MOLECULAR STUDIES ON THE ALCOHOL DEHYDROGENASE GENE OF DROSOPHILA MELANOGASTER

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Abstract

In this thesis, I describe the isolation of the alcohol dehydrogenase (Adh) gene of *Drosophila melaogaster* and some preliminary biochemical characterizations of the gene and its expression. The isolation of the Adh gene was accomplished by screening a bacteriophage λ library containing inserts of *Drosophila* DNA with cDNA probe made from size selected mRNA. One clone which showed hybridization in the initial screen was shown to contain Adh sequences by virtue of its lack of hybridization to Adh deficiency DNA, *in situ* hybridization, translation of ADH protein by mRNA selected by hybridization to the clone, and by partial DNA sequence analysis. Using the clone, approximately 35 kb of the Adh chromosomal region was isolated. This region was found to be composed largely of single-copy sequences, showed limited polymorphism between strains, and encoded only one RNA transcript prevalent in larvae and adults - the Adh mRNA. Two intervening sequences within the Adh coding region were demonstrated by S1 nuclease mapping.

In order to identify sequences important in Adh expression, the cloned Adh gene was transformed into the *Drosophila* germ line by utilizing the hybrid dysgenesis P element vector of Spradling and Rubin. The correct developmental expression of the Adh gene was retained by the transformed gene, even though it had integrated into many locations. These results delimit the sequences and chromosomal enviroment necessary for correct developmental expression of the Adh gene. In addition, the 'transient' expression of cloned DNA in larvae and adults directly grown from injected embryos was investigated. In most instances, ADH activity was found only in tissues that normally express ADH, although low level of activity was observed in some cells which do not normally produce detectable levels of

Adh. Together, these results form the basis for assay systems that may be combined with *in vitro* mutagenesis in order to determine in detail which sequences are necessary for correct developmental expression of the Adh gene.

Table of Contents

		Page
Introduction	. . . .	1
Chapter 1:	Isolation and partial characterization of the alcohol dehydrogenase gene of *Drosophila melanogaster*	29
Chapter 2:	Correct developmental expression of a cloned alcohol dehydrogenase gene transduced into the *Drosophila* germline	35
Appendix:	Initial results of a rapid assay for expression of cloned Adh DNA	51

Introduction

Control of gene expression in higher eucaryotes appears to involve processes not observed in procaryotic and simple eucaryotic organisms. For instance, two bacteria of the same strain generally will have identical morphology and composition, and will react predictably and consistently to the environment. In contrast, two cells located adjacent to one another in the same higher eucaryotic organism, and which may have shared the same ultimate progenitor cell, often have quite dissimilar shapes and biochemical compositions, and react quite differently to the same stimulus. It is the factors that control these differences in cell composition, shape and behavior which I am interested in.

The investigation of the genetic regulatory elements that govern the 'differential gene expression' characteristic of eucaryotic organisms has progressed only slowly (see below), and their study in the case of the Drosophila alcohol dehydrogenase (Adh) gene forms the basis for this thesis. This Introduction will contain a description of the expression of the Adh gene, a summary of the techniques that make the Adh system attractive to study, and a short review of our knowledge of cis-acting sequences important in other RNA polymerase II transcribed eucaryotic genes.

Background - Expression of the Adh gene

ADH enzyme activity in Drosophila melanogaster is found at high levels at all developmental stages. ADH detected in embryos is maternally derived, and newly synthesized ADH is first observed in late embryonic or adult stages. ADH specific activity initially peaks around pupation, drops in pupae, and peaks again in 4-5 day old adults (McDonald and Avise, 1976; Ursprung, et al., 1970). A different developmental profile is seen, however, for a number of closely related species, such as the D. melanogaster sibling

species D. simulans. In this species, early larval ADH activity appears to match that in Drosophila melanogaster, but then a gradual decrease in ADH activity is observed (McDonald and Avise, 1976). It is not clear if the temporal differences in activity reflects important developmental regulation, or simply the differential growth of tissues that contain ADH activity relative to those that do not.

Drosophila melanogaster ADH activity is found in some tissues and not in others. By dissecting tissues and determining ADH enzymatic activity, Ursprung et al. (1970) observed that ADH is synthesized in larvae primarily in the fat body and midgut. In addition, Malpighian tubules displayed high specific activities of ADH. In contrast, ADH activity could not be detected in imaginal discs and brain, and much lower specific activities were detected in the carcass, which was attributed to adherent fat body. In adults, ADH expression in male genital apparatus was demonstrated by a histochemical staining technique.

Using this histochemical staining technique, ADH activity can be detected with higher resolution and more sensitively than by dissection and enzymatic activity determination. Thus, while ADH could be detected by histochemical staining in larval brain and ventral ganglion, it could not be detected by enzyme assays on dissected tissues. The results of an analysis of ADH tissue specificity performed by histochemical staining are presented in Table 3 of the second chapter of this thesis, with selected preparations shown in Figure 4 of the same chapter. Organs that are not listed were not studied.

One difficulty with histochemical staining analysis is that the apparent intensity of staining in each organ is probably dependent on a

number of factors, including the thickness of the tissue, the intracellular location of the ADH, and the penetration of stain into organs. Thus, while imaginal discs do not stain, this may be due to the thinness of the imaginal disc cells, difficulties in penetrating the tissue, or low levels of ADH activity. As well, ADH activity may be influenced by the physiological state of the cell, such as the intrinsic pH, which could suppress ADH activity, even if the protein were synthesized in large quantities in that tissue.

While the factors listed above may affect the actual quantitative determination of ADH tissue specificity by histochemical staining, using different substrates, the staining procedure can give qualitatively intense staining levels in many tissues in which ADH activity is not detected (Dickinson, 1971; Lawrence, 1981). ADH histochemical staining procedure is a simple variation of a standard 'dehydrogenase' specific staining reaction in which different dehydrogenases can be observed by varying the substrate. For example, in the case of aldehyde oxidase (Aldox), in which acetaldehyde is used as a substrate, heavy staining is seen in imaginal discs, whereas imaginal discs do not stain when ethanol or secondary butanol are used to determine ADH activity. Many of the organs listed in Table 3 of Chapter 2 of this thesis which are not stained by the ADH specific stain are stained using acetaldehyde as a substrate. Thus, the lack of staining in certain positions in the organs with the ADH specific stain is probably not due to differences between the cells with respect to the stain penetration or morphological differences between the cells. Rather, the difference most likely reflects either differences in the relative amounts of ADH and Aldox or physiological differences in the cell types that are specific to ADH enzymatic activity as opposed to Aldox.

There do seem to be some quantitative differences between ADH expression in different tissues. For example, whereas larval fat body, intestines, and Malpighian tubules stain heavily, brains and ventral ganglia stain much less intensely. In addition, larval somatic muscle also seems to stain much less dramatically. These qualitative, subjective observations have been confirmed in part by dissection of material and determination of ADH specific activity (Ursprung _et al_., 1970). In these cases, little or no ADH could be detected in these tissues, although the presence of the enzyme could be unambiguously demonstrated by histochemical staining.

Thus, ADH is expressed in some tissues and not in others. In addition, it does not seem to be only 'on' or 'off,' but its expression appears to vary over a wide range. Our intention is to understand the factors leading to this differential gene expression. I believe the _trans_-acting factors involved in this regulation will be extremely difficult to identify and characterize. Such _trans_-acting factors have been identified by genetic means in procaryotes and lower eucaryotes, or by analyzing naturally occurring tissue specific variants of the gene under investigation (e.g. Abraham and Doane, 1978; Dickinson, 1980). ADH, however, is synthesized only in internal organs, for which only primitive developmental genetics has been performed, which does not bode well for the genetic analysis of _trans_-acting factors. If _trans_-acting mutations affecting the tissue specificity of ADH expression could be identified from naturally occuring _Drosophila melanogaster_ populations, loci affecting ADH expression might be studied. However, large scale screens for such variants have been largely unsuccessful (Dickinson, personal communication; Chen, personal communication).

On the other hand, the technology for studying the _cis_-acting involved

in Adh gene expression is advancing rapidly. The techniques of molecular cloning have allowed the isolation of the Adh gene (Chapter 1, this thesis, Benyajati et al., 1980). Techniques for the site-directed mutagenesis are well-established, and the Drosophila P element mediated transformation system allows the reintroduction of cloned Adh genes into the germ line (Spradling and Rubin, 1982; Rubin and Spradling, 1982). The second chapter of this thesis demonstrates that in the majority of cases, these 'transduced' genes are expressed at the correct levels and with the proper stage and tissue specificity. Together, these techniques should permit a detailed analysis of the cis-acting sequences involved in ADH expression.

It seems possible that a complex organization of cis-acting regulatory sites may exist. For example, a number of differences in ADH expression have been observed between closely related species of Hawaiian Drosophila (Dickinson and Carson, 1979). In one comparison, between Drosophila orthofascia and Drosophila grimshawi, ADH was expressed in high levels in both species in adult fat body and midgut, and in larval fat body. However, in D. orthofascia, no expression of ADH was observed in larval midgut, whereas high levels were seen in D. grimshawi. By performing the appropriate crosses, it was determined that the factor(s) responsible for this difference in expression were cis-acting in hybrids between the two species and located close to the Adh structural gene. Finally, it was determined that the respective levels of ADH activity were reflected in the levels of ADH mRNA found in the different organs (Rabinow and Dickinson, 1981). This result suggests that the control of ADH expression may be controlled at least in part by cis-acting regulatory sites that are tissue specific.

The observations described in this background section demonstrate a

complex pattern of ADH expression that

1) is organ specific,

2) is expressed at variable levels; i.e. it is not either 'on' or 'off,' but may be expressed at intermediate levels as well,

3) may be under the control of regulatory sequences that are tissue specific.

In addition to its interest as a system for studying the developmental regulation of gene activity, the Adh gene has many technical advantages that should facilitate analysis of the cis-acting sequences, as described below.

Background - Genetics

The Adh locus is located at recombination map position 50.5 and polytene chromosome band 35B2-3 on the left arm of the second chromosome (Woodruff and Ashburner 1979a; O'Donnell et al., 1977). Electrophoretic mobility (Grell et al., 1968; Retzios and Thatcher 1979), temperature-sensitive activity (Vigue and Sofer 1974) and null activity (Schwartz and Sofer 1976) Adh variants have been extensively characterized at the protein level (see also Thatcher, 1980).

Positive selection for ADH activity can be performed with ethanol. Thus, flies lacking ADH activity die within a few hours on food containing 5% ethanol, whereas wild-type flies will survive upwards of 15% ethanol. This survival reflects one of the presumed functions of ADH, the environmental detoxification of ethanol present on their normal food of fermenting rotting fruit. This positive selection could possibly be used to select for 'up-promoter' mutants, revertants of Adh mutations, or the rare wild-type

intragenic recombination products (Vigue and Sofer, 1976).

An unsaturated secondary alcohol, 1-pentyn-3-ol (pentynol), was found to be itself relatively non-toxic to flies, but was metabolized to a more toxic form by ADH (O'Donnell et al., 1975). This differential toxicity serves as the basis for the selection of ADH negative flies, as only flies lacking alcohol dehydrogenase activity will survive exposure to pentynol.

Two groups have performed saturation point mutation and deficiency mapping experiments in the Adh region (Woodruff and Ashburner 1979a,b; O'Donnell et al 1977; Ashburner and colleagues, unpublished results). A genetic map of this region is presented in Figure 1. Centromere-proximal to Adh is a region in which many chromosome rearrangement breakpoints have been localized, followed by the locus br3 , identified by lethal alleles. Centromere-distal to Adh is the locus outspread wings (osp), then no ocelli (noc), both of which are characterized by visible alleles. In fact, homozygous viable deficiencies lacking Adh, osp and noc simultaneously have been reported (Ashburner et al., 1982b). Distal to noc comes br29, a locus without 'point' alleles, but which is presumed to exist on the basis of chromosome rearrangement lethality. While osp appears to be very near to Adh on genetic criteria (recombination mapping and frequency of rearrangement breakpoints), both noc and br3 appear to be well separated from Adh and osp (Ashburner et al., 1982b; Ashburner and colleagues, unpublished results; this work).

The mutant Sco was originally mapped near the right of Adh on the basis of deficiency mapping (O'Donnell et al., 1977). However, upon further investigation, Sco now appears to be a small reciprocal transposition including the Adh gene (Ashburner et al., 1982a). Sco/deficiency

heterozygotes are useful in that larger deletions extending distal to Adh tend to have extreme mutant phenotypes including sex comb reduction and cratered, bulging eyes, whereas smaller deletions have milder phenotypes.

Background - Nucleic Acid Studies

By isolating successively overlapping bacteriophage genomic clones from the region surrounding Adh, approximately 45 kb of contiguous DNA was isolated. A restriction map for these sequences indicating the position of ADH mRNA transcription is shown in Figure 2. DNA sequences in the Adh region appear to be present in a single copy in the genome. In addition, there does not appear to be extensive sequence repetition within the Adh region, as evidenced by the lack of cross-hybridization between different cloned sequences spanning the region.

Studies using Adh genomic and cDNA clones have shown that ADH protein is translated from an abundant RNA approximately 1100 nucleotides in length (Goldberg 1980; Benyajati *et al*., 1980). This RNA is transcribed from a gene containing two small intervening sequences (65 and 70 bp). Following the observation that ADH mRNA changes in size during development, it was determined that the Adh gene is transcribed from two promoters. The following description refers extensively to sequence analysis of the Adh gene reported by Benyajati and others (Benyajati, personal communication; Benyajati *et al*., 1981; Benyajati *et al*., 1983). The promoter which appears primarily responsible for Adh transcription in larvae (the 'larval promoter') expresses an mRNA with a 5' terminus approximately 70 nucleotides upstream of the initiating AUG codon. Twenty-five bp upstream of the larval mRNA 5' end is the sequence TATAAATA which, in sequence and position relative to transcription initiation, is similar to sequences found in a variety of

other eucaryotic promoter regions, the so-called Goldberg-Hogness box (Goldberg, 1979; Benoist _et al_., 1980).

The 5' end of adult ADH mRNA is located approximately 700 bp upstream from the larval mRNA 5' end. A 653 bp intervening sequence is present in this transcribed region, with a splice acceptor site located between the larval 5' end and the initiating AUG codon. Approximately 25 bp upstream of the adult mRNA 5' end is a sequence TATTTAA, which is similar to the consensus TATAAATA sequence in being highly A-T rich, but which shows little exact sequence homology.

The developmental stage at which transcription switches between the two promoters has not been extensively characterized. In first and second instar larvae, only 'larval' mRNA is observed, but by late third instar larvae, both adult and larval forms can be seen in variable, but roughly equal proportions (Benyajati _et al_., 1983; Goldberg _et al_., 1983).

The two _Adh_ promoters were demonstrated by a number of experiments. Firstly, the size of ADH mRNA observed by RNA blot hybridization increases in size by approximately 100 nucleotides during mid- to late pupal stages (Benyajati _et al_., 1983). S1 nuclease analysis of adult RNA yielded a seemingly anomalous result when compared with the nucleotide sequence of the _Adh_ gene. Whereas the DNA sequence predicted that the exon containing the initiating AUG should by 170 nucleotides in size (on the basis of the position of the TATAATA sequence), S1 nuclease analysis showed a major spot at 140 nucleotides with only a minor component in the region of 170 nucleotides (Goldberg, 1980). The most direct evidence of dual promoters comes from the reverse transcriptase extension on both larval and adult mRNAs of a restriction fragment primer originating near the N-terminal region of the protein

sequence. Analysis of the products formed in the presence of different dideoxynucleotides on appropriate gel systems allowed direct nucleotide sequence determination of the different RNAs (Benyajati _et al_., 1983). A composite of the available DNA and RNA sequence information is presented in Figure 3.

