TWO ALTERNATE TRANSCRIPTS CODING FOR ALCOHOL DEHYDROGENASE ACCUMULATE WITH DIFFERENT DEVELOPMENTAL SPECIFICITIES IN DIFFERENT SPECIES OF *PICTURE-WINGED* DROSOPHILA

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Manuscript received January 6, 1986
Revised copy accepted June 14, 1986

ABSTRACT

Two alternate transcripts of the single copy *Alcohol dehydrogenase* (*Adh*) gene accumulate with developmental specificity in all of 12 species of Hawaiian *picture-winged* Drosophila which have been examined. Relative to the paradigm species *D. affinidisjuncta*, the *Adh* transcript normally restricted to larvae is found to accumulate in both larval and adult tissues in *D. formella*. The other *Adh* transcript, which normally accumulates only in adults, accumulates in third-instar *D. prostopalpis* larvae as well. In species hybrids, the *D. formella* phenotype shows additive inheritance. These observations document the existence of a novel type of genetic variability. Furthermore, such variants suggest specific properties for the biological systems that regulate ADH expression in Drosophila, and they should facilitate further experimental investigations.

DROSOPHILA alcohol dehydrogenase (ADH) is encoded by a single gene (*Adh*) which produces two different transcripts. In the distantly related species *D. melanogaster* (BENYAJATI *et al.* 1983; SAVAKIS, ASHBURNER and WILLIS 1985) and *D. affinidisjuncta* (ROWAN, BRENNAN and DICKINSON 1986; R. G. ROWAN and W. J. DICKINSON, unpublished results), and two *Adh* RNAs are almost completely segregated by developmental stage. *Adh* transcripts in larvae are apparently transcribed from a proximal *Adh* promoter, and the products of a putative distal *Adh* promoter accumulate in adults. These "proximal" and "distal" (respectively) *Adh* transcripts are easily discriminated by their different 5′ end untranslated leader sequences (see Figure 1). Both types of mRNA predict the same ADH amino acid sequence.

Transcription from multiple promoters has been recognized in other higher organisms. These observations clearly indicate that the sequence, as well as the abundance of an mRNA, may be subject to regulation, and also suggest (*e.g.*, SCHIBLER *et al.* 1983) or demonstrate (*e.g.*, ROBERT *et al.* 1984) mechanisms for the control of gene activity. These descriptive data, in turn, direct attention to those biochemical processes that determine where or when each mRNA

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form accumulates. Genetic variants that specify atypical distributions of the same mRNA transcripts would help to resolve these phenomena, as genetic analyses have revealed the biological correlates of multiple promoters in prokaryotic genes (*e.g.*, REICHART and KAISER 1971; RUSSO *et al.* 1977). In higher organisms, technical considerations argue against the recovery of such regulatory mutants from conventional mutagenesis experiments; the interesting phenotypes are not casually observed. A simpler approach is to screen for extant, naturally occurring variation. Both *cis*-acting (*e.g.*, PAIGEN 1961, 1979; SCHWARTZ 1971; DICKINSON 1978; DICKINSON and CARSON 1979) and *trans*-acting (*e.g.*, ABRAHAM and DOANE 1978; LUSIS and PAIGEN 1975; DICKINSON 1980c) modifiers of the tissue distribution of several enzyme activities have been discovered in this way.

Previous studies of ADH expression in the Hawaiian *picture-winged* Drosophila species group have documented considerable interspecific variability at the enzyme activity level (DICKINSON 1980a) and have described the sequence (R. G. ROWAN and W. J. DICKINSON, unpublished results) and transcript organization (ROWAN, BRENNAN and DICKINSON 1986) of the *Adh* gene from a single species, *D. affinidisjuncta*. Using cloned *D. affinidisjuncta* genomic DNA, the present study compares the developmental accumulation of proximal and distal *Adh* transcripts in four *picture-winged* species, and also in species hybrids. Each species' pattern differs from the other three in some manner. These data define a novel class of regulatory variants; they suggest properties of the mechanics of ADH regulation in Drosophila, and they demonstrate that natural systems can contribute to an understanding of transcriptional complexity.

MATERIALS AND METHODS

Drosophila stocks and RNA preparation: The origins of the *D. affinidisjuncta* (S36G1), *D. formella* (M87G1), *D. prostopalpis* (S15B33), *D. heteroneura* (T94B18), *D. differens* (U43V1), *D. silvestris* (T94B7), *D. punalua* (U72Y15), *D. silvarentis* (U87G6), *D. crucigera* (U72Y8) and *D. grimshawi* (G1) stocks that were used in this study have been described (DICKINSON 1980a). *D. hirtipalpus* (J10B5) was obtained from the Mid-America Drosophila Stock Center, Bowling Green, Ohio, under the name *D. orthofascia*. *D. hawaiiensis* (J14B8) is also from the Bowling Green collection. Culture conditions were according to WHEELER and CLAYTON (1965) at 18–20°. Tissue samples obtained by dissecting living larvae (actively feeding third instar) or adults (aged 4–6 wk posteclosion) were frozen immediately in liquid nitrogen. RNA was extracted from the combined tissues of 40 individuals by phenol extraction as previously described for individual larvae (ROWAN, BRENNAN and DICKINSON 1986), except that the volumes were increased fourfold and yeast tRNA (20 μg; Boehringer Mannheim Biochemicals) was added as carrier. RNA preparation from groups of whole larvae or adults was according to BRENNAN *et al.* (1984).

DNA techniques: *D. affinidisjuncta Adh* clones pUC8-9 and pBR322SH1 are diagrammed in Figure 1. The Drosophila DNA in *Adh* clone pBR328E1 (BRENNAN *et al.* 1984) is indicated in Figure 7. Partial restriction enzyme maps of the *D. formella*, *D. hirtipalpus* and *D. prostopalpis* genomic *Adh* regions were obtained using radiolabeled (RIGBY *et al.* 1977) pUC8-9 and pBR328E1 genomic insert DNA to probe agarose gel blots of digested total genomic DNAs (SOUTHERN 1975) as previously described (BRENNAN, ROWAN and DICKINSON, 1984).

Primer extension and S1 nuclease analysis: A 121-nucleotide (nt) *Hin*cII-*Taq*I DNA fragment, ^{32}P-labeled at the 5′ *Taq*I end, was prepared from clone pUC8-9, annealed

to *Adh* RNA and extended with reverse transcriptase as described (ROWAN, BRENNAN and DICKINSON 1986). This analysis discriminates between the proximal and distal *Adh* transcripts from *D. affinidisjuncta*, and the primer is diagrammed in Figure 1. From 15 to 50 μg (see figure legends) of whole organism (larval or adult) RNA was used for a single determination. Tissue-specific RNA samples were not quantitated for nucleic acid content. Rather, these assays used an amount of RNA equivalent to the combined tissues from the following number of organisms: Larval fat body, 1; larval carcass (includes the hypoderm; all other tissues removed), 5; larval midgut, 7.5; larval Malpighian tubules, 15; adult head, 2.5; adult empty abdomen (abdominal carcass, including fat body), 2.5; adult midgut, 10; adult hindgut, 10; adult Malpighian tubules, 15.

