each mutant is shown in the triangle.

(Fig. 4). The *in vivo* ADH transcript of adult flies is also initiated specifically 61 bp downstream from the *Sal*I site, as determined by S1 nuclease mapping plus primer extension experiments and by direct sequencing of the ADH mRNA from adult flies (C. Benyajati *et al.*, in preparation). It appears then that the HeLa extract transcription system correctly recognizes the *Drosophila* *Adh* promoter that is active in adult flies. To investigate whether the *Adh*^{fn4} and *Adh*^{fn6} cloned genes can also promote transcription initiation *in vitro* with the same efficiency as the cloned *Adh*^D gene, we transcribed each DNA (cloned into pBR327 in the orientation shown in Fig. 4), after first cleaving each template with *Pvu*II and *Eco*RI restriction endonucleases. As shown in Fig. 5 (lanes 2,3,4), the cloned wild-type and mutant genes each can promote a specific runoff transcript of 1100 bases. This is the size expected if transcription is initiated at the proper site 61 bp from the *Sal*I site. As shown by its sensitivity to α -amanitin at 1 μ g/ml, the transcription is mediated by RNA polymerase II (Fig. 5, lane 1). A number of fainter products of higher molecular weight can be seen. These presumably result from end-to-end transcription of the DNA template fragments (26). The presence of the 1100 base *in vitro* transcript also indicates the lack of RNA processing activity in this *in vitro* transcription system. From this *in vitro* transcription study and our detailed restriction mapping, it appears that the

Figure 4. Restriction map and the *Drosophila* *Adh* gene organization on a *Drosophila* 4.8-kb *Eco*RI fragment. About 2.2 kb of DNA covering the entire *Adh* gene has been sequenced (5). The solid box represents the protein encoding region; the hatched box represents the *in vivo* 5' and 3' transcribed untranslated regions. The arrow denotes the length of the *in vitro* transcript (1100 bp) (see Fig. 5), initiating about 60 bp from the *Sal*I site (which is also the *in vivo* initiation site) and ending at the *Pvu*II site.

Figure 5. Gel electrophoresis of *in vitro* runoff transcripts of the *Drosophila* *Adh* genes. Plasmid DNAs, each containing the cloned *Adh* wild-type and mutant 4.8-kb *EcoRI* fragments, were cleaved with *PvuII* and *EcoRI* to generate DNA fragments of 3.27, 2.5, and 2.3 kb in length which were used as templates in 50 μ l *in vitro* transcription reaction mixtures with [α - 32 P]UTP as label. The RNAs were analyzed on 8 M urea 4% polyacrylamide gel. Fragments of of ϕ x174 DNA cleaved with *HaeIII* and end-labeled, were used as size markers on the same gel. The templates were: lane 1, 2 μ g of *Adh^D* plasmid DNA with 1 μ g/ml of α -amanitin to inhibit RNA polymerase II in the reaction mixture; lane 2, 2 μ g of *Adh^{fn4}* plasmid DNA, no α -amanitin; lane 3, 2 μ g of *Adh^{fn6}* plasmid DNA, no α -amanitin, and lane 4, 2 μ g of *Adh^D* plasmid, no α -amanitin.

Adh promoter regions of *Adh^{fn4}* and *Adh^{fn6}* from adult flies are indistinguishable from that region of *Adh^D*.

DISCUSSION

In this report we have presented the evidence that both *Adh^{fn4}* and *Adh^{fn6}* have lesions in one IVS. In addition, they accumulate larger-than-normal-size RNA that hybridizes with an *Adh* gene probe. Yet on these mutant genes, *in vitro* ADH RNA synthesis is initiated normally. The above observations taken together suggest that *Adh^{fn4}* and *Adh^{fn6}* flies are defective in mRNA processing. The 1900-base RNA detected in these mutants seems to be a precursor transcript containing the 654-bp IVS. Its appearance in both mutants and its absence in wild-type flies indicates an above-normal accumulation. We presume that the lack of mature-size ADH mRNA in *Adh^{fn4}* and *Adh^{fn6}* flies is caused by a failure in the processing of intervening sequences from the precursor RNA.

Based on the DNA sequence analysis, we conclude that the defect in RNA processing is the consequence of the deletions, which remove the 3' splice site sequences from the 65-bp IVS in *Adh^{fn4}* and the 5' splice site sequences from the same IVS in *Adh^{fn6}*. We have not detected any other changes, either in the coding region or in the other IVS. The accumulation of a precursor-

size RNA in both mutants, which we presume still retains the 654-bp IVS, however, leads us to speculate that splicing of ADH RNA may proceed in a specific order such that mutations in the 65-bp IVS appear to affect the removal of the 654-bp IVS.

Although transcription of the cloned Adh^{fn4} and Adh^{fn6} is normal in vitro, the overall in vivo levels of RNA detected with an Adh gene probe are only 5-10% of the wild-type level. One possible explanation for this finding is that the mutant precursor RNA is less stable and thus turns over more rapidly than mature ADH mRNA. It is also possible that transcriptional efficiency is somehow affected in vivo by the deletions in the 65-bp IVS. Our existing data do not distinguish between these two possibilities.

Our studies on these two independently derived Adh mutants point to the importance of the nucleotide sequences at the splice junctions for normal mRNA processing and stabilization in Drosophila. Mutations involving splice junction sequences that affect the expression of other eucaryotic genes by altering RNA processing and RNA stability have recently been identified in other systems, e.g., the yeast actin gene (31), the SV40 late gene (32), the adenovirus early region genes (33,34), the mouse immunoglobulin λ I gene (35), and several human α - and β -globin genes in α - and β -thalassemias (36-39). Similar molecular lesions result in similar molecular phenotypes in these diverse gene systems, suggesting a possible common pathway of RNA processing and subsequent gene expression.

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