Studies on cis-acting sequences in other systems

A generation of molecular biologists have made extensive and fruitful studies on the regulation of expression of procaryotic genes, and have obtained detailed insights into sequences which are _cis_-acting on gene expression. In general, _cis_-acting sequences are grouped into two categories: promoters, which are necessary to obtain initiation of transcription and which bind to RNA polymerase, and regulatory sequences, which modulate the level of transcription. These regulatory sequences may act, among other means, on the basis of DNA sequence recognition and subsequent RNA polymerase exclusion (e.g. regulation of the lac operon, refs. in Zipser and Beckwith, 1977), transcription termination or attenuation (e.g. Platt, 1981), or enhancement of transcription by protein-protein interactions with RNA polymerase (Meyer and Ptashne, 1980). This rather dry description does not convey the true complexity of many situations, such as the bacteriophage λ right operator, in which a variety of regulatory proteins interact with a small region of DNA in an elaborate system of positive and negative regulation (Ptashne _et al_., 1980). In addition, other forms of regulation are known to act through _cis_-acting sites, such as the use RNA polymerases with novel promoter specificities, used to program the temporal use of genes in certain bacteriophage (e.g. Chamberlain _et al_., 1970; Tijian and Pero, 1976).

In many of the cases studied, the precise arrangement of promoter and regulatory sequences has been determined. In contrast, in eucaryotic systems, very little can be said with certainty about the _cis_-acting sequences. It seems likely, however, given the large number of modes of control in procaryotes, that similar diversity will be found in eucaryotes. At the present low level of resolution, three types of sequences have been identified in eucaryotes, each with different effects on transcription and different effects. However, the functions of these sequences are obscure, and tend to have different effects depending on the system used to assay gene transcription.

The first sequence identified that appears to be a part of eucaryotic promoters was the 'Goldberg-Hogness' or TATA box (Goldberg, 1979), which shows homology to the 'Pribnow' box in procaryotes, and which is located approximately 25 bp upstream of the site of transcription initiation. The TATA box appears to be necessary for efficient transcription and for positioning transcription initiation (Wasylyk _et al_., 1980; McKnight and Kingsbury, 1982), with the exception of SV40 early transcription (Gluzman _et al_., 1980).

Less well characterized are the 'upstream' sequences, which appears to greatly enhance gene transcription in some systems. These 'upstream sequences' are located typically between the TATA sequence and 100 bp upstream from the start point of transcription. Unlike the -35 sequence of procaryotic promoters which is found a set distance from the Pribnow box, these upstream elements are a variable distance from the TATA sequence.

Four cases in which these sequences have been identified in higher eucaryotic promoters give an indication as to the heterogeneity in structure

and function of these upstream elements. In the case of the Herpes Simplex Virus thymidine kinase gene, the upstream element has a bipartite organization, with components at -47 to -61 and -80 to -105 bp from the start of transcription. These two components appear to functionally interact (McKnight, 1982). In the β globin genes, a sequence conserved among a number of different species (consensus GG(C/T)CAATCT), the CAAT box, is present at approximately -80 bp (Efstradiatis et al., 1980). Mutations in this box greatly reduced transcription levels (Dierks et al.. 1983). In the virus SV40, between -100 bp and the TATA homology, are 3 repeats of a 21 bp sequence which is internally repititious. At least one of these sequences appears to be required in order to retain efficient RNA synthesis (Fromm and Berg, 1982, 1983). Lastly, in the Drosophila glue protein gene, Sgs4, two sites located far upstream of the transcription initiation site, at approximately -330 and -405 to -480, appear to be essential for promoter utilization. These sites also correspond to regions of DNAase I-hypersensitivity (Shermoen and Beckendorf, 1982).

Some of these upstream elements appear to have some role in the regulation of transcription. For instance, in the Drosophila hsp70 heat shock gene, a sequence between -47 and -60 bp has been identified that is both necessary and sufficient to allow heat shock induction of promoters to which the sequence is abutted. Other upstream sequences affecting transcriptional regulation have also been identified in yeast in the his3 (Struhl, 1982) and cytochrome c (Guarente and Ptashne, 1981) genes, and in the human and mouse β-globin genes (Chao et al. 1983). In each case mentioned above, the upstream sequences do not share sequence homology, are located at different distances from the TATA box, and have variable effects on transcription.

A third type of *cis*-acting element affecting gene expression are called 'enhancer' sequences. These sequences are defined by their ability to affect transcription efficiency when located at variable distances and either orientation with respect to the promoter. Their presence can be demonstrated in the early promoter of SV40 (Fromm and Berg, 1983). In this case, both the orientation and relative position of one of the 72 bp repeats is necessary to obtain efficient expression of T antigen. The effects of the enhancer sequence is easily transfered between genes, and in fact, the first demonstration of enhancer function was obtained by linking the SV40 enhancer to the β-globin (Banerji *et al*., 1981) and conalbumin (Moreau *et al*., 1981) genes. The effect of the enhancer sequence on transcription could be observed even when the enhancer was 1400 bp 5' or 3300 bp 3' of the β-globin gene promoter.

The functions of the three elements described above (the TATA box, upstream sequences, and enhancers) are presently obscure. In particular, it is uncertain which if any of these sequences act in controlling the tissue specific expression of genes, although many of these sequences can be deduced not to have developmental importance. For instance, many of the genes which have been studied do not appear to be developmentally regulated (such as the SV40 early promoter, the Herpes Simplex Virus thymidine kinase gene, or the yeast genes). Many of the other sequences function in assay systems which do not mimic developmental characteristics. Thus, the CAATT box can be shown to have importance in fibroblast transformation assays of β-globin genes, eventhough the β-globin gene is not normally transcribed in fibroblasts (Dierks *et al*., 1983).

In some cases, however, the importance of some of these elements on

developmental regulation or response is better demonstrated. Thus, a hybrid mouse-human β-globin gene has been transfered into mouse erythroleukemia (MEL) cells, which retain certain differentiated characteristics, and can be induced to express globin genes by treatment with certain chemicals. In this system, the induction of transformed globin genes has been demonstrated, albeit at an overall transcriptional level approximately 100-fold lower than the endogenous genes (Chao *et al*., 1983).

In addition, it has recently been determined that sequences within the immunoglobulin heavy chain constant region act as enhancers, although only in lymphocyte (myeloma) cell culture (Banerji *et al*., 1983; Gillies *et al*., 1983). This enhancer can confer up to 200-fold increased transcription to β-globin or SV40 T antigen genes in myeloma cell lines, but has no effect on gene transcription in fibroblast cell lines.

The difficulty in studying the promoter and regulatory elements described above is basically the problem of finding an assay system in which a gene is correctly regulated. This problem is especially acute if the developmental regulation of gene expression is under study. As described above and in the discussion section of Chapter 2, few higher eucaryotic systems are appropriate for such an investigation. We hope, however, that the *Adh* gene, in conjunction with P element mediated transformation and *in vitro* mutagenesis will allow such a study to be undertaken.

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Figure 1 - Genetic organization of the Adh region

The heavy line represents the linear organization of the Adh region derived by O'Connell *et al*., (1979) and Ashburner *et al*. (1980). Loci named *br* are characterized by lethal alleles, while other loci have visible phenotypes (*Sco* is a recessive lethal in addition to its dominant visible phenotype). *br29* is postulated to exist on the basis of chromosome rearrangement lethality complementation. Broad spaces divide loci widely separated by physical distance with many intervening genetic loci. Recombination map positions are presented below selected loci.

Lines above the genetic organization chart the extents of deficiencies. Distances depicted in this map are not drawn to physical or genetic scale.

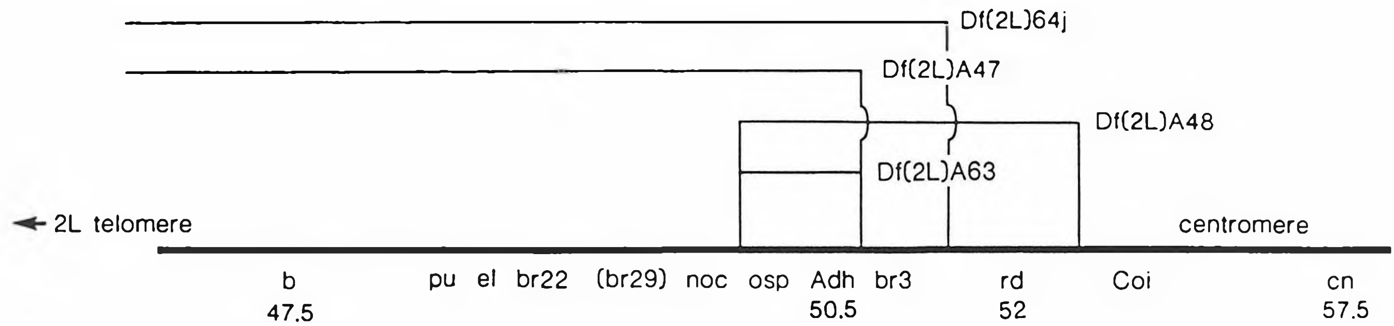
Df(2L)64j
Df(2L)A47
Df(2L)A48
Df(2L)A63
2L telomere
centromere
b
47.5
pu
el
br22
(br29)
noc
osp
Adh
50.5
br3
rd
52
Coi
cn
57.5

Figure 2 - Physical map of the *Adh* region

The heavy line presents a map of the Eco RI (E), Hind III (H) and Sal I (S) restriction enzyme recognition sites in the *Adh* region. The sites are present in clones obtained from Canton-S DNA. Nucleotide and insertional polymorphisms have been described among *Drosophila* strains which may affect this distribution of restriction sites (Goldberg 1980; Langley *et al.*, 1982).

Above this map are displayed the extents of the Adh protein coding sequence and the largest region showing significant hybridization to labeled cDNA to total RNA (Adh RNA transcription may represent only part of this region). Below the map are presented regions of *Drosophila* DNA present on plasmid (pAA, sAC1, sAS1) and bacteriophage lambda (gAC1, gAC2, gAC3) vectors used in the construction of this map.

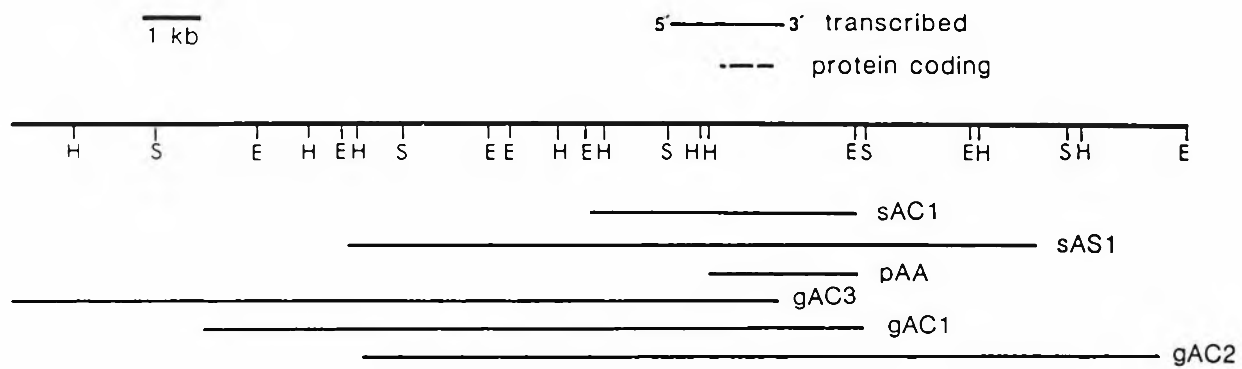
1 kb
5' 3' transcribed
protein coding
H S E H EH S EE H EH S HH ES EH SH E
sAC1
sAS1
pAA
gAC3
gAC1
gAC2

Figure 3

The sequence of the Adh gene as determined by C. Benyajati is presented. Data related to the positions of the larval and adult trancsripts is from Benyajati *et al*., 1983. The following symbols were used to denote sequences of special function:

↑↑↑↑ - sequences thought to act in a fashion similar to the TATAAATA Goldberg-Hogness box. This symbol also indicates the AAUAAA sequence thought to be important in polyadenylation of mRNA.

~~ - the 5' end of Adh transcripts as determined by a combination of primer extension analysis, S1 analysis, and position relative to TATAAATA-like sequences.

[** **] - position of intervening sequences; the large intervening sequence extending from nucleotides 354-1007 is indicated only at its extremities.