Adh transcripts mapping to the distal *Adh* promoter (distal transcripts in Figure 1) were also identified by primer extension from a *Sal*I recognition site within the 5′ leader as follows. *Adh* genomic insert DNA of pBR322SH1 (*Sal*I-*Hin*dIII fragment) was end labeled at the *Sal*I site using α-(^{32}P)dCTP (New England Nuclear) and DNA polymerase I large fragment (Boehringer Mannheim Biochemicals) and was purified by gel electrophoresis as described (ROWAN, BRENNAN and DICKINSON 1986). Approximately 5 ng of this fragment (specific activity approximately 10^6 cpm/pmol DNA 3′ end) was annealed with total Drosophila RNA and was extended with reverse transcriptase as described for the *Hin*cII-*Taq*I primer (above). The cDNA sequences were removed from the primer sequence with *Sal*I as described (ROWAN, BRENNAN and DICKINSON 1986) and were analyzed by electrophoresis.

The probe for S1 nuclease mapping (BERK and SHARP 1978) was an *Eco*RI-*Hin*cII fragment of pUC8-9 (Figure 1) that had been labeled throughout the RNA-complementary DNA strand using α-(^{32}P)dCTP (New England Nuclear) and T4 DNA polymerase (New England Biolabs) by replacement synthesis (O'FARRELL 1981; MANIATIS, FRITSCH and SAMBROOK 1982) as previously described (ROWAN, BRENNAN and DICKINSON 1986). Labeled probe DNA was annealed to *Adh* RNA at 47° for 12 hr and was digested with S1 nuclease (New England Nuclear) at 19° for 30 min according to FAVALORO, TREISSMAN and KAMEN (1980), as previously described (ROWAN, BRENNAN and DICKINSON 1986). Extension products and S1 nuclease-resistant probe DNAs were analyzed by electrophoresis through denaturing polyacrylamide gels containing 8.3 M urea (MAXAM and GILBERT 1980); these gels were fixed in 10% acetic acid and dried before fluorography at −80°. pBR322 DNA cleaved with *Hpa*II and labeled with α-(^{32}P)dCTP by DNA polymerase large fragment served as DNA size standards (SUTCLIFFE 1978).

ADH activity determinations: Crude homogenates were prepared by grinding living larvae or adults in 40 μl of distilled water, then they were analyzed for ADH activity by electrophoresis in (NAD^+)-containing agar noble gels (URSPRUNG and LEONE 1965) as described by DICKINSON and CARSON (1979).

RESULTS

The transcript map of *D. affinidisjuncta Adh*, as determined by S1 nuclease and primer extension mapping (ROWAN, BRENNAN and DICKINSON 1986) and by DNA and RNA sequencing (R. G. ROWAN and W. J. DICKINSON, unpublished results), is presented in Figure 1. Because the *Adh* gene from the distantly related species *D. melanogaster* is organized in exactly the same manner (BENYAJATI *et al.* 1983), we anticipated a conserved *Adh* gene structure among the closely related *picture-winged* flies. A preliminary examination of total RNA from larvae and adults of 12 species showed no obvious differences in *Adh* RNA size (determined by denaturing electrophoresis and blot hybridization; data not presented) and indicated the presence of two *Adh* RNA 5′ ends [determined by primer extension analysis (Figures 2, 3 and 4), see below]. This

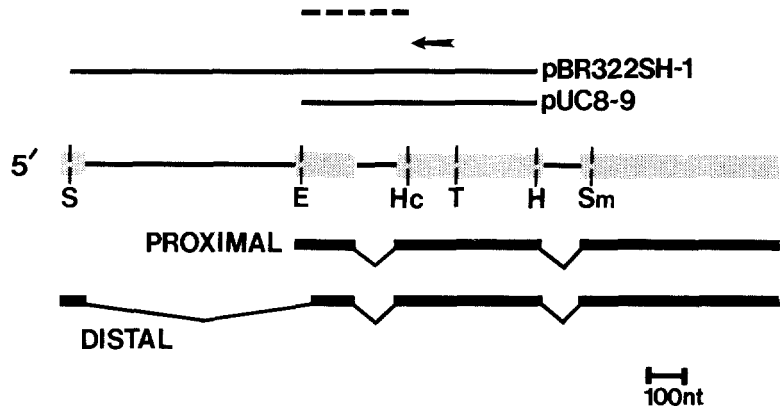

FIGURE 1.—Transcription map of the *D. affinidisjuncta Adh* gene (ROWAN, BRENNAN and DICKINSON 1986). Shaded bars represent exons placed on a partial restriction map of the gene. The 5′ end is to the left. Restriction enzyme recognition sites are abbreviated as follows: S = *Sal*I, E = *Eco*RI, Hc = *Hinc*II, T = *Taq*I, H = *Hin*dIII, Sm = *Sma*I. Two *Adh* RNAs, "proximal" and "distal" are diagrammed by heavy lines (exons) and joined diagonal lines (intervening sequences) in relative alignment to the restriction enzyme map. *D. affinidisjuncta* genomic DNA subclones pBR322SH-1 and pUC8-9 are also aligned with the restriction map. An arrow shows the location of a 121-nt *Hinc*II-*Taq*I DNA fragment used to prime cDNA syntheses (primer extension analyses). A scale bar (100 nt) is given.

report considers only four of these species: *D. affinidisjuncta*, *D. hirtipalpus*, *D. formella*, and *D. prostopalpis*, in detail.

From these four species, *Adh* transcripts in larval fat body, carcass, midgut and Malpighian tubules and in adult empty abdomen (primarily fat body lining the abdomen), head, midgut, hindgut and Malpighian tubules were examined by primer extension. ADH enzyme activity is not reliably detected in any other tissues from the four species examined (DICKINSON 1980b; R. G. ROWAN, unpublished observations). Larval and adult data are presented in Figures 3 and 4, respectively. *Adh* RNA from eight other species has been characterized from whole organisms (larval *vs.* adult comparisons; Figure 2) only. In general (see Figures 2, 3 and 4), larval RNAs direct the synthesis of a 290-nt *Adh* cDNA, and the predominant adult *Adh* cDNA is 320 nt in size. Extension products of these sizes identify the proximal and distal (respectively) transcripts in *D. affinidisjuncta* (ROWAN, BRENNAN and DICKINSON 1986).

Conservation of the *D. affinidisjuncta Adh* transcription map was further established by examining larval and adult *Adh* RNA from *D. formella*, *D. hirtipalpus* and *D. prostopalpis* more closely. S1 nuclease analysis of the 5′ ends of these molecules also indicates the presence of two *Adh* transcripts (Figure 5). As predicted from the *D. affinidisjuncta Adh* transcript map (Figure 1), the protected probe DNA fragments are 132 and 122 nt in size. These DNAs indicate the proximal and the distal *D. affinidisjuncta Adh* transcripts, respectively (ROWAN, BRENNAN and DICKINSON 1986). Note from Figure 5 that *D. prostopalpis* third-instar larvae and *D. formella* adults are exceptional in containing both *Adh* transcripts in similar abundance. This situation is also apparent from primer extension (see below). An analysis, by primer extension, of

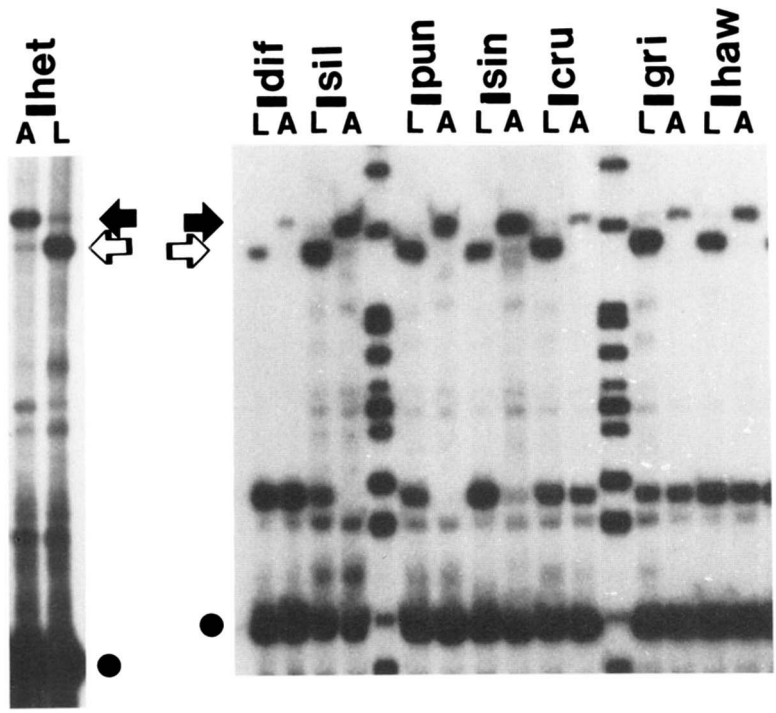

FIGURE 2.—Primer extension analysis of *Adh* transcripts from larvae (L) and from adults (A) of *D. heteroneura* (het), *D. differens* (dif), *D. silvestris* (sil), *D. punalua* (pun), *D. silvarentis* (sin), *D. crucigera* (cru), *D. grimshawi* (gri) and *D. hawaiiensis* (haw). ^{32}P end-labeled primer (5×10^4 cpm) was annealed with 50 μg of total RNA from each sample and was extended with reverse transcriptase. An amount equivalent to between 15 and 50 μg of the input RNA is displayed. Closed circles indicate the position of the 121-nt primer DNA fragment. Open and closed arrows indicate the extension products directed by proximal RNA (size = 290 nt) and by distal RNA (size = 320 nt), respectively.

the total RNA from 18 individual adult *D. formella* and from eight individual *D. prostopalpis* larvae did not show individual variation (data not shown). Thus, proximal and distal *Adh* transcripts are concomitantly expressed in these two instances.

A second primer extension analysis compares the 5′-terminal exon of the distal *Adh* transcript from these four species. In *D. affinidisjuncta* this exon contains a *Sal*I site (see Figure 1) that is convenient for transcript mapping (ROWAN, BRENNAN and DICKINSON 1986; R. G. ROWAN and W. J. DICKINSON, unpublished results). Genomic Southern maps (see Figure 7, below) imply that this *Sal*I recognition sequence is conserved in the related species. Accordingly, RNA samples were tested for the presence of distal *Adh* transcripts by examining cDNA syntheses primed from the exon 1 *Sal*I site. In each instance, including the distal *Adh* RNA in *D. prostopalpis* larvae, extensions of 22, 21, 20 and 17 nt from the *Sal*I site are predominant (Figure 6). These data agree with previous mapping of the *D. affinidisjuncta* distal *Adh* transcript (ROWAN, BRENNAN and DICKINSON 1986; R. G. ROWAN and W. J. DICKINSON, unpub-

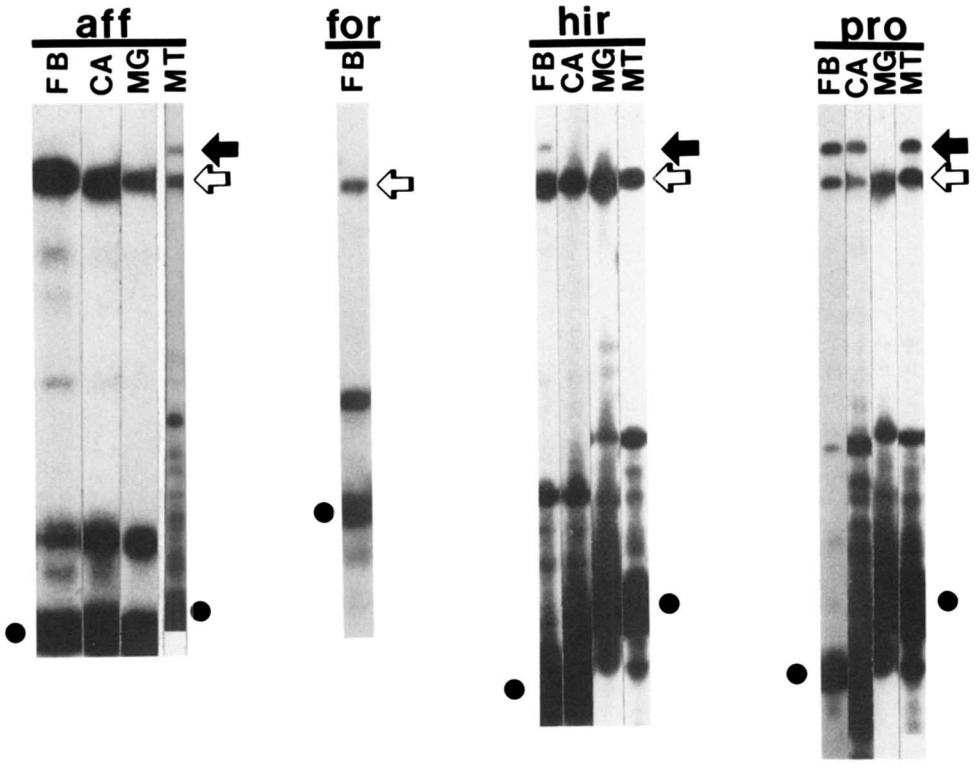

FIGURE 3.—Primer extension analyses of larval fat body (FB), larval carcass (CA), larval midgut (MG) and larval Malpighian tubules (MT) *Adh* RNA from *D. affinidisjuncta* (aff), *D. formella* (for), *D. hirtipalpus* (hir) and *D. prostopalpis* (pro). Closed circles indicate the position of the 121-nt primer DNA fragment. Open and closed arrows indicate the extension products directed by proximal RNA (size = 290 nt) and by distal RNA (size = 320 nt), respectively. Tissues that are not represented lack detectable ADH RNA (data not shown).

lished results). Larval RNA from *D. affinidisjuncta*, *D. formella* and *D. hirtipalpus* had no detectable template activity in this assay (data not shown). These data strongly imply that all four species express homologous distal *Adh* transcripts.