pA - the position of mRNA polyadenylation as determined by sequence analysis of a cDNA clone

```
            10        20        30        40        50        60        70        80        90

0     GACTCTTTTTTGATTTTGGAATATTTTCGTTCGTTTTATGTTTTTACGTTTTCGCATATTTGTTTCACAGTGACATTTCTGGTGTTCCATTTTCTATTGG

100   GCTCTTAACCCCGCATTTGTTTGCAGATCACTTGCTTGCGCATTTTTATTGCATTTTACATATTACACATTATTTGAACGCCGCTGCTGCTGCATCCGTC

200   GACGTCGACTGCACTCGCCCCCACGAGAGAACAGTATTTAAGGAGCTGCGAAGGTCCAAGTCACCGATTATTGTCTCAGTGCAGTTGTCAGTTGCAGTTC
                                        ^^^^^^^
300   AGCAGACGGGCTAACGAGTACTTGCATCTCTTCAAATTTACTTAATTGATCAAGTAAGTAGCAAAAGGGCACCCAATTAAAGGAAATTCTTGTTTAATTG
                                                               [***
400   AATTTATTATGCAAGTGCGGAAATAAAATGACAGTATTAATTAGTAAATATTTTGTAAAATCATATATAATCAAATTTATTCAATCAGAACTAATTCAAG

500   CTGTCACAAGTAGTGCGAACTCAATTAATTGGCATCGAATTAAAATTTGGAGGCCTGTGCCGCATATTCGTCTTGGAAAATCACCTGTTAGTTAACTTCT

600   AAAAATAGGAATTTTAACATAACTCGTCCCTGTTAATCGGCGCCGTGCCTTCGTTAGCTATCTCAAAAGCGAGCGCGTGCAGACGAGCAGTAATTTTCCA

700   AGCATCAGGCATAGTTGGGCATAAATTATAAACATACAAACCGAATAACTAATATAGAAAAAGCTTGCCGGTACAAAATCCCAAACAAAAACAAACCGTG

800   TGTGCCGAAAAATAAAAATAAACCATAAACTAGGCAGCAGCGCCGTCGCCGGCTGAGCAGCCTGCGTACATAGCCGAGATCGCGTAACGGTAGATAATGA

900   AAAGCTCTACGTAACCGAAGCTTCTGCTGTACGGATCTTCCTATAAATACGGGGCCGACACGAACTGGAAACCAACAACTAACGGAGCCCTCTTCCAATT
                                                ^^^^^^^^
1000  GAAACAGATCGAAAGAGCCTGCTAAAGCAAAAAAGAAGTCACCATGTCGTTTACTTTGACCAACAAGAACGTGATTTTCGTTGCCGGTCTGGGAGGCATT
         ***]                                  IN1SerPheThrLeuThrAsnLysAsnValIlePheValAlaGlyLeuGlyGlyIle

1100  GGTCTGGACACCAGCAAGGAGCTGCTCAAGCGCGATCTGAAGGTAACTATGCGATGCCCACAGGCTCCATGCAGCGATGGAGGTTAATCTCGTGTATTCA
      GlyLeuAspThrSerLysGluLeuLeuLysArgAspLeuLys[*************************************************************

1200  ATCCTAGAACCTGGTGATCCTCGACCGCATTGAGAACCCGGCTGCCATTGCCGAGCTGAAGGCAATCAATCCAAAGGTGACCGTCACCTTCTACCCCTAT
      ******]AsnLeuValIleLeuAspArgIleGluAsnProAlaAlaIleAlaGluLeuLysAlaIleAsnProLysValThrValThrPheTyrProTyr

1300  GATGTGACCGTGCCCATTGCCGAGACCACCAAGCTGCTGAAGACCATCTTCGCCCAGCTGAAGACCGTCGATGTCCTGATCAACGGAGCTGGTATCCTGG
      AspValThrValProIleAlaGluThrThrLysLeuLeuLysThrIlePheAlaGlnLeuLysThrValAspValLeuIleAsnGlyAlaGlyIleLeuA

1400  ACGATCACCAGATCGAGCGCACCATTGCCGTCAACTACACTGGCCTGGTCAACACCACGACGGCCATTCTGGACTTCTGGGACAAGCGCAAGGGCGGTCC
      spAspHisGlnIleGluArgThrIleAlaValAsnTyrThrGlyLeuValAsnThrThrThrAlaIleLeuAspPheTrpAspLysArgLysGlyGlyPr

1500  CGGTGGTATCATCTGTAACATTGGATCCGTCACTGGATTCAATGCCATCTACCAGGTGCCCGTCTACTCCGGCACCAAGGCCGCCGTGGTCAACTTCACC
      oGlyGlyIleIleCysAsnIleGlySerValThrGlyPheAsnAlaIleTyrGlnValProValTyrSerGlyThrLysAlaAlaValValAsnPheThr

1600  AGCTCCCTGGCGGTAAGTTGATCAAAGGAAACGCAAAGTTTTCAAGAAAAACAAAACTAATTTGATTTATAACACCTTTAGAAACTGGCCCCCATTACC
      SerSerLeuAla[*****************************************************************]LysLeuAlaProIleThr

1700  GGCGTGACCGCTTACACCGTGAACCCCGGCATCACCCGCACCACCCTGGTGCACAAGTTCAACTCCTGGTTGGATGTTGAGCCCCAGGTTGCTGAGAAGC
      GlyValThrAlaTyrThrValAsnProGlyIleThrArgThrThrLeuValHisLysPheAsnSerTrpLeuAspValGluProGlnValAlaGluLysL

1800  TCCTGGCTCATCCCACCCAGCCATCGTTGGCCTGCGCCGAGAACTTCGTCAAGGCTATCGAACTGAACCAGAACGGAGCCATCTGGAAACTGGACCTGGG
      euLeuAlaHisProThrGlnProSerLeuAlaCysAlaGluAsnPheValLysAlaIleGluLeuAsnGlnAsnGlyAlaIleTrpLysLeuAspLeuGl

1900  CACCCTGGAGGCCATCCAGTGGACCAAGCACTGGGACTCCGGCATCTAAGAAGTGATAATCCCAAAAAAAAAACATAACATTAGTTCATAGGGTTCTGCG
      yThrLeuGluAlaIleGlnTrpThrLysHisTrpAspSerGlyIleTER.

2000  AACCACAAGATATTCACGCAAGGCAATAAGGCTGATTCGATGCACACTCACATTCTTCTCCTAATACGATAATAAAACTTTCCATGAAAAATATGGAAAA
                                                                             ^^^^^^
2100  ATATATGAAAATTGAGAAATCCAAAAAACTGATAAACGCTCAACTTAATTAAATAGATAAATTGGGAGCGGCAGGAATGGCGGAGCATAGGCCAAGTTCC
                        pA
2200  AACCAGCCAATCAGTCGTAAAACAGAAGTCGTGGAAGCGAATAAGAAAAGATGTTC
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Chapter 1

Isolation and partial characterization of the alcohol dehydrogenase gene of *Drosophila melanogaster*

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Biochemistry

Isolation and partial characterization of the *Drosophila* alcohol dehydrogenase gene

(molecular cloning/polymorphism/intervening sequence)

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ABSTRACT **The alcohol dehydrogenase (ADH; alcohol: NAD$^+$ oxidoreductase, EC 1.1.1.1) gene (*Adh*) of *Drosophila melanogaster* was isolated by utilizing a mutant strain in which the *Adh* locus is deleted. Adult RNA from wild-type flies was enriched in ADH sequences by gel electrophoresis and then used to prepare labeled cDNA for screening a bacteriophage λ library of genomic *Drosophila* DNA. Of the clones that hybridized in the initial screen, one clone was identified that hybridized with labeled cDNA prepared from a wild-type *Drosophila* strain but did not hybridize with cDNA prepared from an *Adh* deletion strain. This clone was shown to contain ADH structural gene sequences by three criteria: *in situ* hybridization, *in vitro* translation of mRNA selected by hybridization to the cloned DNA, and comparison of the ADH protein sequence with a nucleotide sequence derived from the cloned DNA. Comparison of the restriction site maps from clones of three different wild-type *Drosophila* strains revealed the presence of a 200-nucleotide sequence in one strain that was absent from the other two strains. The ADH mRNA sequences were located within the cloned DNA by hybridization mapping experiments. Two intervening sequences were identified within *Adh* by S1 nuclease mapping experiments.**

The *Drosophila* alcohol dehydrogenase (ADH; alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) locus (*Adh*) provides an interesting system for studying the molecular basis of differential gene expression during development because it is a developmentally regulated gene for which considerable genetic and biochemical data are available.

ADH activity varies during development and is restricted to certain tissues (1). Activity is first detected during the first larval instar, increases throughout larval development, and gradually declines during the pupal stage. It increases again just prior to adult emergence and peaks 5–10 days later. ADH activity is limited to the fat body, intestines, Malpighian tubules of larvae and adults and to the accessory genital structures of adults. The enzyme cannot be detected in the imaginal discs, larval brain, salivary glands, ovaries, or testes. Thus, ADH expression is developmentally regulated and those elements responsible for its tissue- and stage-specific expression may be accessible to genetic and biochemical analysis.

ADH is one of the best biochemically characterized enzymes in *Drosophila*. It is an abundant protein comprising approximately 1% of the total protein of the adult fly (2). The complete amino acid sequence for the wild-type protein has been determined, as well as the amino acid replacements in a number of genetically variant proteins (3).

Adh is among the best characterized genetic loci in *Drosophila*. The *Adh* locus has been mapped to within two bands (35B 2-3) on the second chromosome (4, 5). Flanking loci with visible and lethal alleles have been identified and their cytogenetic positions have been determined by using deletion mapping of chemically induced mutations (4–6). ADH protein from various strains may be distinguished by electrophoretic mobilities of the enzyme on starch gels. *cis*- and *trans*-acting elements affecting *Adh* expression have been identified (7, 8). The isolation of flies that express or lack ADH activity is possible because both positive and negative selections have been devised (9, 10). These selections are useful both in the isolation of *Adh* null mutants (10) and for the isolation of rare wild-type recombinants generated during fine structure genetic analysis (11).

This paper reports the molecular cloning and initial characterization of the *Drosophila* gene for ADH. The region surrounding this gene is present as a single copy within the *Drosophila* genome and the only adult transcript that is observed to originate from the region is the ADH mRNA. The presence of intervening sequences within the mRNA coding region of the *Adh* gene is demonstrated.

MATERIALS AND METHODS

Strains and Materials. The following bacterial strains were used: K802 for propagation of bacteriophage clones (12); HB101 for propagation of bacterial plasmids (13); JM101 for propagation of M13 clones (14). The following bacterial vectors were used: Charon 9 and Charon 10 bacteriophage (12); pBR322 (15); M13mp2 (14); pSV2 [constructed by placing a central *Eco*RI fragment from Charon 9 into pBR322 and subsequently removing the internal *Sac* I fragments (unpublished data)]. The *Drosophila* bacteriophage λ library was constructed by J. Lauer (see ref. 16 for a description of this library).

Adh^S and w;*Adh*^{F} *Drosophila* strains were obtained from W. Sofer. Strains carrying *Adh*^{fn2}*pr* and *Sco*$^{R+1}$ chromosomes were obtained from R. Woodruff (4).

Restriction endonucleases and T4 DNA ligase were purchased from commercial sources and used as directed. Avian myeloblastosis reverse transcriptase was obtained from J. Beard (Office of Program Resources and Logistics, Viral Cancer Program, National Institutes of Health).

Construction and Propagation of M13 Cloned DNA. mACl-3 was constructed by transferring the *Drosophila* 4.75-kilobase (kb) *Eco*RI fragment of sACl to the M13 vector mp2 (see Fig. 3 for a description of *Adh* clones mentioned in this paper). Three independent clones with the *Drosophila* insert in the desired orientation were plaque-purified, and replicative form DNA isolated from 10-ml cultures was analyzed. Two clones deleted the insert with a high frequency; the third clone maintained the insert largely intact as shown by restriction analysis. This phage clone was grown directly in large scale. Single-stranded phage DNA was prepared from virions and fractionated on a 0.7% agarose gel. The intact clone DNA was electroeluted and used in S1 nuclease mapping experi-

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Abbreviations: nt, nucleotide(s); kb, kilobase(s); *Adh*, alcohol dehydrogenase genetic locus; ADH, alcohol dehydrogenase.

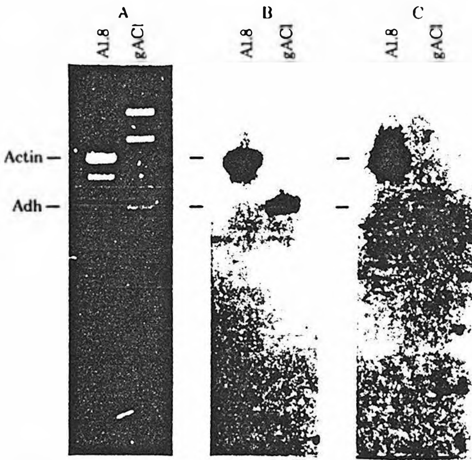


FIG. 1. Hybridization of wild-type and deficiency cDNAs to cloned *Drosophila* DNA. Duplicate samples of DNA prepared from gACI and a plasmid clone containing a *Drosophila* actin gene [A1.8, obtained from E. Fyrberg (26)] were digested with *Eco*RI, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose (18). The pattern of the ethidium stained gel is shown in *A*. Separate blots were then hybridized with ^{32}P-labeled cDNA (23) prepared from total adult RNA from wild-type (Urbana S) flies (*B*) or total RNA obtained from flies genetically deleted for *Adh* [Adh^{fn2}/Sco^{R+4} (4)] (*C*).

ments. Supercoiled replicative form DNA prepared from the large-scale culture was analyzed by restriction endonuclease digestion, and no deletion products could be detected.

S1 Nuclease Mapping Experiments. These experiments (17) were performed under different conditions depending on whether mACI-3 or sAS1 DNA was used.

mACI-3. mACI-3 single-stranded phage DNA (100 ng) was hybridized with 2–10 μg of total adult *Drosophila* RNA for 1 hr at 37°C in 20 μl of 50% formamide/0.4 M NaCl/40 mM 1,4-piperazinediethanesulfonic acid, pH 6.8/1 mM EDTA. The reaction was diluted into 200 μl of S1 nuclease buffer (300 mM NaCl/30 mM NaOAc, pH 4.5/3 mM $ZnCl_2$) and digested with 2000–5000 units of S1 nuclease (Miles) at room temperature or 37°C for 1 hr. After digestion, the mixture was ethanol precipitated and run in two dimensions with a 2.0% or 2.5% agarose nondenaturing dimension and a 2.5% agarose alkaline dimension (17). The gel was transferred to a nitrocellulose filter (18) and probed with nick-translated sAS1 (19).

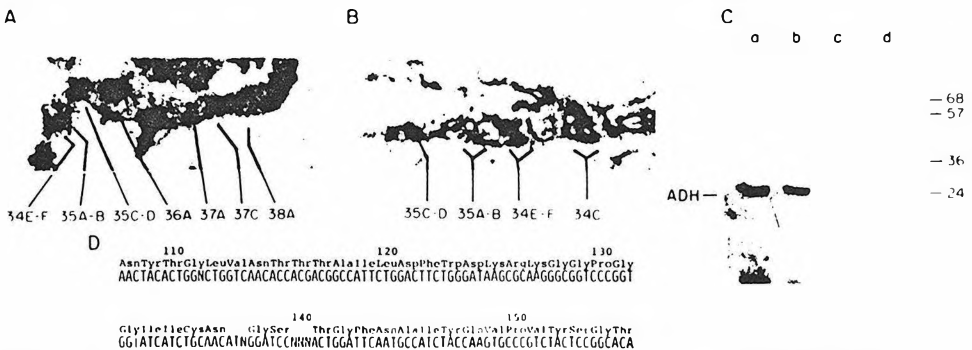
sAS1. Bgl II-digested sAS1 DNA (100 ng) was hybridized with 2–10 μg of total adult *Drosophila* RNA for 3 hr at 48 or 49°C in 20 μl of 80% formamide/0.4 M NaCl/40 mM 1,4-piperazinediethanesulfonic acid, pH 6.8/1 mM EDTA. The reaction was diluted into 200 μl of ice-cold S1 nuclease buffer and digested with 2000–5000 units of S1 nuclease at room temperature. The rest of the procedure was carried out as described above for mACI-3 DNA.

RESULTS

Isolation of the ADH Gene. To obtain a hybridization probe enriched for ADH mRNA sequences, total polyadenylylated RNA from 1- to 3-day-old *Drosophila* adults was fractionated by agarose gel electrophoresis, and RNA in the size range 600–1500 nucleotides (nt) was recovered [ADH protein is encoded in approximately 760 nt (3)]. The fractions containing ADH mRNA were identified by their ability to stimulate incorporation of ^{3}H-labeled amino acids (20) into protein that could be specifically immunoadsorbed by an ADH antibody coupled to Sepharose (21). (We thank W. Sofer for providing ADH antibody and purified ADH protein.) The immunoadsorbed protein comigrated with purified ADH protein on a $NaDodSO_4$/polyacrylamide gel (22).

^{32}P-Labeled cDNA was prepared (23) from the ADH mRNA-enriched fraction and used to screen (24) a bacteriophage λ library of random, high molecular weight (>15 kb) *Drosophila* DNA (16). Of 50,000 plaques screened, 31 hybridized strongly.

To determine which of the phage clones carried *Adh* se-



140 150
GlyIleIleCysAsn GlySer ThrGlyPheAsnAlaIleTyrGlnValProValTyrSerGlyThr
GGIATCATCTGCAACATNGGATCCNNNACTGGATTCAATGCCATCTACCAAGTGCCCGTCTACTCCGGCACA

FIG. 2. Confirmation of the presence of *Adh* sequences in gACI DNA. (*A* and *B*) Salivary gland polytene chromosomes were prepared from wild-type (Oregon R) third-instar larvae. gACI, 2E2, and 11E4 DNAs were ^{3}H-labeled by nick-translation and hybridized *in situ* to the chromosome preparations (25). 2E2 and 11E4 are cloned DNAs that hybridize only to regions 38A and 37C of *Drosophila* polytene chromosomes, respectively (J. Hirsch, personal communication). (*C*) A1.8, a *Drosophila* actin clone (25), sACI, and gACI DNAs were cleaved with *Eco*RI and bound to nitrocellulose as described (26). Approximately 30 μg of total adult RNA was hybridized with each nitrocellulose filter. The filter was washed and the RNA was eluted as described (26) and translated in a rabbit reticulocyte *in vitro* translation system (20). The translation products were immunoadsorbed to ADH antibody immobilized on Sepharose (21) and fractionated on a $NaDodSO_4$/polyacrylamide gel (22). The filter hybridizations were performed with 26 μg of sACI DNA (lane a), 12 μg of gACI DNA (lane b), 10 μg of A1.8 DNA (lane c), and no DNA (lane d). Protein standard molecular weights ($\times 10^{-3}$) are listed at the right. (*D*) The nucleotide sequence was read in both directions from a *Bam*HI site in the sAS1 *Drosophila* DNA insert (27). The sequence was read from only one strand and only those sequences that were independently read from the autoradiogram by three people are presented. Nucleotides listed as N could not be read unambiguously. The amino acid residue numbers are shown.