To summarize, the *Adh* mRNA from 12 species of *picture-winged* Drosophila exists in two forms identified by different 5′ ends. Homology of four proximal and four distal *Adh* transcripts (those from *D. affinidisjuncta*, *D. formella*, *D. hirtipalpus* and *D. prostopalpis*) is indicated by transcript mapping. However, one of the two *Adh* RNAs in *D. formella* and *D. prostopalpis* does not accumulate with typical developmental specificity.

The tissue distribution of *Adh* transcript types was investigated by primer extension. In *D. prostopalpis* third-instar larvae (Figure 3) the distal *Adh* RNA predominates in the carcass, but only the proximal *Adh* RNA is detected in the midgut. *D. prostopalpis* larval fat body and Malpighian tubules yield approximately equal amounts of both *Adh* transcripts. *D. formella* adult (Figure

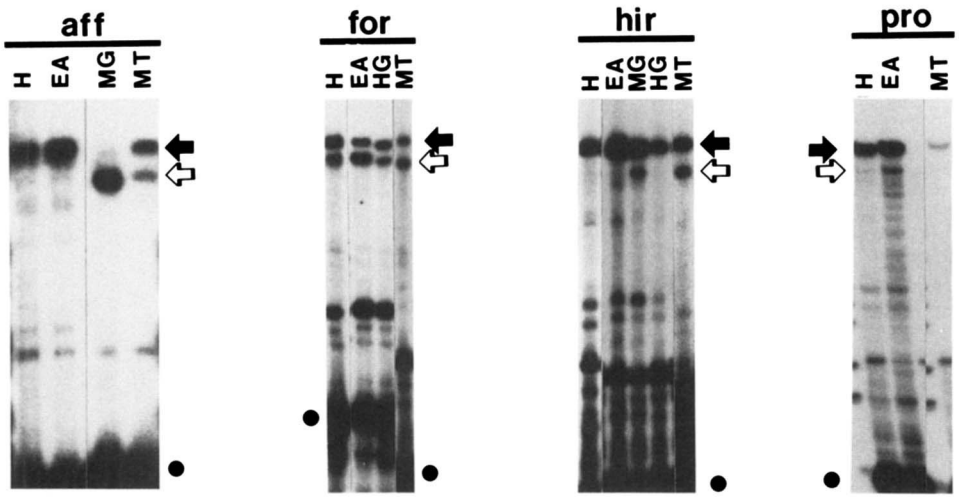

FIGURE 4.—Primer extension analyses of adult head (H), adult empty abdomen (EA), adult midgut (MG), adult hindgut (HG) and adult Malpighian tubule (MT) *Adh* RNA from the same species presented in Figure 3 (abbreviations unchanged). Tissues that are not represented lack detectable *Adh* RNA. Closed circles indicate the position of the 121-nt primer DNA fragment. Open and closed arrows indicate the extension products directed by proximal RNA (size = 290 nt) and by distal RNA (size = 320 nt), respectively.

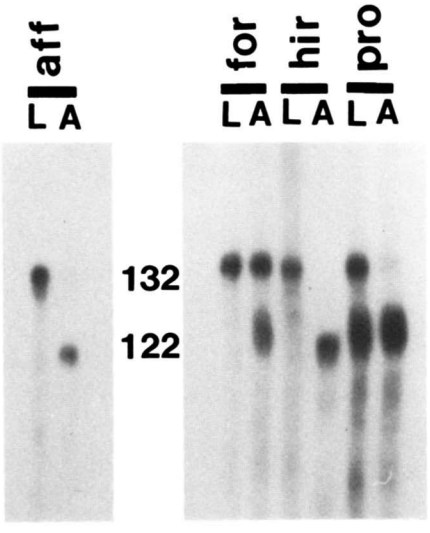

FIGURE 5.—S1 nuclease analysis of *Adh* RNA from larvae (L) and from adults (A) of *D. affinidisjuncta* (aff), *D. formella* (for), *D. hirtipalpus* (hir) and *D. prostopalpis* (pro). A ^{32}P-labeled DNA fragment (*Eco*RI-*Hin*cII; see Figure 1) from the *D. affinidisjuncta* clone pUC8-9 (5×10^4 cpm) was annealed to *Adh* RNA (supplied as 40 μg of total RNA) and digested with S1 nuclease. An amount equivalent to between one-tenth and one-half of the total sample is displayed. The sizes of the S1 nuclease-resistant probe DNA (132 and 122) are given in nucleotides.

4) abdominal fat body and Malpighian tubules appear to contain a slight excess of the proximal *Adh* RNA, relative to the adult head and hindgut samples.

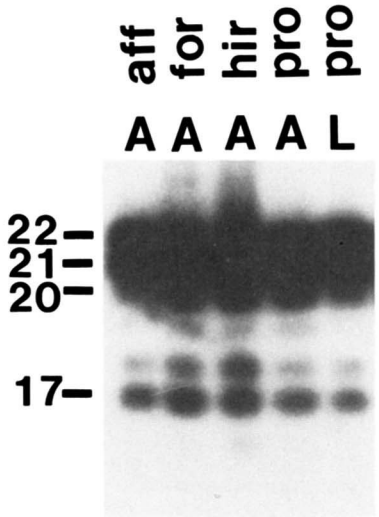

FIGURE 6.—Mapping the 5′ end of distal *Adh* RNAs by primer extension. The 1159-nt genomic DNA insert from *D. affinidisjuncta* clone pBR322SH1 (*Sal*I-*Hin*dIII; see Figure 1) was ^{32}P end-labeled and was annealed to ADH RNA from adult (A) *D. affinidisjuncta* (aff), *D. formella* (for), *D. hirtipalpus* (hir) and *D. prostopalpis* (pro) and from *D. prostopalpis* larvae (L). In each instance, 50 μg of total RNA was analyzed (see text for further methods). Sizes of the extension products are given in nucleotides.