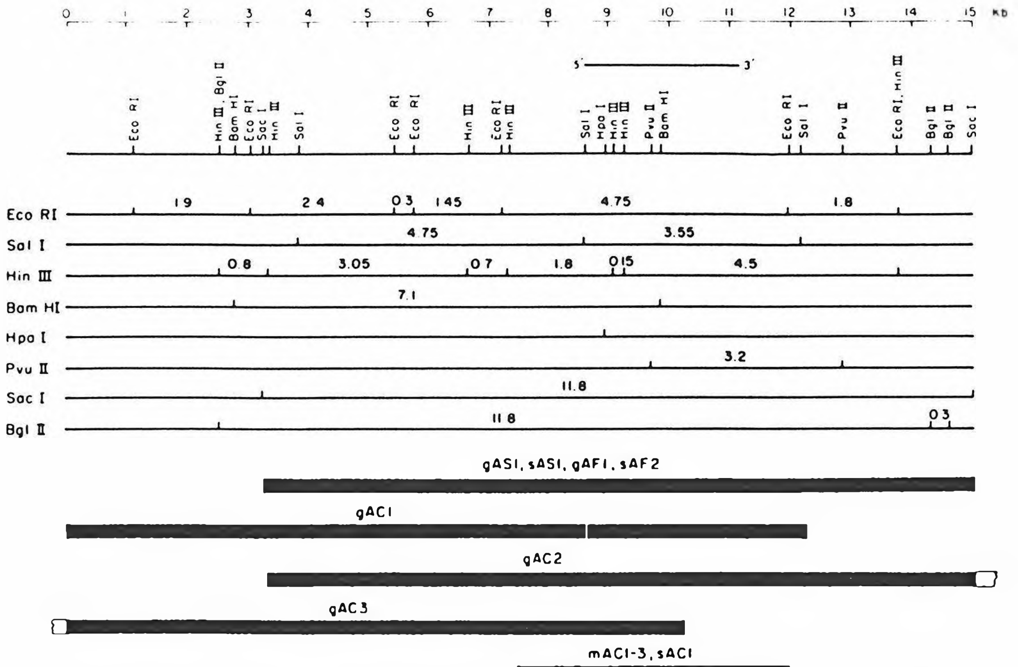


FIG. 3. Restriction map of the *Adh* region. A 4.7-kb *Eco*RI fragment of gAC1 that hybridizes to ADH mRNA was subcloned into pBR322 (sAC1). Two additional *Adh* clones (gAC2 and gAC3) were obtained by screening the *Drosophila* library with sAC1. Also, Adh^S (slow electrophoretic mobility/low specific activity) and Adh^F (fast electrophoretic mobility/high specific activity) alleles were cloned as 11.8-kb *Sac* I fragments in the bacteriophage vector Charon 10. These *Sac* I fragments were subcloned in a pBR322-derived *Sac* I vector (pSV2) to give clones sAS1 (from Adh^S) and sAF2 (from Adh^F). A restriction map was determined for the *Drosophila* inserts in gAC1 and sAS1. Some restriction site polymorphisms exist between the Canton S, Adh^S, and Adh^F strains from which gAC1, sAS1, and sAF2 were derived; however, these are not listed on the map. The restriction sites in the region overlapped by sAS1 and gAC1 are the same in both clones. Bars at the bottom of the figure denote the regions cloned in the respective recombinant molecules. gAC2 extends 2.0 kb to the right and gAC3 5.8 kb to the left of the region depicted in the figure. No cDNA hybridization to cloned restriction fragments was observed outside of the bar located above the composite restriction site map. The 5′- to -3′ orientation of the ADH mRNA is from left to right. Fine structure genetic mapping within and around the *Adh* locus and protein sequencing of *Adh* null and electrophoretic variants has established the orientation of the ADH protein coding sequences on the *Drosophila* second chromosome (11). Together, the physical and genetic data orient the cloned DNA with respect to the second chromosome centromere. Thus, the centromere lies to the left of this figure with *outspread wings* (*osp*) being the closest proximal genetic locus. The telomere lies to the right, with *no ocelli* (*noc*) being the closest distal genetic locus. The physical distances of these adjacent genetic loci to *Adh* is not known.

quences, DNA was prepared from each clone, digested with *Eco*RI, fractionated on two agarose gels, and transferred to nitrocellulose filters (18). The duplicate filters were hybridized with cDNA prepared from total RNA of a wild-type strain (Oregon R) or a strain genetically deficient for ADH protein ($Adh^{\text{n2}}/Sco^{\text{R+4}}$). The *Adh* deficiency strain is heterozygous for two different deletion chromosomes that overlap in the *Adh* region. By cytogenetic analysis these flies appear to be lacking the *Adh* locus (4). DNA from one phage, gAC1, hybridized to cDNA from the wild-type strain but not to cDNA from the *Adh* deficiency strain (Fig. 1).

Three experiments were carried out to confirm that gAC1 contains the *Adh* structural gene (Fig. 2). First, ^{3}H-labeled gAC1 DNA was hybridized *in situ* to salivary gland polytene chromosomes (25). Hybridization was observed at 35 A-B only, which agrees with the cytogenetic localization of *Adh* (4). Second, total adult *Drosophila* RNA was hybridized to nitrocellulose filters containing DNA from gAC1 or a *Drosophila* actin gene (26, 28). The RNA that hybridized was eluted, and translated *in vitro*, and the products were immunoadsorbed and fractionated by NaDodSO$_4$/polyacrylamide gel electrophoresis. ADH was translated only from RNA derived from the gAC1 DNA filter. Third, the nucleotide sequence of a region of the cloned DNA that hybridized to ADH-enriched mRNA was determined (27). As shown in Fig. 2, this DNA sequence agreed with the ADH protein sequence derived by Thatcher (3). In addition, C. Benyajati and W. Sofer (personal communication) have isolated and characterized an *Adh* cDNA clone and have determined that the cDNA clone hybridizes specifically to gAC1.

Characterization of *Adh* Genomic DNA. To characterize *Adh* genomic DNA, a number of different *Adh* clones and subclones were prepared and a map of their restriction sites was derived (Fig. 3). Southern blots (18) of various restriction enzyme digests of *Drosophila* DNA were probed with *in vitro*-labeled DNA from a number of different *Adh* clones. The *Adh* gene and its flanking sequences were present only once in the *Drosophila* genome, and no detectable rearrangements occurred during cloning.

Comparisons of the restriction maps of gAC1, 2, 3 (from

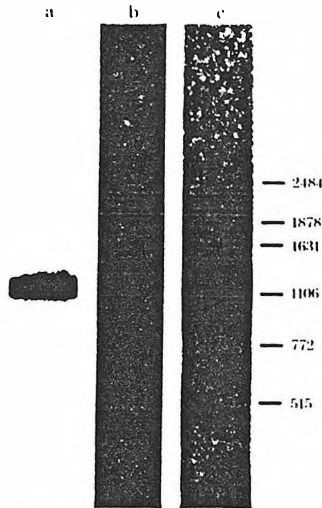


FIG. 4. Hybridization of cloned *Adh* DNA to total *Drosophila* adult RNA. Four micrograms of total adult *Drosophila* RNA was run in each lane of a 1% agarose formaldehyde gel (29). The electrophoresis buffer was 40 mM morpholinopropanesulfonic acid, pH 7.2, 10 mM Na acetate/1 mM EDTA. The gel contained 6% formaldehyde. Samples were heated at 55°C for 30 min in gel buffer containing 50% dimethyl sulfoxide and 6% formaldehyde before they were loaded onto the gel. After the run, the gel was washed three times with an excess of H_2O and then twice with 0.2 M ammonium acetate; the second wash contained ethidium bromide (0.5 μg/ml). After photography of unlabeled DNA size markers under short-wave UV illumination, the gel was soaked in 1.5 M NaCl/0.15 M Na citrate and the RNA was transferred to nitrocellulose filter paper. After transfer, the filter was baked for 3 hr at 80°C under reduced pressure and then hybridized with labeled DNA essentially as described (30). Lanes a, b, and c are total adult RNA hybridized with nick-translated gAS1, gAC2, and gAC3, respectively. (Transfer of RNA to nitrocellulose is an unpublished observation of E. Butler, B. Seed, and D. Goldberg.)

Canton S, an Adh^S strain), sAS1 (from Schwenk Forest, an Adh^S strain), and sAF2 (from an Adh^F strain) revealed very few differences. However, sAF2 contains a 200-base-pair insertion relative to both sAS1 and gAC2. It is not known whether this represents a deletion in the Adh^S strains or the insertion of DNA in Adh^F strains from elsewhere in the genome. This insertion has been mapped between the *Pvu* II and *Sal* I sites at 12.2 and 12.9 kb on the map of Fig. 3. This lies 3′ to the mRNA coding sequences, and its effects, if any, on ADH expression are unknown.

Transcription Mapping of the *Adh* Gene. There appears to be only one major adult RNA transcript originating from the region encompassed by the cloned *Adh* region. Three clones (gAC2, gAC3, sAS1) spanning approximately 21 kb were used to probe total adult *Drosophila* RNA which was run on denaturing formaldehyde agarose gels and transferred to nitrocellulose (Fig. 4). Only one hybridizing species, 1100–1150 nt, was detected. Because only 760 nt are required to code for the ADH protein, the ADH mRNA contains approximately 400 nt of noncoding sequences including poly(A). This experiment does not rule out low-abundance adult transcripts or transcripts produced at other stages of development.

To map the transcribed regions of the *Adh* clones, labeled cDNA prepared from adult poly(A)$^+$ RNA was used to probe blots of restriction digests of different cloned DNAs spanning the *Adh* region. The region of hybridization is shown in Fig. 3. The sequence of DNA surrounding the *Bam*HI site was determined (Fig. 2). This sequence corresponds to the amino acid sequence near the center of the protein and establishes the orientation of 5′- to -3′ transcription as left to right on the map in Fig. 3.

To determine whether the *Adh* gene is interrupted by intervening sequences, S1 nuclease mapping studies were performed using either linearized sAS1 plasmid DNA or a subclone (mAC1-3) of the 4.75-kb *Eco*RI fragment in the single-strand phage vector M13mp2 (14). The results of two mapping experiments are shown in Fig. 5. In both experiments RNA was hybridized to DNA, and the resultant hybrid was digested with S1 nuclease. The digested nucleic acid was run on a two-dimensional gel system, nondenaturing in the first dimension and alkaline denaturing in the second dimension. Migration in the nondenaturing dimension indicates the total hybrid length [mRNA less poly(A)]; migration in the denaturing dimension indicates the sizes of DNA fragments left intact by the digestion. Spots off the diagonal indicate the presence of intervening sequences in the genomic DNA. No significant hybridization was seen in control experiments with tRNA hybridized to mAC1-3 DNA or sAS1 DNA (data not shown). With adult RNA hybridized to mAC1-3 DNA (*A*) or sAS1 DNA (*B*), two major

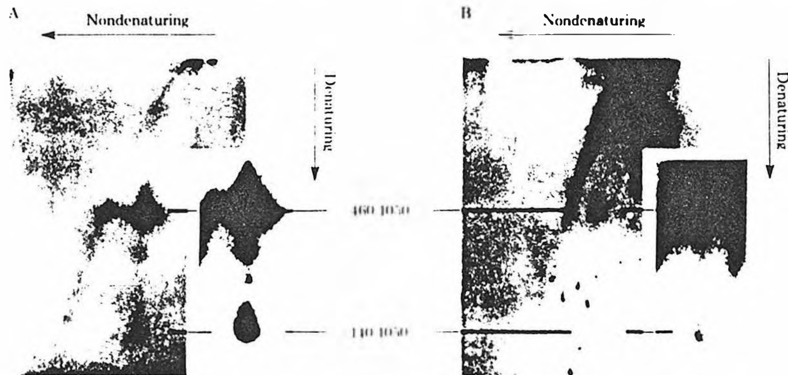


FIG. 5. S1 nuclease mapping analysis of ADH mRNA/genomic DNA hybrids. Each spot is identified by two numbers separated by a slash. The first number refers to the spot mobility in the alkaline denaturing dimension, and the second number refers to the mobility in the neutral dimension. (*A*) mAC1-3 DNA (100 ng) hybridized to 5 μg of polyadenylylated adult RNA. (*B*) sAS1 DNA (100 ng) cleaved with *Bgl* II and hybridized with 5 μg of polyadenylylated adult RNA. The minor spots in *A* have not been explained.

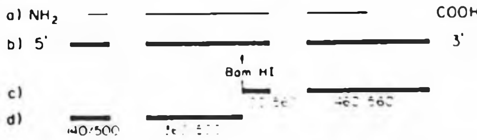


FIG. 6. Map of the *Adh* exons. Line a, approximate position of the regions of ADH mRNA coding for ADH protein. Line b, organization of exons within the genomic DNA. The relative positions of the intervening sequences are shown as blank spaces, although the size of the space is *not* indicative of the length of the intervening sequence. Lines c and d, interpretation of the results of the S1 nuclease mapping experiments performed with isolated *Bam*HI fragments of sAC1. These experiments were performed with the *Bam*HI fragment covering the 3' end (line c) and the 5' end (line d) of the *Adh* gene. Each DNA fragment is denoted by the spot it was associated with in the actual experiment according to the nomenclature of Fig. 5.

spots corresponding to 140 and 460 nt were observed in the second dimension. Both spots were derived from a 1050-nt RNA as determined from the first-dimension mobility. This size is the same as that determined for ADH RNA (Fig. 4). The 460- and 140-nt spots, which correspond to exons of ADH RNA, cannot account for the entire mRNA length of 1100–1150 nt.

In order to position the exon sequence on the restriction map of Fig. 3, S1 nuclease mapping experiments were performed with purified *Bam*HI fragments of sAC1 (Fig. 6). With the *Bam*HI fragment spanning the 5' end of the RNA, spots corresponding to species 140/500 and 360/500 were observed (data not shown; see Fig. 5 for spot nomenclature). With the *Bam*HI fragment spanning the 3' end of the RNA, spot 100/560 was observed. The expected complementary fragment 450/560 would be obscured in this experiment by hybridizing sequences along the diagonal. These data suggest that *Bam*HI cuts a 460-nt exon into 360- and 100-nt fragments (5' and 3', respectively). This interpretation assumes that the 460-nt spot found in S1 nuclease mapping experiments with mAC1-3 and sAS1 is composed of two fragments of similar size. In some experiments, two spots have been observed in the region of the gel corresponding to 460 nt.

The presence of an intervening sequence located 100 nucleotides 3' to the *Bam*HI site was confirmed by comparing the location of restriction sites mapped on the cloned DNA with the predicted restriction sites determined from the amino acid sequence. Fine structure restriction endonuclease mapping studies revealed *Hpa* II and *Hae* III sites that are inconsistent with the amino acid sequence 165, 180, and 204 nt from the *Bam*HI site. Thus, an intervening sequence is located less than 165 nt from the *Bam*HI site. C. Benyajati and W. Sofer have determined sequences of regions from an *Adh* genomic clone and have found intervening sequences in positions consistent with the S1 nuclease mapping experiments presented in this paper (personal communication).

Hybridization with complex cDNA probes including or lacking *Adh* sequences was used to identify a clone containing the *Drosophila Adh* gene. Similar approaches have been used by others (31, 32) using cDNAs prepared from mRNA populations from cells of different developmental or physiological states. In this study, mutant flies lacking the *Adh* gene were constructed genetically. Only a small genetic region was deleted in the mutant flies relative to the wild-type flies, conferring fine selectivity to the screen. Additionally, single-copy *Adh* sequences can be detected by hybridizing nick-translated total *Drosophila* DNA to nitrocellulose filters containing cloned *Adh* DNA (unpublished observations). Thus, it may be possible to use differential screens to identify clones from various genetic loci at which homozygous viable deficiencies have been isolated, such as the *white* locus and the *dunce* locus (cyclic AMP phosphodiesterase).

There are more people who helped in various aspects of this work than can be acknowledged in this space. Special thanks go to Brian Seed and Randy Smith for their trenchant criticism and humor, Mark Silver for his inimitable technical skills, Jennie Cortenbach for preparing media, and Tip Benyajati, Bill Sofer, Mike Ashburner, and Bob Karp for making *Adh* a pleasant system to work on. Tom Maniatis, in particular, helped in getting me started, getting me thinking, and getting me space and money (a National Institutes of Health Program Project Grant and a National Science Foundation Predoctoral Fellowship).

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Chapter 2

Correct Developmental Expression of a Cloned Alcohol Dehydrogenase Gene Transduced into the *Drosophila* Germ Line

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Summary

We have used P-element-mediated transformation to introduce a cloned Drosophila alcohol dehydrogenase (*Adh*) gene into the germ line of ADH null flies. Six independent transformants expressing ADH were identified by their acquired resistance to ethanol. Each transformant carries a single copy of the cloned *Adh* gene in a different chromosomal location. Four of the six transformant lines exhibit normal *Adh* expression by the following criteria: quantitative levels of ADH enzyme activity in larvae and adults; qualitative tissue specificity; the size of stable *Adh* mRNA; and the characteristic developmental switch in utilization of two different *Adh* promoters. The remaining two transformants express ADH enzyme activity with the correct tissue specificity, but at a lower level than wild type. These results demonstrate that an 11.8 kb chromosomal fragment containing the *Adh* gene includes the *cis*-acting sequences necessary for its correct developmental expression, and that a variety of chromosomal sites permit proper *Adh* gene function.

Introduction

The Drosophila alcohol dehydrogenase (*Adh*) gene provides a well-characterized example of developmentally regulated gene expression. ADH activity is limited to certain tissues and displays characteristic variations during development. The enzyme can be detected in tissues such as larval and adult fat body and midgut, but is not present in many other tissues, such as imaginal discs (Ursprung et al., 1970). In addition, the level of ADH expression appears to be modulated between tissues. For instance, ADH is present in high specific activities in the larval fat body, and at lower activities in larval midgut and Malpighian tubules (Ursprung et al., 1970). Furthermore, the distribution of ADH activity within certain organs is not uniform. For example, ADH is observed in the anterior and posterior larval midgut, whereas it is absent in foregut, middle midgut, and hindgut.