These variant phenotypes are stage-specific: The other stage of both species, *i.e.*, *D. prostopalpis* adults (Figures 4 and 5) and *D. formella* larvae (Figures 3 and 5), is conventional with respect to the type of *Adh* transcript that accumulates. Both *Adh* transcript types are generally observed in adult Malpighian tubules (Figure 4 and our unpublished data from additional species). The apparent absence of proximal *Adh* RNA in this tissue from *D. prostopalpis* (Figure 4) is not conclusive because distal *Adh* RNA is present at a level only slightly above the limit of detection.

The following comparison detects another possible regulatory variant. Among the 12 species examined in this study, *D. affinidisjuncta* and *D. hirtipalpus* are exceptional in having detectable ADH activity in the adult midgut (DICKINSON 1980a; R. G. ROWAN, unpublished observations). These phenotypes clearly differ, however, in that *D. affinidisjuncta* adult midgut *Adh* RNA is proximal, whereas *D. hirtipalpus* adult midgut *Adh* RNA is a mixture (predominantly distal) of both transcripts (Figure 4). It is not yet clear whether both species actually express *Adh* RNA in the same region of this complex tissue, or if individual *D. hirtipalpus* all show the same phenotype. *In situ* hybridization methods are being developed in order to address these questions. For the present, the existence of only the proximal *Adh* transcript in an adult tissue is noteworthy.

The developmental regulation of *Adh* transcript type in the Malpighian tubules is also species-variable. *D. affinidisjuncta* and *D. hirtipalpus* adults (Figure 4) contain both *Adh* transcripts in this tissue. Larval (Figure 3) Malpighian tubules, in these instances, contain either just the proximal transcript (*D. hir-*

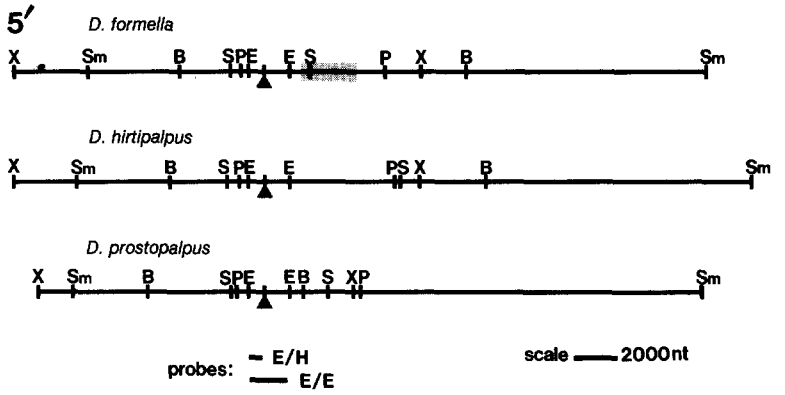

FIGURE 7.—Partial restriction enzyme map of the *Adh* genomic DNA region of *D. formella*, *D. hirtipalpus* and *D. prostopalpis*. To the left is 5′ (defined by the chemical polarity of ADH RNA). Genomic RNAs were digested singly with *Xho*I (X), *Sma*I (Sm and solid triangle), *Bgl*II (B), *Sal*I (S), *Pvu*II (P) and *Eco*RI (E), and also doubly with these enzymes plus *Sma*I. After electrophoretic separation and transfer to nitrocellulose filters, *Adh* gene-containing DNA fragments were visualized by sequential hybridization with the E/H probe from pUC8-9 and with the E/E probe from pBR328E1 (indicated below the maps; see also Figure 1). By this method, for any restriction enzyme, only the recognition site nearest to the *Adh* gene is detected. These sites were mapped relative to the central *Sma*I site (solid triangle) by the double digestions. An apparent deletion of DNA 3′ to the *Adh* transcribed region was observed in approximately one-half of the genomic DNA from *D. formella*. The approximate position of these deleted sequences is given by the shaded bar in the *D. formella* map. A scale bar (2000 nt) is given.

tipalpus) or both (*D. affinidisjuncta*) *Adh* transcripts. Again, some or all of this variability could be the result of anatomical or individual differences in ADH expression.

The possibility that the two *Adh* RNAs detected in *D. formella*, *D. hirtipalpus* and *D. prostopalpis* are transcribed from separate *Adh* loci was assessed by genomic restriction enzyme mapping (SOUTHERN 1975). Similar but more complete data from *D. affinidisjuncta* have been previously discussed (BRENNAN *et al.* 1984; ROWAN, BRENNAN and DICKINSON 1986). As summarized in Figure 7, approximately 30,000 base pairs of genomic DNA were examined. Two *Adh* restriction maps were detected in our laboratory stock of *D. formella*, but an analysis of 24 individual diploid genomes showed the three classes predicted by two segregating alleles (data not shown). Although segregation has not been demonstrated, the obvious relationship between the two *D. formella Adh* genes (approximately 2300 base pairs of DNA deleted from the *Adh* 3′ nontranscribed region; see Figure 7) is also not inconsistent with the presence of only a single *Adh* locus per haploid *D. formella* genome. Only a single *Adh* restriction map was detected in the genomes of *D. hirtipalpus* and *D. prostopalpis*. These data support the conclusion that only a single ADH coding locus exists in each of these species. A related observation is the failure to detect multiple, stage- or tissue-specific ADH isozyme forms in all of the species of *picture-winged* Drosophila which have been surveyed by gel electrophoresis (DICKINSON 1980a; W. J. DICKINSON and R. G. ROWAN, unpublished observations).

A genetic analysis of the *Adh* transcript pattern observed in *D. formella* was

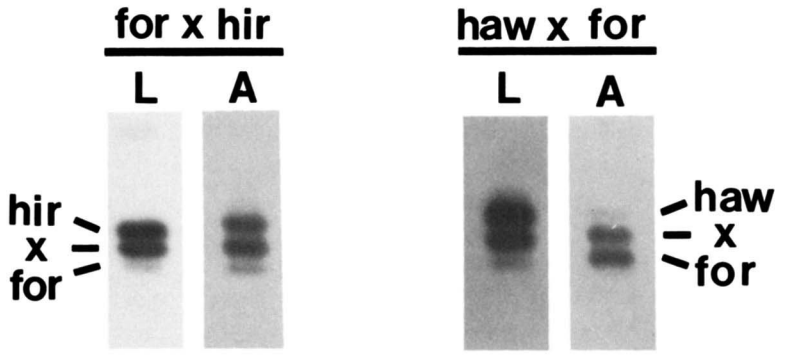

FIGURE 8.—Electrophoretic analysis of ADH activity in Drosophila species hybrids. Whole larval (L) and whole adult (A) isozyme patterns are given for F_1 hybrids between *D. formella* and *D. hirtipalpus* (for × hir) and *D. hawaiiensis* and *D. formella* (haw × for). Homodimeric ADH molecules (hir, for and haw) and interspecific heterodimers (x) are indicated. Migration is from the top. Because the data are taken from separate assays, these four determinations of ADH activity are not directly comparable to one another.

attempted by crossing *D. formella* females with *D. hirtipalpus* males, and *D. hawaiiensis* females with *D. formella* males. Of the three parents, only *D. formella* contains appreciable amounts of proximal *Adh* RNA as adults; larvae of each species contain only proximal *Adh* transcripts (see Figures 2, 3 and 4). Because the ADH enzyme from *D. formella* is electrophoretically distinct from the allele carried by *D. hirtipalpus* and *D. hawaiiensis*, the contribution of each parental allele to ADH activity expressions in these species hybrids may be determined.