The isolation and characterization of the *Adh* gene (Goldberg, 1980) and *Adh* cDNA clones (Benyajati et al., 1980) and subsequent transcriptional mapping studies have provided insights into the temporal regulation of *Adh* gene expression. Two different *Adh* mRNAs which are transcribed from different promoters are detected during Drosophila development (Benyajati et al., 1983b). One mRNA is expressed primarily in larval tissues, the other in adult tissues. The "larval" mRNA is synthesized from a precursor containing two introns internal to the ADH protein coding sequence, whereas the "adult" mRNA precursor contains an additional intron in the 5′ noncoding region (see Figure 5A). This additional intron apparently has no function as the ADH proteins made from the two mRNAs are identical (Thatcher, 1980; Benyajati et al., 1983b). The temporal restriction of promoter utilization is not absolute; "adult" mRNA is observed in third instar larvae. This "adult" RNA is present in the larval fat body, whereas the "larval" RNA is present in both larval fat body and midgut (Benyajati et al., 1983b).

An understanding of the mechanisms of *Adh* gene regulation will require the identification and characterization of both *cis*- and *trans*-acting control elements. Such elements were first identified in bacterial systems by genetic studies. Extensive genetic analysis of the *Adh* locus has led to the isolation of a large number of ADH null mutations (Grell et al., 1968; O'Donnell et al., 1975; Ashburner et al., 1982). Characterization of many of these mutants has revealed interesting defects in translation and splicing, but no *cis*-acting mutations that alter developmental regulation (Schwartz and Sofer, 1976; Kubli et al., 1982; Benyajati et al., 1982).

An alternative approach to the functional characterization of DNA sequences adjacent to the *Adh* structural gene is to introduce mutations into the cloned gene in vitro and then examine the functional consequences of these alterations in an appropriate expression assay. Such an approach would theoretically allow the investigation of the effects of mutations in every nucleotide singly or in combination, but it requires a suitable assay for developmental gene expression. While procedures for site-directed mutagenesis are now well-established, it has not been possible until recently to develop expression systems in higher eucaryotes that allow cloned eucaryotic genes to be expressed in a manner which is quantitatively and qualitatively indistinguishable from that of the natural chromosomal gene.

The development of P element-mediated transformation of Drosophila with cloned DNA (Spradling and Rubin, 1982; Rubin and Spradling, 1982) provides the opportunity for assaying the effects of mutations in the *Adh* gene by directly introducing the mutated genes into the Drosophila germ line. (We will refer to genes introduced by this method as "transduced" genes in order to indicate explicitly that the gene integration was mediated by a biologically active element.) Here we examine the expression of a cloned 11.8 kb region containing the *Adh* gene which has been introduced into the germ line of genetically ADH null embryos. Transformants were selected on the basis of their acquired resistance to ethanol. In most cases, the transduced *Adh* gene is expressed in the same tissue-specific manner as the endogenous gene, and at roughly the same levels. In addition, the promoter utilization at different stages is indistinguishable from the normal *Adh* gene.

Results

Construction of *Adh*-P Element Hybrid Plasmids

Adh-P element hybrid plasmids were constructed by inserting an 11.8 kb Sac I fragment containing the *Adh* gene (Goldberg, 1980) into the unique Sac I site within the P element boundaries of the plasmid pπ25.1 (O'Hare and Rubin, 1983; see Figure 1). The Sac I fragment carries the *Adh'* allele, which encodes a high-activity, fast electrophoretic variant of ADH. As shown in Figure 1, the *Adh'* gene is flanked by about 6 kb of genomic DNA on the 5' side and about 4 kb on the 3' side. Two hybrid plasmids, pAP-2 and pAP-5, representing both orientations of the *Adh* gene fragment within the P element, were used in these studies.

Transformation of ADH Null Embryos

Homozygous ADH null mutants are viable when grown on medium lacking ethanol. It is therefore possible to microinject ADH null embryos with a cloned *Adh* gene, grow them under nonselective conditions, and then impose an ethanol selection (Vigue and Sofer, 1976) on the progeny of injected flies to detect germ-line transformants. In this study, we used *Adh*^{fn6} *pr cn* as the recipient strain. The *Adh*^{fn6} allele contains a 34 bp deletion in the third coding exon of the gene (Benyajati et al., 1983a). The mutant gene produces nearly normal amounts of stable *Adh* mRNA, which is translated to yield a protein that is cross-reactive with anti-ADH antibody but is completely inactive as an enzyme (Benyajati et al., 1982).

Embryos (G0 embryos) of this strain were injected prior to pole cell formation with a mixture of the plasmids pAP-2, pAP-5, and pπ25.1 (see Experimental Procedures). Adult flies (G0 adults) that developed from the injected embryos were mated in individual vials to flies of the recipient strain. The adult progeny (G1 adults) of each mating were then tested for resistance to 6% ethanol, which is lethal to ADH null flies. Resistant G1 flies were used to construct homozygous or balanced stocks, as described in Experimental Procedures. Six independent transformants (designated tAP-1 to tAP-6) were identified and established as stocks in these experiments.

Location and Structure of the Transduced DNA

An analysis of the genetic segregation of the ethanol resistance phenotype was carried out in order to identify which chromosome(s) in each transformed line carries a functional *Adh* gene (or genes). The results are shown in Table 1. In each transformant, a single chromosome was found to carry the trait: the X chromosome in two cases, the second chromosome in three cases, and the third chromosome in one case.

The location of the transduced DNA sequences with respect to the cytological map of Drosophila salivary gland polytene chromosomes was determined by in situ hybridization with an *Adh* gene probe. In each transformed line only a single site of hybridization was observed in addition

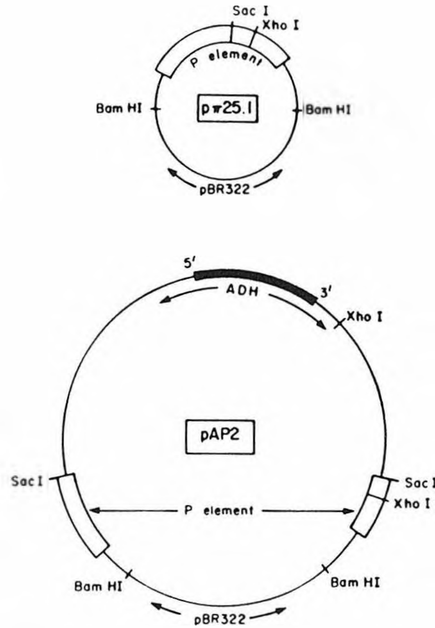


Figure 1. Diagram of Plasmids Used for Transformation of Drosophila Embryos with the *Adh* Gene

Top: pπ25.1 (Spradling and Rubin, 1982; O'Hare and Rubin, 1983), which carries an intact P element. Bottom: pAP-2, one of two plasmids constructed by inserting an 11.8 kb Sac I restriction fragment containing the wild-type *Adh'* gene (Goldberg, 1980) into the unique Sac I site of pπ25.1 (top). The second plasmid constructed in this way, pAP-5 (not shown), differs in the orientation of the *Adh* gene fragment with respect to the P element. The two orientations are conveniently distinguished by digestion with Xho I, which has one site in the P element and one (asymmetrically placed) in the *Adh* fragment. A mixture of all three plasmids was used in the transformation experiments.

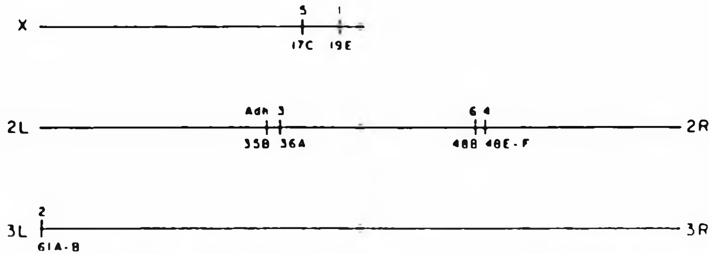
to the endogenous gene (Table 1). These results are in complete agreement with the genetic segregation analysis.

The integrated *Adh* DNA sequences were studied in detail by Southern blot analysis of genomic DNA from transformed flies. An example of the results is shown in Figure 2. Hybridization of a suitable probe to genomic DNA digested with Xho I (which cuts only once within the 11.8 kb *Adh* gene fragment) indicated the presence of a single integrated *Adh* gene in each transformant, in addition to the endogenous gene. The same result was observed in genomic DNA digested with Bam HI, which likewise cuts once within the *Adh* fragment (data not shown). The conclusion from this analysis, the in situ hybridization, and the genetic segregation of ethanol resistance is that each transformed line carries a single functional *Adh* gene; no nonfunctional transduced copies were detected.

Digestion of the genomic DNA with Xho I permitted a

Table 1. Characteristics of Integrated *Adh* Gene P Element Transposons

Transformant[a]	Chromosomal[b] Segregation	Cytological[c] Location	Number of[d] Integrated Gene Copies	Orientation[e] of *Adh* Gene within P Element
tAP 1	X	19E	1	A
2	3	61A-B	1	A
3	2	36A	1	A
4	2	48E-F	1	A
5	X	17C	1	B
6	2	48B	1	A



[a] Transformant strains are designated by tAP (for transformant *Adh* P) followed by a number.
[b] Chromosomal segregation of ethanol resistance phenotype (see Experimental Procedures).
[c] Determined by in situ hybridization to salivary gland polytene chromosomes (see Experimental Procedures). See diagram above. In the diagram, horizontal lines represent chromosome arms, solid dots represent centromeres. The number (1-6) above each site is the number of the corresponding transformant line. The location of the endogenous *Adh* gene is also indicated. The designation below each site is the cytological location according to the standard Bridges map of Drosophila salivary gland polytene chromosomes (Lefevre, 1976).
[d] Per haploid genome, determined by Southern blot analysis of genomic DNA and by in situ hybridization to salivary gland polytene chromosomes (see Experimental Procedures and Figure 2).
[e] Determined by Southern blot analysis of genomic DNA (see Experimental Procedures and Figure 2). Orientation A is that found in the plasmid pAP 5, B is that found in pAP 2 (see Figure 1).

determination of the orientation of the *Adh* gene with respect to the P element in each transformant (Figure 2). Five of the transformants carry the transduced *Adh* gene oriented as in the plasmid pAP 5, while only one (tAP 5) carries the gene in the other orientation (Table 1 and Figure 2). Other restriction digests of genomic DNA were used to detect possible rearrangements of the integrated DNA, but none were observed. For example, as shown in Figure 2, Eco RI digestion yields only the 4.7 kb fragment known to include the ADH coding region in both the recipient strain and donor DNAs (Goldberg, 1980).

Expression of the P-element-transduced *Adh* Genes

The genomic DNA blotting and in situ hybridization experiments described above demonstrate the presence of P element transduced *Adh* DNA sequences and their chromosomal locations in the transformed flies. In addition, the ability of these flies to survive exposure to 6% ethanol indicates that the transduced *Adh* genes are expressed at a level that is adequate to detoxify the ethanol. Here, we examine the quantitative and tissue-specific expression of the transduced *Adh* genes, and the developmental switch in utilization of the larval and adult promoters. As indicated by the data presented below, the expression of most of the transduced *Adh* genes is indistinguishable from that of the endogenous *Adh* gene by all criteria examined.

ADH Enzyme Activity

In order to compare the level of expression of transduced *Adh* genes with a wild type, endogenous *Adh* gene, each of the transformed strains was mated with a wild-type *Adh* strain. The ADH homodimer made by this strain can be distinguished electrophoretically from the ADH homodimer produced by the *Adh* strain from which the transduced *Adh* gene was derived (the recipient *Adh* strain produces a shortened ADH protein that has no detectable activity and which does not participate in ADH heterodimer formation; W. Sofer, personal communication). Larval and adult extracts from the resulting heterozygotes were prepared and electrophoresed on polyacrylamide gels, and the electrophoretic mobilities and amounts of ADH were displayed by treating the gels with a stain specific for ADH activity. The *Adh* protein serves as a control for variations

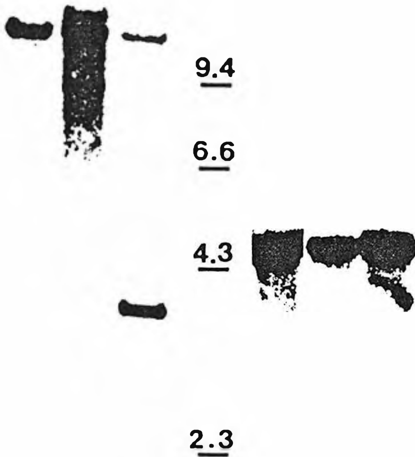


Figure 2 Southern Blot Analysis of Genomic DNAs

Genomic DNA from adult flies of the recipient strain (R) and two transformant lines (tAP 3 and tAP 5) was digested with the indicated restriction enzyme and electrophoresed on 0.7% agarose gels. After transfer to nitrocellulose, the DNA was hybridized with the following probes: Xho I digest — a 300 bp Bgl II fragment from the extreme 3' end of the 11.8 kb Sac I *Adh* gene fragment in the plasmid sAF2 (see Goldberg, 1980). Eco RI digest — a 160 bp Hind III fragment from the adult intron of *Adh* (Benyajati et al., 1983b). Size scale shows the positions of marker bands in a Hind III digest of bacteriophage λ DNA, run in a parallel lane. The appearance of the 3.7 kb band in the Xho I digest of tAP 5 DNA indicates that the orientation of the *Adh* gene fragment with respect to the P element in the transformant is the same as that in the plasmid pAP 2 (see Figure 1). The band has one terminus in the *Adh* Sac I fragment and the other in the P element (O'Hare and Rubin, 1983). In the other orientation, the corresponding band has a minimum size of 5.1 kb, with one terminus in the *Adh* gene fragment and one in the flanking chromosomal DNA.

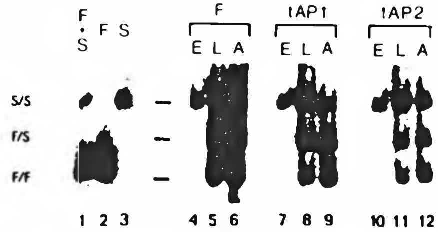


Figure 3. Electrophoretic Analysis of ADH Expression in Transformed Flies

Adh^S females were mated with males from strains Adh^f, tAP 1, and tAP 2. Protein extracts from 6–18 hr embryos, late third instar larvae, and 5–8 day old adults were prepared and electrophoresed on polyacrylamide gels which were stained for ADH activity. The mobilities of the Adh^S and Adh^f homodimers and Adh^S/Adh^f heterodimers are indicated at the left of the figure. Lane 1: extracts from homozygous Adh^S and Adh^f strains were mixed, and incubated for 30 min on ice before electrophoresis. Lane 2: extract prepared from a homozygous Adh^f strain. Lane 3: extract prepared from a homozygous Adh^S strain. Lanes 4–12 show the patterns observed in extracts from the Adh^S crosses. In each series (S/F, S/tAP 1, S/tAP 2) the results are shown for extracts from embryos (E), larvae (L), and adults (A).

in ADH activity that are the result of environmental or genetic factors.

A typical result of this analysis is presented in Figure 3. Lane 1 shows that when Adh^S and Adh^f extracts are mixed, S/F heterodimers do not form. Lanes 2 and 3 display the electrophoretic mobility of ADH homodimers obtained from extracts prepared from Adh^f (F) or Adh^S (S) extracts. Lanes 4–12 show the electrophoretic patterns of extracts obtained from matings of Adh^S females with Adh^f, tAP 1, and tAP 2 transformants. Bands corresponding to F homodimers are observed in all three matings, demonstrating that both transformants express ADH protein with the F electrophoretic mobility characteristic of the injected cloned DNA. In addition, bands corresponding to S/F heterodimers are present in larval and adult extracts, indicating that both S and F proteins are produced in the same cells, because heterodimers cannot be formed by mixing extracts from homozygous Adh^S and Adh^f strains (Figure 3, lane 1). In both control Adh^f and transformant tAP crosses, Adh^S protein, but not Adh^f protein, is detected in the embryo (Figure 3, lanes 4, 7, 10). ADH protein detected in embryos is maternally derived (D. A. G., unpublished data).

Comparison of the levels of ADH activity in the homo- and heterodimer bands between different crosses and between larvae and adults of the same cross shows that in most of the six transformants, ADH is expressed at roughly equivalent levels to the Adh^f strain from which the transforming DNA was originally obtained (Figure 3 and data not shown). In addition, the relative levels of Adh^f and Adh^S protein within each transformed line is the same in larval and adult extracts in all three crosses shown in Figure 3. A lower level of Adh^f protein was detected in tAP 4 larvae and tAP 6 adults.

A more quantitative estimate of ADH expression was

Table 2 ADH specific Activity in Transformed Strains

Strain	Larval Male	Larval Female	Adult Male	Adult Female
Adh[f]	1.00 (± .11)[a]	1.00 (± .17)[a]	1.00 (± .06)[a]	1.00 (± .07)[a]
tAP 1	.92 (± .20)	.70 (± .18)	.85 (± .15)	1.04 (± .15)
tAP 2	.55 (± .01)[b]	.73 (± .27)	1.04 (± .08)	.89 (± .12)
tAP 3	1.14 (± .19)	.55 (± .08)	.84 (± .12)[a]	1.24 (± .17)[a]
tAP 4[c]	.19 (± .03)[b]	.10 (± .03)[b]	.65 (± .05)[ab]	.88 (± .01)[a]
tAP 5	1.27 (± .05)[a]	1.14 (± .14)[a]	.80 (± .30)[a]	.97 (± .04)[a]
tAP 6	.61 (± .18)	.41 (± .09)	.22 (± .03)[ab]	.28 (± .05)[ab]

Protein extracts were prepared from larval and adult males and females of control and transformed strains, and ADH-specific activity (units per milligram protein per ADH gene) was determined and normalized to the amounts of protein made in *Adh*[f] flies of the particular sex and developmental stage noted. Each number represents the average of normalized specific activity measurements from two extracts (protein and activity measurements were performed twice on each extract), and the spread between the two relative specific activities is indicated in parenthesis.

[a] This developmental stage of the indicated strain shows no dosage compensation with a t test probability of ≤ .1. Calculated by comparing the female values with one half the male values.

[b] These values are distinct from the *Adh*[f] value of the same sex and developmental stage with a Student's t test probability of ≤ .05.

[c] Because the lethal period of the tAP 4-bearing chromosome is not known, the genotypes of these larvae were uncertain (see Experimental Procedures).

obtained by preparing protein extracts from both male and female larvae and adults of each of the transformants, and determining the specific activity of ADH. The results of this analysis are presented in Table 2 as the specific activities per gene of the transformed *Adh* strains relative to the wild-type *Adh*[f] strain of the same sex and developmental stage. In most cases, the transduced *Adh* gene is expressed in both males and females at levels that cannot be distinguished from wild type by standard statistical methods (Student's t test). The exceptions are noted in Table 2. Of particular interest are tAP-4, in which larval expression is reduced about 5–10 times from wild-type levels, and tAP-6, in which adult expression is down approximately 4 times. We do not know the reason for the specific reduction in tAP-4 larval and tAP-6 adult ADH expression. We note that the chromosome carrying the transduced *Adh* gene in the tAP-4 strain carries a lethal mutation that could be associated with the integration of the *Adh*–*P* element transposon.

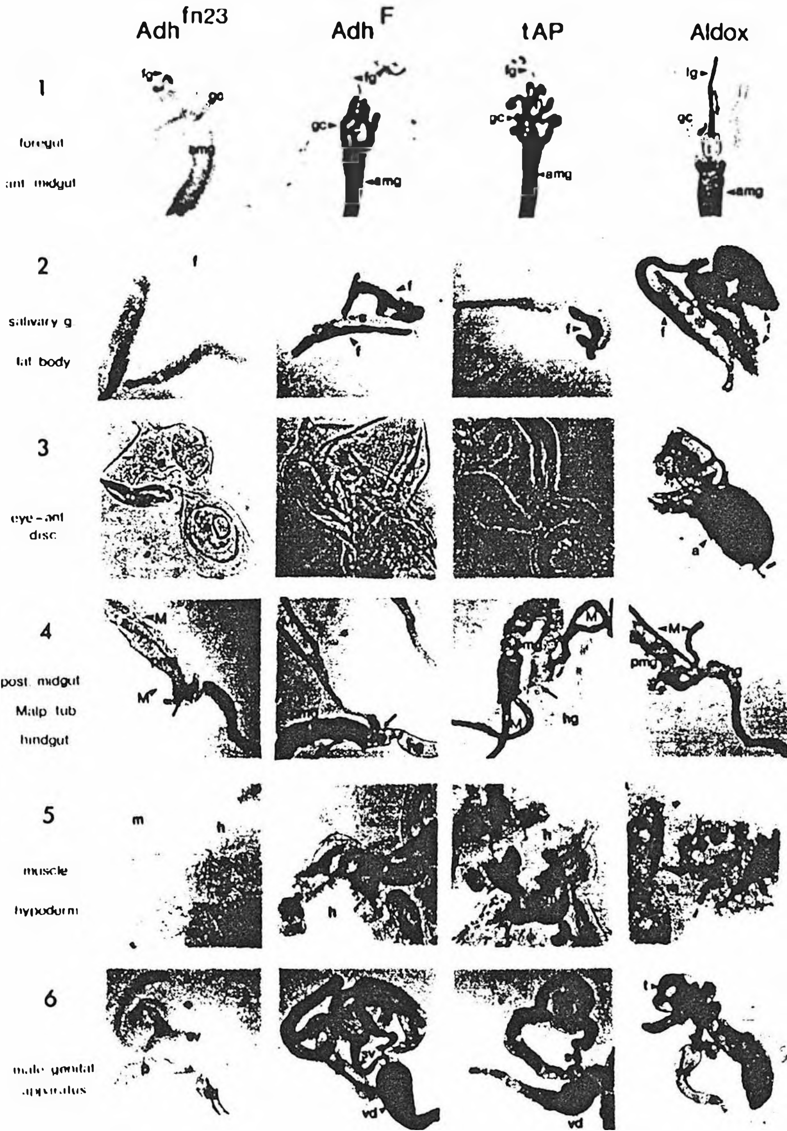
Genes located on the X chromosome are normally regulated so that males and females show equivalent amounts of gene product, even though the gene dosage in females is twice that in males. This regulatory phenomenon is called dosage compensation (reviewed in Lucchesi, 1978). Because the *Adh* gene in transformants tAP-1 and tAP-5 is located on the X chromosome, if these genes were subject to normal X-chromosome dosage compensation, the level of their expression in males would be twice that observed in females of the same strain (on a per gene basis). However, we find that ADH levels in females of the tAP-1 and tAP-5 transformants are approximately twice that in males, or equivalent on a per gene basis (Table 2). Thus these transformants do not appear to display complete (2-fold) dosage compensation, though the data are not statistically significant in this regard (confidence levels of 0.85 and 0.94 for tAP-1 and tAP-5, respectively). Whether tAP-1 and tAP-5 exhibit partial dosage compensation, such as observed by Spradling and Rubin (1983) for several *rosy*[+] transformants and by Scholnick et al. (1983) for a dopa decarboxylase transformant, cannot be determined from these data.

Tissue-specific Expression of ADH

In wild-type flies, ADH activity is found in certain tissues and not in others, as determined both by histochemical staining of whole organs and by measurement of ADH activity in extracts of dissected tissues (Ursprung et al., 1970; our unpublished data). These techniques have revealed that the major sites of ADH activity are the larval and adult midguts, fat bodies, and Malpighian tubules, although substantial activity is found in larval somatic muscles, adult hindgut, some visceral muscles, genital disc derivatives, and the crop. No activity, however, can be detected in larval hypoderm, imaginal discs, trachea, foregut, hindgut, salivary glands, germ cells, or adult somatic muscles.

We used histochemical staining of intact organs to determine the tissue-specific distribution of ADH activity. This technique is advantageous because it is sensitive and has a high degree of spatial resolution. In contrast, determination of ADH specific activity from extracts of dissected tissues is relatively insensitive, and gives information only about ADH activity in an organ as a whole. The primary disadvantage of histochemical staining is that it does not readily allow comparison of relative ADH activities between different organs because it is not a quantitative assay.

The pattern of histochemical staining of wild-type *Adh*[f], ADH null, and transformed strains is presented in Figure 4 and Table 3. We also examined the histochemical staining of organs with the same coupled dye system used for ADH staining, using acetaldehyde rather than secondary butanol as the substrate. The acetaldehyde allows the detection of the enzyme aldehyde oxidase (Aldox), which has a very different tissue-specific distribution from ADH



Adh fn23
Adh F
tAP
Aldox
1
foregut
ant. midgut
fg
gc
amg
lg
2
salivary g.
fat body
f
3
eye-ant
disc
a
4
post. midgut
Malp. tub
hindgut
M
pmg
hg
5
muscle
hypoderm
m
h
6
male genital
apparatus
sv
vd
t

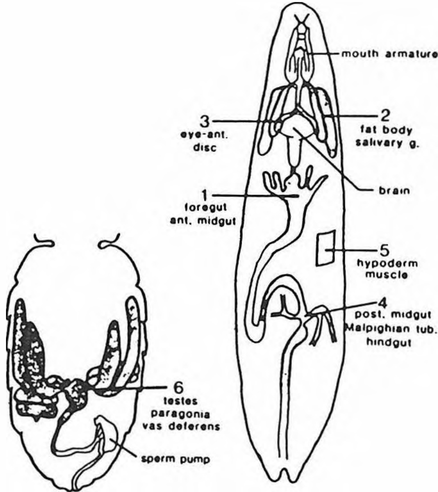


Figure 4. Histochemical Staining Patterns of Transformed and Control Strains

Preparations of tissues from different Drosophila strains treated with histochemical stains specific for ADH or aldehyde oxidase (Aldox). The deposition of a purple formazan dye is indicative of the presence of the respective dehydrogenase (ADH or Aldox). In the leftmost column, preparations from the recipient strain Adh^{fn23} *pr cn* stained with an ADH specific stain are presented. The next column shows the staining of tissues from Adh^f, the strain from which the transforming ADH gene was obtained. The third column shows the staining from transformants IAP 1 or IAP 3. The rightmost column shows the patterns obtained from Adh^{fn23} *pr cn* larvae treated with a stain specific for Aldox, which differs from the ADH specific stain only in the substitution of the substrate acetaldehyde for sec-butanol. The results of the Aldox staining are similar to those reported earlier by Dickinson (1971).

The results obtained for different tissues are given in succeeding rows and are discussed below. As a reference, the black and white composite presents the approximate locations and sizes of the tissues described and is keyed by the numbers in the color figure. The embryological origins of these organs was described by Poulson (1950). The differences in sizes between analogous tissues in the different boxes of a given row reflects either slight differences in the age of the larvae, environmental factors, or the strains used. A more complete listing of the results of the histochemical staining analysis is presented in Table 3.

(1) Larval foregut and anterior midgut (63×). This row shows the staining of anterior larval gut structures, including the anterior midgut (amg) and the foregut (fg). These gut structures are of separate embryonic origins. The gastric cecae (gc) are diverticula emanating from the anterior midgut. The proventriculus, seen most clearly in the Adh^{fn23} and Aldox boxes, is a bulb connecting the foregut and the anterior midgut. It is thought to be of partly anterior midgut and partly foregut origin. There is no staining of gut tissue evidence in the Adh^{fn23} preparation. The staining cells at the crown of the proventriculus are of unknown origin. In the Adh^f and IAP preparations, the anterior midgut and gastric cecae are stained heavily, the foregut is unstained, and the proventriculus is mostly unstained, except for a circumferential band approximately three cells wide and located midway along its length. In certain specimens of both Adh^f and IAP the gastric cecae are stained lightly if at all, especially in immature larvae. In the Aldox preparation, the foregut and anterior midgut are heavily stained, and the gastric cecae and proventriculus are unstained. Individual anterior midgut cells are easily resolved in this figure. In Aldox preparations, the gastric cecae are never stained.

(2) Larval salivary gland and fat body (63×). This figure shows the larval salivary gland (s) and attached fat body (f). There are considerable amounts of additional fat body in the larvae other than shown here, though these results are characteristic of the entire larval fat body. In Adh^{fn23} larvae, no staining of either the salivary gland or the fat body is observed. In Adh^f and IAP preparations, heavy staining of the fat body is seen, while the salivary gland is unstained. In Aldox preparations, heavy staining of the fat body is accompanied by very light staining of the salivary gland. In all the preparations shown, individual salivary gland cells are resolved.

(3) Eye-antennal disc (160×). The eye-antennal disc is divided into two parts, roughly corresponding to those parts giving rise to eye (e) or antennal (a) structures. The eye specifying region is relatively flat, whereas the antennal region is considerably folded. No staining is seen in Adh^{fn23}, Adh^f, or IAP preparations of eye-antennal or other imaginal discs. In Aldox preparations, heavy staining of only the antennal region is seen. Many other imaginal discs are stained.

(4) Larval posterior midgut, Malpighian tubules, and hindgut (63×). This row shows staining in the larval posterior midgut (pmg), Malpighian tubules (M), and hindgut (hg). Each of these tissues are of separate embryonic origin. The Malpighian tubules insert into the gut approximately at the junction of the posterior midgut and hindgut (shown by arrow with tail). No staining is seen in the Adh^{fn23} tissue. Adh^f and IAP preparations show no staining of the hindgut, and variable staining in the other tissues. For instance, in the IAP preparation shown, the posterior midgut is heavily stained, while the Malpighian tubules are lightly stained. In contrast, in the IAP preparation, the posterior midgut is variably stained along its length, and the Malpighian tubules are heavily stained. Variation of this type is commonly seen within larvae of the same strain, and we can discern no systematic differences between Adh^f and the transformed IAP strains. In Aldox preparations, the posterior midgut and Malpighian tubules are moderately stained, and the hindgut has a line of staining cells running down its length. Variability of Aldox staining in this region of the larva is also commonly seen.

(5) Larval hypoderm and somatic muscles (160×). These preparations of larval hypoderm (h) and somatic muscle (m) are obtained from the carcass of larva stripped of internal organs. Bands of somatic muscle line the carcass, whose inner surface contains hypodermal cells. Muscles are evidenced by their rippled appearance, and hypoderm by the presence of large nuclei. In the Adh^{fn23} preparations, no staining of either tissue is observed. In Adh^f and IAP preparations, staining of muscles but not hypoderm is evident. The staining of the muscles is variable, and appears most dramatically in muscles which are torn or damaged. This likely reflects a difficulty in penetration of the stain. In the Aldox preparations, hypoderm and not muscles is stained. Note the hypodermal nuclei surrounded by stained cytoplasm, which is seen underneath unstained muscle.

(6) Adult male genital apparatus (63×). This row demonstrates the staining of adult male genital tissues, composed of the vas deferens (vd), seminal vesicle (sv), paragonium (p), and testis (t) (the sperm pump is not shown). The vas deferens, seminal vesicle, and paragonium are derived from the genital imaginal disc. The testis is composed of germ cells as well as sheath cells, which are thought to arise as part of the mesoderm. The nomenclature is from Bodenstein (1950). In Adh^{fn23} individuals, no staining is observed. In Adh^f and IAP preparations, the vas deferens and seminal vesicle are heavily stained, and the paragonium and testes are unstained or very lightly stained. In Aldox preparations, the vas deferens is unstained, the seminal vesicle and paragonia are heavily stained, and the testis is variably stained. Specks of stain are often seen at the extremities of the Aldox prepared paragonia.

(Dickinson, 1971). For example, unlike alcohol dehydrogenase, Aldox is readily observed in larval foregut, hindgut, hypoderm, and imaginal discs. The Aldox staining serves as a control to demonstrate that the compositionally similar ADH-specific stain could penetrate and stain a variety of tissues other than the ones normally seen. However, this analysis is incomplete because certain tissues do not stain intensely with either stain (e.g., brains).

In most cases, the tissue-specific staining of the transformed strains is identical to that seen in the wild-type Adh^f strain. This is observed for both larval and adult tissues. In addition, the relative staining between each tissue type in a transformant appears to be of similar intensity, and comparisons between strains with different ADH activities indicates that the staining intensity qualitatively reflects relative ADH activity. For example, the tissue staining levels of the *Adh* alleles Adh^f, Adh^S, and Adh^{n5}, which in adults of our lab stocks have relative activities per milligram protein of 1:0.28:0.05, were compared. A clear difference in intensity could be discerned between Adh^f and Adh^S, and Adh^{n5} stained only slightly above *Adh* null controls. Thus the staining intensity may be used for qualitative comparison of levels of activity in the same organ.