Several (5–10) hybrid larvae and adults of each type were individually tested for ADH activity, and typical data are presented in Figure 8. Hybrids exhibit three distinct bands of ADH activity corresponding to two parental ADH homodimers and a single interallelic ADH heterodimer. These data document the expression of both *Adh* alleles in these hybrids. Also note that, in *D. hawaiiensis* × *D. formella* hybrids, the two alleles are expressed at different relative levels in larvae (*D. hawaiiensis* enzyme predominates) and in adults (*D. formella* enzyme predominates). These same alleles are differentially expressed in this same manner in individuals of the parental stocks (data not shown). In contrast, the ADH isozyme pattern in *D. formella* × *D. hirtipalpus* hybrids is not stage-specific (Figure 8). These data are also consistent with ADH activity measurements on the parental species (data not shown). Taken together, these and related observations (DICKINSON, 1980a,b; W. J. DICKINSON and R. G. ROWAN, unpublished observations) imply that the expression of Drosophila ADH activity can vary independently in larvae and in adults.

Proximal and distal *Adh* transcripts were detected in species hybrids by primer extension (Figure 9). Only proximal transcripts are apparent in hybrid larvae, and hybrid adults accumulate both transcript types. Predominance of distal *Adh* RNA in these files indicates that the parental phenotypes are expressed additively. (Recall that *D. hawaiiensis* and *D. hirtipalpus* adults express essentially only distal transcripts and that approximately one-half of the *Adh*

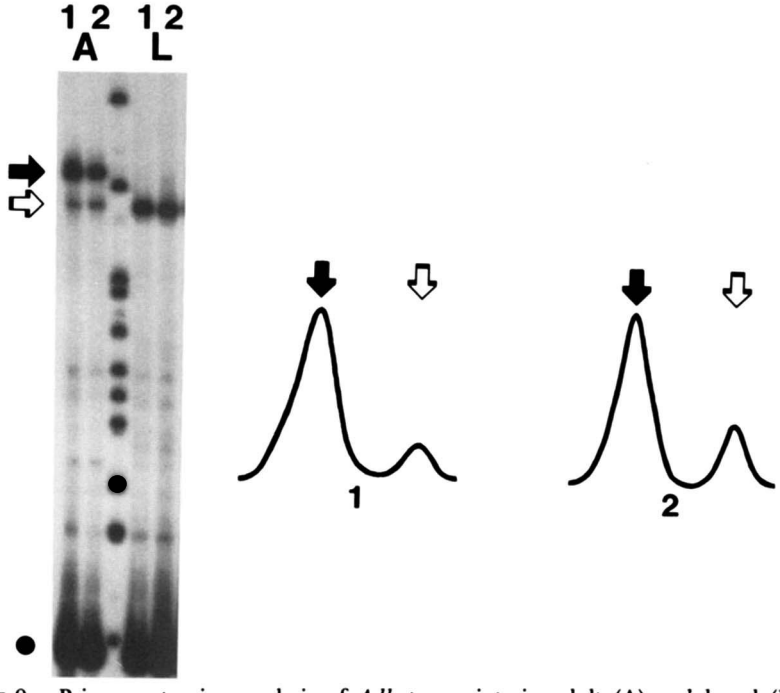

FIGURE 9.—Primer extension analysis of *Adh* transcripts in adult (A) and larval (L) species hybrids. In each instance, 25 μg of total RNA was analyzed. Symbols (open and solid arrows, circles) are as in Figure 2. Hybrid 1 is *D. formella* × *D. hirtipalpus* F_1 progeny, and hybrid 2 is *D. hawaiiensis* × *D. formella* F_1 progeny. Densitometric scans are from the hybrid adult patterns and are similarly labeled. The two scans were normalized to show equivalent signals for the cDNAs of distal *Adh* transcripts (solid arrows).

RNA in adult *D. formella* is proximal.) Further inspection of the hybrid adult patterns shows that the relative abundance of the proximal *Adh* transcript correlates with the relative expression of the *D. formella Adh* activity allele (compare the densitometric scans presented in Figure 9 and the isozyme patterns presented in Figure 8). This observation indirectly (the two parental mRNAs in the hybrids have not been distinguished) suggests *cis*-dominant regulation of *Adh* transcript accumulation, *i.e.*, that proximal transcripts are produced only from the *D. formella* allele in the adult hybrids. Of course, these data are not actually diagnostic of any regulatory model, but they do indicate that the variant *D. formella* pattern is neither completely recessive nor completely dominant to the conventional phenotypes shown by *D. hirtipalpus* and *D. hawaiiensis*.

DISCUSSION

Proximal and distal *Adh* transcripts from *D. melanogaster* (BENYAJATI *et al.* 1983) and from *D. affinidisjuncta* (ROWAN, BRENNAN and DICKINSON 1986; R. G. ROWAN and W. J. DICKINSON, unpublished results) have been mapped using cloned *Adh* genomic DNA from these species. Cloned *D. affinidisjuncta Adh* genomic DNA has been used in the present study to investigate the 5′ end

organization of *Adh* RNAs from 11 other species of Hawaiian *picture-winged* Drosophila. Definitive transcript maps cannot be constructed without conspecific genomic DNA clones, but the reported data indicate that the *D. affinidisjuncta Adh* transcript map also describes the expression of the *Adh* gene in at least three other species (*D. formella*, *D. hirtipalpus* and *D. prostopalpis*). These observations include S1 nuclease (Figure 5) and primer extension (Figures 3, 4 and 6) transcript mapping, as well as the physical mapping of genomic *Adh* region DNA with restriction enzymes by Southern blotting (Figure 7). Other species have been examined by primer extension mapping only (Figure 2) and do not appear exceptional by this criterion.

Summarily stated, the salient feature of this map (see Figure 1) is that two promoters of a single *Adh* locus produce transcripts that accumulate with different developmental specificities. Promoter elements ("TATA box" sequences) are apparent from the *Adh* genomic DNA sequence of *D. affinidisjuncta* (R. G. ROWAN and W. J. DICKINSON, unpublished results); homologous elements in other *picture-winged* species may be inferred from the presence of homologous *Adh* RNAs.