Consistent with the low ADH specific activity found in tAP-4 larval extracts, staining in organs of tAP-4 larvae was considerably weaker than that found in wild type and the other transformants (part of this difference may be ascribed to the heterozygous state of the transduced gene in tAP-4). In general, the tissue-specific staining was similar to that found in wild-type larvae, except that the somatic muscles, posterior midgut, and Malpighian tubules were not stained. In addition, the gastric cecae, diverticula emanating from the anterior midgut, are not stained. This different tissue-specific staining pattern may indicate a tissue-specific difference in *Adh* expression, or may reflect the lower overall ADH activity in the tAP-4 strain. This latter interpretation is supported by the observations of stained Adh^{n5} larvae, which have lower ADH specific activity than the Adh^f controls, and in which the staining of Malpighian tubules, somatic muscles, and gastric cecae is inconsistent and weak. The Adh^{n5} strain should have approximately 3–5 times the activity of the tAP-4/CyO^{nl} larvae.

Adh mRNA

Analysis of RNA from the transformed lines tAP-1, tAP-2, tAP-3, and tAP-5 revealed that approximately normal amounts of *Adh* mRNA of the correct size is produced by the transduced *Adh* gene in both adults and larvae (Figure 5B). The endogenous Adh^{fn6} allele of the recipient strain makes nearly normal amounts of *Adh* mRNA (Benyajati et al., 1982) which lacks 34 nucleotides in the 3′ part of the *Adh* coding region (Benyajati et al., 1983a). RNA derived from the transduced gene can therefore be analyzed specifically by probing gel blots of total RNA with a 50-nucleotide Ban I-Dde I fragment from the wild-type gene (Benyajati et al., 1981) that spans the deletion in the Adh^{fn6} allele (see Figure 5A). As shown in Figure 5B (lane 1), the Ban I-Dde I probe (Δ) fails to hybridize to RNA from adults (and larvae; data not shown) of the recipient strain (R). However, the probe detects an mRNA of the same mobility in adults (lanes 2–3) and larvae (lanes 4–5) of both the transformant tAP-5 and the wild-type Adh^f strain. Furthermore, the amount of this mRNA in tAP-5 is comparable to that in the control strain. This same analysis was carried out for the transformants tAP-1, tAP-2, and tAP-3, and the same result was obtained (data not shown). Therefore all of the transduced *Adh* genes thus far examined produce approximately normal amounts of *Adh* mRNA of the correct size in both larvae and adults.

Adh Promoter Utilization

A major feature of the developmental pattern of *Adh* expression is a "switch" in the relative level of stable *Adh* mRNA transcripts originating from two different *Adh* promoters (Benyajati et al., 1983b; Figure 5A). In third instar larvae, the proximal, or larval, promoter is used predominantly; in adults older than four days, the distal, or adult, promoter functions instead. To determine whether the developmental promoter switch occurs for the transduced *Adh* genes, we examined the structure of the *Adh* mRNA produced in several of the transformant lines. Gel blots of total RNA from larvae and adults of the transformants tAP-1, tAP-2, tAP-3, and tAP-5 and of a wild-type Adh^f control strain were hybridized with DNA probes carrying sequences specific to "larval" and "adult" *Adh* mRNAs (that is, mRNAs transcribed from the larval and adult promoters, respectively). Figure 5B shows an example of the results. The adult RNAs (lanes 6–7 and 10–11) of both tAP-5 and the control Adh^f strain hybridize almost exclusively to the "adult-specific" probe, while both larval RNAs (lanes 8–9 and 12–13) show very much stronger hybridization to the "larval-specific" probe. The fact that, as described above, the transduced *Adh* gene in the transformant produces amounts of mRNA comparable to the endogenous gene implies that the normal switch in promoter usage is carried out by both genes. The same result was obtained for the transformants tAP-1, tAP-2, and tAP-3 (data not shown).

A more direct demonstration of the promoter switch was carried out for the transformant tAP-5. By appropriate crosses, a strain was constructed that placed the tAP-5 allele in an Adh^{fn4} background. The Adh^{fn4} allele is a splicing mutant in which only small amounts of stable *Adh* RNA accumulate (Benyajati et al., 1982). This RNA can be distinguished from the wild-type RNA because of putative intron sequences present in the stable RNA species. Promoter utilization in the tAP-5;Adh^{fn4} strain was then compared with the wild-type Adh^f gene using a primer extension assay (Figure 5C). A primer internal to the ADH coding region was labeled, hybridized to total larval or adult RNA, and extended with reverse transcriptase. The length of the products are diagnostic for RNAs initiated at the larval and adult promoters. In adult RNA (lanes 1, 3, 5), a strong band at the length expected for transcripts initiated at the adult promoter is seen in tAP-5;Adh^{fn4} and Adh^f RNAs. In tAP-5;Adh^{fn4} and Adh^{fn4} (lanes 1 and 5), a

Table 3. Histochemical Staining of Transformed Larvae and Adults

Larval ADH Distribution

	Salivary Glands	Foregut	Brain	Imaginal Discs	Fat Body	Anterior Midgut	Somatic Muscles	Posterior Midgut	Malpighian Tubules	Hindgut	Hypoderm
Adh^{fn23}	–	–	–	–	–	–	–	–	–	–	–
Adh^{f}	–	–	–	–	+	+	+	+	+	–	–
tAP-1	–	–	–	–	+	+	+	+	+	–	–
tAP-2/*TM6B*	–	–	–	–	+	+	+	+	±	–	–
tAP-3	–	–	–	–	+	+	+	+	±	–	–
tAP-4/*CyO*	–	–	–	–	+	+[a]	–	–	–	–	–
tAP-5	–	–	–	–	+	+	+	+	+	–	–
tAP-6	–	–	–	–	+	+	+	+	+	–	–
Aldox	±	+	–	+[b]	+	+[a]	–	+	+	+	+

Adult ADH Distribution

	Crop	Midgut	Fat Body	Somatic Muscles	Malpighian Tubules	Rectum	Testes and Paragonia	Seminal Vesicles and Vas Deferens[c]
Adh^{fn23}	–	–	–	–	–	–	–	–
Adh^{f}	+	+	+	–	+	+	–	+
tAP-1	+	+	+	–	+	+	–	+
tAP-2/*TM6B*	+	+	+	–	+	+	–	+
tAP-3	+	+	+	–	+	+	–	+
tAP-4/*CyO*	+	+	+	–	+	+	–	+
tAP-5	+	+	+	–	+	+	–	+
tAP-6	+	+	+	–	+	+	–	+
Aldox	+	+	+	–	+	+	+[d]	+[e]

The results presented in this table are a compilation of the histochemical staining of at least three larvae or adults of each transformant, with both sexes represented at least once. A "+" was scored when staining was present in all specimens, a "–" scored when present in no specimen, and "±" was scored when more than one, but not all specimens showed staining. No indication is given of the relative intensity of staining. The larval Malpighian tubules often showed inconsistent staining, even in control Adh^{S} larvae. Aldox indicates the tissue distribution of the enzyme aldehyde oxidase, which is observed with a stain very similar to that of the ADH stain. Examples of the histochemical staining and detailed description of the staining is presented in Figure 4.

[a] No staining of gastric cecae.

[b] Not all imaginal discs are stained (e.g., see antennal disc in Figure 6).

[c] The nomenclature of Bodenstein, 1950 is used to name the male genital apparatus.

[d] Staining of testes weak. Paragonia are usually speckled at their distal tips, although occasionally there is deep, uniform staining.

[e] The vas deferens is completely unstained, whereas the seminal vesicle is deeply stained.

less intense band is seen at a higher molecular weight, which appears to be a product initiated at the adult promoter, yet contains part of the first coding sequence intron (IVS-1) as a result of the Adh^{fn4} deletion. In larval RNA (lanes 2, 4, 6), shorter products corresponding to larval promoter transcripts are seen in tAP-5;Adh^{fn4} and Adh^{f} (lanes 2 and 4) in addition to almost equal amounts of adult promoter transcripts. No *Adh* larval transcripts are seen in adults. The similarity of the tAP-5;Adh^{fn4} and Adh^{f} experimental lanes demonstrate that in the tAP-5 transformant, both larval and adult promoters are utilized at approximately the wild-type ratios at two different developmental stages.

Discussion

Assays for Developmental Expression of Cloned Eucaryotic Genes

The greatest difficulty in studying *cis*-acting regulatory sequences in higher eucaryotes is establishing meaningful assays for regulated expression. One approach to the problem is to introduce cloned genes into cultured cells that are either differentiated or can be induced to differentiate. Cell-type-specific regulation of gene expression has been reported for the chicken lysozyme gene in chicken oviduct cells (Renkawitz et al., 1982), mouse and human β-globin genes in differentiated mouse erythroleukemia cells (Chao et al., 1983; S. Wright, R. DeBoer, R. Flavell, and F. Grosveld, personal communication), the chick δ-crystallin gene in mouse lens epithelial cells (Kondoh et al., 1983), and mouse immunoglobulin genes and their associated enhancer elements in mouse myeloma cells (Rice and Baltimore, 1982; Oi et al., 1983; Gillies et al., 1983; Queen and Baltimore, 1983; Banerji et al., 1983). Although significant quantitative differences in the expression of the cloned genes and their endogenous counterparts have been observed in these systems (e.g., Chao et al., 1983), the qualitative cell specificity of expression does provide a basis for studying *cis*-acting regulatory sequences.

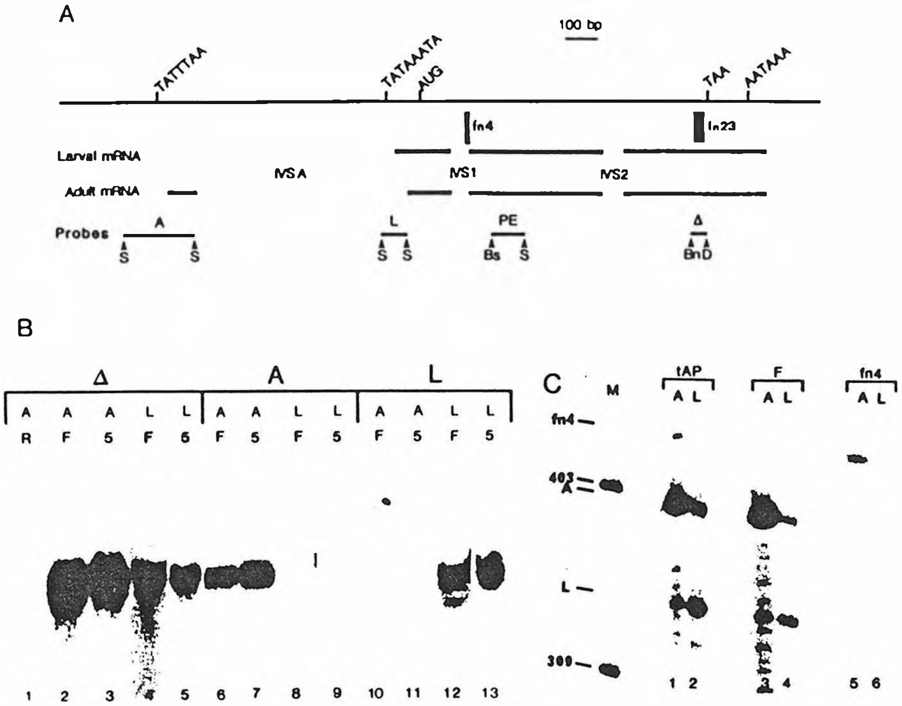


Figure 5. Analysis of *Adh* RNA in the tAP-5 Transformant

(A) Schematic diagram of *Adh* transcripts and probes used in RNA analysis. The top line denotes genomic DNA from the *Adh* region with sequences of potential importance given above the line (Benyajati et al., 1983b). For a general review of the function of these sequences, see Breathnach and Chambon (1981). TATTTAA—"TATA box" homologous sequence thought to function in adult *Adh* mRNA synthesis. TATAAATA—"TATA box" homologous sequence thought to function in larval *Adh* mRNA synthesis. AUG—methionine codon initiating ADH protein synthesis. TAA—terminator codon ending ADH protein synthesis. AATAAA—sequence implicated in polyadenylation of mRNA. The two middle lines show the regions present in larval and adult mRNA. Whereas larval mRNA synthesis involves only intervening sequences IVS-1 and IVS-2, adult mRNA synthesis involves an additional intervening sequence, IVS-A. The vertical bars indicate the sequences deleted in Adh^{fn4} and Adh^{fn23} mutations. The bottom line indicates the probes used in the experiments of parts (B) and (C). S—Sau3A I, Bs—Bst EII; Bn—Ban I, and D—Dde I. (B) RNA gel blots of total RNA of transformed and control strains. Total RNA from adults (A) or larvae (L) of the recipient strain (R), a wild-type strain homozygous for the Adh^F allele (F), or the transformed line tAP-5 (5) was denatured and electrophoresed on 1.2% agarose gels containing 2.2 M formaldehyde. Pairs of lanes for direct comparison were loaded with equal amounts of RNA. Following transfer to nitrocellulose, the RNA was hybridized to the probes indicated at the top (see Figure 5A; Experimental Procedures). Δ: a 50 nucleotide Ban I–Dde I fragment spanning the deletion in the ADH coding region of the recipient strain fn23 allele. A: a 221 nucleotide Sau3A I fragment containing sequences specific to *Adh* mRNA transcribed from the adult promoter. L: a 70 nucleotide Sau3A I fragment containing sequences specific to *Adh* mRNA transcribed from the larval promoter (see Figure 5A). (C) Primer extension assay. A 106 nucleotide single-stranded labeled fragment (see PE, Figure 5A) complementary to sequences within ADH mRNA was hybridized to total larval or adult RNA. The annealed primer was extended with reverse transcriptase and the products were resolved on an acrylamide-urea sequencing gel. On the left-hand margin the sizes of two markers (309 and 403 nucleotides) and the expected lengths of extension products made from wild-type adult (A), larval (L), and adult *Adh* (fn4) RNA are shown. The brackets from left to right indicate the products obtained from tAP-5 in an Adh^{fn4} background (tAP), Adh^F (F), and Adh^{fn4} (fn4) strains, respectively. Lanes marked "A" and "L" used adult and larval RNA, respectively.

Germ-line transformation has a potential advantage over cultured cell systems in that the transduced gene is present in all cells of the organism and is exposed to its entire developmental history. Thus this method should be particularly valuable for investigating tissue-specific gene expression and the temporal regulation of gene activity during development. To date, gene function following germ-line transformation has been studied extensively only in Drosophila and mice. In the latter system, considerable difficulty has been encountered in obtaining correctly regulated expression of germ-line integrated genes. For example, Palmiter et al. (1982) have examined the expression of a mouse metallothionein-herpes virus thymidine kinase fusion gene. No correlation was observed between the number of integrated gene copies and the level of expression in different transformed mouse lines. Although the activity of the fusion gene was inducible by cadmium, dexamethasone was ineffective for induction, in contrast

to observations with the normal mouse metallothionein gene. Expression of the fusion gene was shown to be unstable in succeeding generations. Costantini and Lacy (1981) have obtained lines of mice transmitting integrated copies of the rabbit β-globin gene. No expression of the gene was observed in erythroid cells; however, transcripts were present in the muscle tissue of one transformed line and in the testis of another (Lacy et al., submitted). In five other transformed lines, no expression could be detected. Jaenisch et al. (1981) have studied the expression of single copies of the Moloney leukemia virus (M-MuLV) genome integrated into the germ line of 13 different mouse substrains. Each substrain carries the M-MuLV genome in a different chromosomal position; evidence for rearrangement of the integrated sequences was found in only two cases. Nevertheless, four different patterns of activation of virus expression were observed in the 13 substrains. These examples illustrate the difficulties that have been encountered so far in establishing an assay for developmental expression of genes following germ line transformation in the mouse. They contrast markedly with the results presented here and in the accompanying papers (Scholnick et al., 1983; Spradling and Rubin, 1983), in which P-element-mediated transformation has been used to introduce genes into the Drosophila germ line.

Correctly Regulated Expression of a Cloned *Adh* Gene

The primary conclusions from our study are that an 11.8 kb chromosomal fragment containing the *Adh* gene includes the *cis*-acting sequences necessary for correctly regulated *Adh* expression, and that integration of this fragment into a variety of chromosomal locations is compatible with its proper expression.