Transcript mapping by primer extension shows that distinct proximal and distal *Adh* RNAs accumulate in all 12 species of *picture-winged* Drosophila which were examined. Transcript stage specificity is conserved in ten of 12 species, in which the proximal and the distal *Adh* RNAs are largely or entirely confined to the larvae and to the adults, respectively, which were examined. Of particular interest are two variant phenotypes: *D. prostopalpis* feeding third-instar larvae and adult *D. formella* each accumulate approximately equal amounts of both transcript types. These natural variants are potentially informative in two ways. First, comparative phenotypic analyses might reveal properties of the underlying regulatory system(s), and they could contribute to an understanding of *Adh* gene evolution. Also, natural variants provide material for the experimental analysis of gene regulation. These prospects are discussed in turn below.

The variant *D. formella* and *D. prostopalpis* phenotypes are not obviously complex. In each case, both *Adh* transcripts are concomitantly expressed at a developmental stage characterized by the presence of predominantly a single *Adh* RNA in other species. With the single exception of the *D. prostopalpis* larval midgut, where only proximal *Adh* RNA was detected, neither variant phenotype is strikingly tissue-specific. Thus, parsimony favors the conclusion that altered functions in these species coordinately change the developmental specificity of *Adh* transcript accumulation throughout the organism. One alternative model would postulate that *D. formella* and *D. prostopalpis* simultaneously express multiple independent and tissue-specific variant phenotypes. Other species might exhibit variant phenotypes at other developmental stages; the present study screened only feeding third-instar larvae and mature (aged 4–6 wk) adult flies for *Adh* transcripts.

In this context, it is of interest to examine the relative developmental profiles of proximal and distal *Adh* transcripts more closely. The switch from proximal to distal *Adh* RNA expression is abrupt in *D. affinidisjuncta* (ROWAN, BRENNAN

and DICKINSON 1986) and in *D. melanogaster* (SAVAKIS, ASHBURNER and WILLIS 1985): Proximal *Adh* transcripts are abundant in feeding larvae of all three instars, but *Adh* RNA becomes relatively nonabundant when distal *Adh* transcripts appear in late third-instar larvae (operationally defined as larvae which have left the food and, in the case of *D. affinidisjuncta*, have begun a prepupational wandering). Little *Adh* RNA is then detectable until eclosion, at which time distal *Adh* transcripts are abundantly expressed. As determined by S1 nuclease mapping and blot hybridization (data not shown), *Adh* transcript switching in *D. formella* occurs similarly: Only proximal *Adh* RNA is detected in feeding larvae of each instar, and approximately equal amounts of each *Adh* transcript are present in prepupational larvae and at all subsequent stages. As in *D. affinidisjuncta*, the abundance of *Adh* RNA in *D. formella* is very low in prepupational larvae, pupae and pharate adults, relative to feeding larvae and adults. One simple interpretation of these data maintains that the stage-specific regulation of distal *Adh* transcripts, and of *Adh* RNA accumulation *per se*, is similar in *D. affinidisjuncta* and *D. formella*, but the proximal transcript fails to undergo a developmentally regulated switch in *D. formella*.

Comparable descriptions of *Adh* transcript expression are lacking for *D. prostopalpis*, and our laboratory stock no longer exists. In addition to feeding third-instar larvae (Figure 2) and mature adults (Figure 3), total RNA from second instar larvae has also been examined (data not shown). Detectable *Adh* transcripts are predominantly (approximately 7/8) proximal at this stage. To a first approximation, therefore, a slightly precocious expression of the distal *Adh* transcript distinguishes *D. prostopalpis* from the other species (see above). However, because the relevant developmental stages have a behavioral definition (larvae which are feeding *vs.* larvae which have commenced a prepupational wandering) conclusions must be tentative. Blot hybridization analyses (data not shown) of the available *D. prostopalpis* RNA samples do show that the appearance of distal *Adh* transcripts in these third instar larvae is not associated with a decrease in the relative abundance of ADH RNA, as is the case in *D. melanogaster* (SAVAKIS, ASHBURNER and WILLIS 1985) and in *D. affinidisjuncta* (ROWAN, BRENNAN and DICKINSON 1986) and *D. formella* (above). These observations, like the observations on *D. formella* (above), favor the conclusion that the regulation of *Adh* transcript quality (proximal *vs.* distal) and the stage-specific regulation of *Adh* transcript quantity are separable phenomena.

The expression of ADH activity in *D. formella* adults and in *D. prostopalpis* third-instar larvae is conservative in the context of a broad species survey (DICKINSON 1980a). Thus, the simultaneous accumulation of both *Adh* transcript types does not incur obvious changes in the tissue-specificity of ADH expression. On the other hand, the atypical regulation of the proximal transcript in adult *D. formella* is correlated with an atypical regulation of ADH in larvae. As mentioned in the legend to Figure 3, *D. formella* third-instar larvae express detectable quantities of *Adh* RNA (and ADH activity; see DICKINSON 1980b) in only a single tissue, the larval fat body. Consistent with the general rule (Figures 2 and 3), this is proximal transcript. Of 26 species of *picture-winged* Drosophila examined as larvae, 23 contain detectable levels of ADH

activity in both the fat body and at least one other tissue (DICKINSON 1980a; W. J. DICKINSON and R. G. ROWAN, unpublished observations). Thus, proximal *Adh* transcripts in *D. formella* are expressed with disordinate restriction in larvae, but without restriction in adults. Perhaps these phenotypes are due to a single mutation. Unfortunately, the two other species which also exhibit a restricted larval ADH activity (DICKINSON 1980a) no longer exist in culture.

Like the naturally occurring differences in ADH activity expression that have been described by DICKINSON (1980a,b,c), variant *Adh* transcript expression patterns are of questionable adaptive significance. The relative strength of ADH expression in adult, as compared to larval, *D. formella* (measured relative to the expression of the *Adh* gene from *D. hawaiiensis* in Figure 8) might be attributable in part to the utilization of both *Adh* transcripts rather than only the distal one. Note, however, that the *D. hirtipalpus Adh* allele produces, in adults, still more ADH activity (Figure 8) and *Adh* RNA (data not shown) by accumulating only the distal *Adh* transcript (Figure 4).

It is of interest to relate the present observations to a description of ADH expression in the distantly related *repleta* species group (BATTERHAM *et al.* 1983, 1984). Two similar but nonallelic isozymes, ADH-1 and ADH-2, contribute to the expression of ADH activity in some of these species. Equating *D. mojavensis* (BATTERHAM *et al.* 1983) or *D. mulleri* (BATTERHAM *et al.* 1984) ADH-1 with proximal *picture-winged Adh* RNA, and ADH-2 with the distal transcript, *D. prostopalpis* and *D. mojavensis/D. mulleri* have very similar ADH expression patterns. This correspondence includes both the absence of ADH-2 in the *D. mojavensis* third-instar larval midgut (recall that only the proximal transcript is detected in the *D. prostopalpis* larval midgut; Figure 3) and the appearance of only ADH-1 in second instar *D. mojavensis* larvae. Also, the appearance (in third instar larvae) of both ADH-2 (BATTERHAM *et al.* 1983) and of the distal *D. prostopalpis Adh* transcript (above) occurs without the drop in overall *Adh* RNA accumulation that characterizes this event in other species (above). *D. mojavensis* adults express only ADH-2 except in the ovary, where only ADH-1 is detected. In the *picture-winged* flies, this situation is paralleled by *D. affinidisjuncta* adults which contain only the proximal *Adh* RNA in their midgut (Figure 3). These two phenotypes are related by being exceptional in two ways. In each case, an *Adh* transcript that is typically restricted to larval tissues is expressed in an adult tissue that does not typically (in the context of closely related species' phenotypes; see BATTERHAM *et al.* 1984; DICKINSON 1980b) express ADH at all. These correlations strengthen the inference that the two *repleta* ADH isozymes and the proximal and distal *Adh* transcripts in other Drosophila are homologously regulated (BENYAJATI *et al.* 1983; ROWAN, BRENNAN and DICKINSON 1986).

Distinct ADH isozymes in the *repleta* group species probably arose by gene duplication (BATTERHAM *et al.* 1983, 1984), and it appears that the extant *Adh-1* and *Adh-2* genes each retain only one promoter. Because the divergence between *D. melanogaster* and *D. affinidisjuncta* predates the divergence between *D. affinidisjuncta* and *D. mojavensis* (see THROCKMORTON 1975), the two-promoter motif is considered ancestral (see also BATTERHAM *et al.* 1984). The

observations discussed above suggest homologies between the functional *Adh-1* promoter and the proximal promoter, and between the functional *Adh-2* promoter and the distal promoter. Both of the *Adh* promoters from *D. affinidisjuncta* and *D. melanogaster* show putative interspecific homologies at the DNA sequence level (R. G. ROWAN and W. J. DICKINSON, unpublished results). Interestingly, a DNA sequence comparison of the *Adh* promoters from *D. mulleri* and *D. melanogaster* reveals relatively little interspecific similarity (FISCHER and MANIATIS 1985).

An independence of the larval and adult stages is implicit in much of the comparative data on ADH expression in the *picture-winged* Drosophila. Stage-specific representation of the products of the proximal and distal *Adh* promoters is a general rule (Figures 2, 3 and 4). Also, related species can have similar distributions of ADH activity as larvae, but divergent patterns as adults, and vice versa (DICKINSON 1980a,b; DICKINSON and CARSON 1979). Analogous data from species hybrids has been presented in Figure 8. Relative to *D. formella* ADH, *D. hawaiiensis* ADH activity is high in larvae but is low in adults. *D. hirtipalpus* ADH activity is high in both larvae and adults relative to *D. formella*. The descriptions of proximal and distal *Adh* transcript expression in *D. formella* and in *D. prostopalpis* also provide examples of stage-specific variation in the pattern of ADH regulation.

ADH activity is expressed with different tissue-specificity in each of the four species considered in this report (DICKINSON 1980a; R. G. ROWAN, unpublished observations). Tissue-specific quantitations of ADH activities and of *Adh* RNA are positively correlated in these and in other species of *picture-winged* Drosophila (R. G. ROWAN, unpublished observations). These observations argue against several hypotheses, *e.g.*, that the lack of ADH activity in a tissue results from the accumulation of the "wrong" *Adh* RNA in that tissue. However, no data demonstrate that the proximal *Adh* transcripts in adult *D. formella*, or the distal transcripts in *D. prostopalpis* larvae, are actually translated. Initial attempts to address this question by an analysis of polysomal RNA from adult *D. formella* have not been technically successful. Proximal *Adh* transcripts can apparently be translated in at least one adult tissue that contains ADH activity, the adult midgut, because only proximal transcripts are detectable in this tissue from *D. affinidisjuncta*.

Molecular models of *Adh* gene regulation which entertain the dichotomy of separate proximal and distal *Adh* transcripts remain vague. As noted previously (BENYAJATI *et al.* 1983; ROWAN, BRENNAN and DICKINSON 1986), the production of distinct RNA populations might be regulated in a variety of ways, either transcriptionally (*e.g.*, different *Adh* promoters functioning in different developmental contexts) or posttranscriptionally (*e.g.*, at the levels of RNA processing or degradation, or both). Descriptions of variant phenotypes demonstrate the mutability of these processes, but reveal little about their physical nature. More promising is the prospect of using natural variants in experimental analyses. Although the Hawaiian *picture-winged* Drosophila are not amenable to formal genetic analysis, the feasibility of utilizing *P* transposable element-mediated transformation (RUBIN and SPRADLING 1982) to study gene regula-

tion in these flies has been demonstrated (BRENNAN, ROWAN and DICKINSON 1984). Also, transgenic *D. melanogaster* express an introduced *D. affinidisjuncta Adh* locus (M. D. BRENNAN, personal communication). If ADH regulatory phenotypes cotransform with genomic DNA from the *Adh* region, it should be possible to map regulatory functions using interspecific recombinant *Adh* loci constructed *in vitro*. Various species-specific patterns of ADH regulation are preserved in the atypical genetic background of species hybrids (DICKINSON and CARSON 1979; DICKINSON 1980b; this communication), perhaps including the adult expression of proximal RNA by the *D. formella Adh* locus (Figure 9). These data provide some pretense for expecting regulatory information to be correctly expressed in interspecific gene transformants. Because cloned *D. melanogaster* genomic *Adh* DNA exhibits normal transcriptional (or posttranscriptional?) regulation after reintroduction by *P* element-mediated transformation (GOLDBERG, POSAKONY and MANIATIS 1983), and since the *Adh* genes of the *picture-winged* Drosophila and *D. melanogaster* are identically organized (ROWAN, BRENNAN and DICKINSON 1986; R. G. ROWAN and W. J. DICKINSON, unpublished results; this communication), this general approach is warranted. Indeed, the analysis of natural variants may prove to be more direct than a conventional study of a cloned "wild-type" *Adh* locus DNA by mutational analysis.

Valuable advice and ideas were provided by MARK BRENNAN. We thank C. CARTER and P. FEY for maintaining Drosophila stocks, and GLENN HERRICK for a critical review of an earlier draft of the manuscript. This work was sponsored by grant HD10723 from the National Institutes of Health. R.G.R. was supported by a National Institutes of Health predoctoral service award trainee grant (5-T32-GM7464).

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Communicating editor: V. G. FINNERTY