Four of the six *Adh* transformant strains (tAP-1, tAP-2, tAP-3, and tAP-5) exhibit normal expression by four major criteria. One, quantitative levels of ADH enzyme activity in larvae and adults. Two, qualitative tissue specificity. Three, the size of stable *Adh* mRNA. Four, the developmental switch in larval and adult promoter utilization. These strains carry the *Adh* gene integrated at four different genomic locations (on all three major chromosomes). In addition, correct *Adh* expression is independent of the orientation of the gene within the P element transposon. We also note that we have thus far obtained no evidence of instability in the structure, chromosomal location, or regulated expression of transduced *Adh* genes over many generations.

The levels of ADH enzyme activity in two strains (tAP-4, and tAP-6) are more than two times lower that of wild-type controls (in larvae and adults, respectively). Although the reasons for this lower level of expression are not known, it is possible that the local chromosomal environment at the site of integration of the *Adh* gene is responsible.

Selection for *Adh* Gene Function in Transformants

The transformants obtained in these studies were selected by the ethanol resistance conferred upon adult flies by the transduced gene, and four of the six transformants display normal *Adh* expression. The ethanol selection may have biased our results in a number of ways. For example, transformants expressing no or low levels of ADH would have been lost and transformants that expressed substantial levels of ADH but in incorrect tissues might similarly have been selected against. It is not known what tissues must contain ADH activity in order for an adult fly to survive exposure to ethanol. We can, however, estimate that the lower limit of ADH activity necessary for reliable selection under our conditions is perhaps 10% of the wild-type Adh^{F} level. This is based on the fact that flies homozygous for the Adh^{nS} allele, which at 25°C have about 5% of wild-type activity (Vigue and Sofer, 1974), show only about 20% survival of the ethanol treatment used here.

Analysis of Tissue-specific Expression of ADH

The analysis of the tissue specificity of ADH expression in this study was based upon a histochemical staining procedure (Ursprung et al., 1970). Certain tissues (such as larval salivary glands and imaginal discs) exhibit no detectable ADH activity by this assay in either wild-type strains or transformants. This result is not likely to be due to the failure of the staining reagents to penetrate these tissues. First, Ursprung et al. (1970) carried out ADH activity measurements on homogenized samples of dissected tissues; those which were null by histochemical staining also lacked detectable activity in homogenates. Second, in the present study, the same histochemical reagents (except for the substrates) have been used to detect both ADH and aldehyde oxidase activity in dissected tissues. The latter activity was clearly evident in tissues (e.g., larval foregut and imaginal discs) that do not stain for ADH.

It is possible that the restriction of ADH activity to certain tissues is mediated to some degree posttranscriptionally, and we have not addressed this issue here. However, it should be noted that Rabinow and Dickinson (1981) have explicitly demonstrated that in Drosophila orthofascia the presence of ADH activity in larval fat body and its absence in larval gut correlate respectively with the presence or absence of stable *Adh* mRNA in these tissues.

Conclusion

The *Adh* gene shows a complex pattern of activity during development, including tissue-specific expression and a temporal switch in promoter utilization. The results presented in this paper demonstrate that the *cis*-acting sequences required to control this pattern of expression are present on a defined DNA fragment, and that integration of this fragment into a variety of chromosomal sites is compatible with correctly regulated *Adh* expression. These observations provide the necessary basis for a detailed analysis of *Adh* regulatory sequences in vivo.

Experimental Procedures

General Methods

The following procedures were carried out using standard methods as described (Maniatis et al., 1982, and references therein): preparation of

plasmid DNAs, restriction enzyme digestion, polyacrylamide and agarose gel electrophoresis, DNA ligation, bacterial transformation, screening of transformant colonies, repair synthesis labeling of DNA fragments with Klenow polymerase, isolation of labeled probe fragments, blotting and hybridization of DNA and RNA on nitrocellulose, and autoradiography. Flies were maintained on standard cornmeal-yeast extract-sucrose medium at 18°C or 25°C.

Fly Strains

The following fly strains were used in the experiments described in this report: *Adh^{fn23} pr cn*, the recipient strain for transformation injections. Obtained from W. Sofer. The *Adh^{fn23}* allele has a 34 bp deletion in the third coding exon of the gene (Benyajati et al., 1983a), and produces nearly normal amounts of *Adh* mRNA, which is translated to yield a nonfunctional polypeptide (Benyajati et al., 1982). *Adh^{fn4} pr cn*, obtained from W. Sofer. The *Adh^{fn4}* allele produces very low levels of stable *Adh* RNA (Benyajati et al., 1982). *w; Adh^F*, a wild-type strain homozygous for an allele of *Adh* which encodes an F, or fast, electrophoretic variant of ADH. *Adh^S*, a wild-type strain homozygous for an allele of *Adh* which encodes an S, or slow, electrophoretic variant of ADH. *b Df(2L)A47 cn bw/CyO^{nB}; Ubx e^4/MKRS*, an ADH null balancer chromosome stock used for testing genetic segregation of *Adh^+* chromosomes in transformant lines and for homozygosing *Adh^+* second and third chromosomes. *Df(2L)A47* is a deficiency for the *Adh* region (Aaron, 1979; Ashburner et al., 1982). *CyO^{nB}* is a balancer chromosome, *In(2LR)O, Cy dp^{lv1} pr cn^2* (marked with Curly [*Cy*], a dominant wing mutation), carrying a null allele (nB) of *Adh* (Gerace and Sofer, 1972; Kubli et al., 1982). *MKRS, Tp(3)MRS, M(3)S34 kar ry^2 Sb* (McCarron et al., 1974; Gelbart and Chovnick, 1979) is a multiply rearranged third chromosome carrying the dominant bristle marker Stubble (*Sb*). It is useful as a balancer for some parts of the third chromosome. All other mutations are described in Lindsley and Grell (1968).

Construction of *Adh* Gene–P Element Hybrid Plasmids

The 11.8 kb Sac I fragment containing the *Adh^F* gene was isolated from the plasmid sAF2 (Goldberg, 1980) and ligated to Sac I digested DNA of the P element plasmid pπ25.1 (Spradling and Rubin, 1982; O'Hare and Rubin, 1983). The ligated DNA was used to transform E. coli strain MM294. Clones were identified which contain the *Adh* gene fragment in either orientation with respect to the P element; two plasmids, pAP-2 and pAP-5, were used in the experiments described here. In pAP-2 (see Figure 1), the direction of *Adh* transcription is away from the unique Sal I site in the P element and toward the unique Xho I site (O'Hare and Rubin, 1983); the opposite orientation is found in pAP-5.

For transformation experiments, embryos were injected with a solution containing 150-300 μg/ml of pπ25.1 and 300-500 μg/ml each of pAP-2 and pAP-5 in 5 mM KCl, 0.1 mM sodium phosphate, pH 6.8 (Spradling and Rubin, 1982).

Microinjection of Drosophila Embryos

Microinjection of Drosophila embryos was carried out as follows. Flies of the recipient strain *Adh^{fn23} pr cn* were placed in glass milk bottles, the bottoms of which had been removed and replaced with gauze and cotton stoppers. The bottles were kept at 25°C. Yeasted collection plates were placed into the mouths of the bottles, and embryos collected every 30-45 min. Subsequent steps were carried out at 19°C. Embryos were dechorionated in groups of 10-20 as follows. They were transferred from the collection plate to a strip of double stick tape on a glass microscope slide. A second taped slide was then sandwiched together with the first. A length of wire of appropriate diameter at each end of one of the slides prevented crushing the embryos. The slides were tapped lightly in the region of the embryos and separated. Embryos were removed from their ruptured chorions with watchmaker's forceps and transferred, posterior end outward, to another taped slide. Following desiccation in a closed chamber containing Drierite for 5-10 min, the embryos were covered with halocarbon oil (Series 700, Halocarbon Products Corp., Hackensack, NJ). The slide containing the embryos was then placed on the stage of a Zeiss microscope equipped with a 40× long working distance objective.

Needles for injection were pulled on a Kopf needle puller (David Kopf Instruments, Tujunga, CA), using double walled "dot tubing" (#30-31-1, Frederick Haer and Co., Brunswick, ME). The needle was back filled with DNA solution, connected by Intramedic tubing to an air-filled plastic syringe (50 ml), and mounted on a holder attached to a Narishige micromanipulator (equipped with a vernier control for movement in the vertical axis; Narishige Scientific Instrument Lab., Tokyo, Japan). The needle tip was broken to a useful diameter by running it gently into the edge of the slide containing the embryos. The actual injection of the embryos was carried out with the microscope stage controls. Embryos were injected prior to pole cell formation; older or damaged embryos were subsequently destroyed under a dissecting microscope. The slides were kept in plastic refrigerator boxes lined with wet sponges until the embryos hatched. Larvae were then transferred to standard food in individual vials and kept at 19°C until eclosion. Our injection protocol is a composite of techniques and advice from Germeraad (S. Germeraad, Ph.D. thesis, University of California, Irvine, California, 1976), S. Artavanis-Tsakonas (personal communication), Spradling and Rubin (1982), and an unpublished manual prepared by M. Zalokar and P. Santamaria (Syllabus of Experimental Methods in Drosophila Embryology, 1979).

Selection of Transformants

Adult flies (G0 adults) developing from injected embryos (G0 embryos) were individually mated at 25°C to flies of the recipient strain. The progeny of these matings (G1 adults) were aged for 4 days, and then tested for survival on a 6% solution of ethanol in water, as described by Vigue and Sofer (1976). Small cotton balls were placed in the bottoms of empty vials and saturated with 2.5 ml of 6% ethanol; care was taken to minimize wetting of the vial walls. Flies to be tested were transferred into the vials, which were then stoppered with cotton and sealed with Parafilm. The vials were kept at 25°C for 14-24 hr. Under these conditions, flies of the recipient strain showed 100% mortality, while wild-type flies showed complete survival. In various experiments, transformation frequencies of 5%-10% were obtained, that is, approximately 5%-10% of fertile G0 adults gave rise to at least one ethanol-resistant G1 progeny fly.

Genetic Segregation Analysis and Homozygosis

The genetic segregation of functional *Adh* genes in the transformants was analyzed as follows. Individual ethanol-resistant G1 or G2 transformant flies (genotypically *Adh^{fn23} pr cn*) were mated to flies of the balancer stock *b Df(2L)A47 cn bw/CyO^{nB}; Ubx e^4/MKRS*. Male and virgin female progeny carrying both the *Cy* and *Sb* markers (i.e., both balancer chromosomes) were selected with 6% ethanol as described above. A small number of surviving males (Cy, Sb, $6\%^R$) were then mated individually to virgin females of the recipient strain *Adh^{fn23} pr cn*. The progeny of each mating were separated into the four phenotypic classes Cy; Sb; Cy, Sb; and neither, and tested for resistance to 6% ethanol. In this scheme, transmission of a functional *Adh* gene on the X chromosome is revealed by the survival of all of the females and none of the males in all four phenotypic classes. Transmission on chromosome 2 is indicated by the death of all progeny carrying Cy and the survival of the other two classes. Transmission on chromosome 3 is shown by the death of all progeny carrying *Sb* and the survival of the remainder.

The *MKRS* chromosome is a poor balancer for the left end of the third chromosome. Consequently, the above analysis only tentatively identified chromosome 3 as the one carrying the functional *Adh* gene(s) in the transformant tAP-2, as a result of recombination between the tested chromosome and the *MKRS* chromosome. The genetic segregation of the *Adh^+* phenotype in tAP-2 was confirmed by an analysis similar to that described above, using instead the third chromosome balancer *TM6B* (*In(3LR)TM6B, Hu e Tb ca*; L. Craymer, personal communication).

To construct homozygous stocks of the transformants tAP-3, tAP-4, and tAP-6, the Cy, Sb, $6\%^R$ male and virgin female flies derived as described above were mated, and the progeny examined for those lacking both dominant markers. Such homozygous progeny were recovered for tAP-3 and tAP-6, but in the case of tAP-4, no progeny lacking the Cy marker survived, presumably because of a recessive lethal mutation on chromosome 2. This transformant line was thus maintained as a balanced stock carrying *CyO^{nB}*. Similarly, attempts to make chromosome 3 of tAP-2 homozygous were unsuccessful, and this transformant was maintained as a balanced stock carrying *TM6B*. Homozygous stocks of tAP-1 and tAP-5 were established as follows. Virgin females which could be either heterozygous or homozygous for *Adh^+* on the X chromosome were obtained by

backcrossing These were mated individually to ethanol-resistant males The progeny of these pairwise matings were then tested for ethanol resistance to identify homozygous stocks

Protein Analysis

Proteins were prepared from 5-20 larvae or adults by homogenization in 50 mM Tris-phosphate (pH 8.6) in Eppendorf tubes using custom made epoxy pestles. Protein concentration was determined by the procedure of Lowry et al. (1951), and ADH specific activity was determined as described by Sofer and Ursprung (1968), using secondary butanol as a substrate. Activity units were calculated from the absolute change in OD_{340} per fixed time interval. Because the tAP-2 and tAP-4 alleles were maintained as balanced stocks with balancer chromosomes, care was taken to select larvae and adults of the correct genotype. This was not possible, however, in the case of the tAP-4/CyO^{n8} strain, because the genotype of the larvae could not be determined, and the stage at which the tAP-4 bearing chromosome is lethal was not determined. However, since the CyO^{n8} chromosome itself is lethal before the late third instar (D. Goldberg, unpublished data), the resulting third instar larvae had a tAP-4 gene dosage between one and two per diploid cell. If the tAP-4-bearing chromosome allowed larval survival, this could underestimate the tAP-4 larval activity per gene by a factor of at most two, and by 33% on the average. For the purposes of Table 2, it was assumed that there was one copy per diploid cell. Polyacrylamide gel electrophoresis and staining for ADH activity was performed according to Grell et al. (1968).

Histochemical Staining

Tissue dissection and histochemical staining was carried out essentially as described by Ursprung et al. (1970). Staining was performed for 20 min, and tissues were left in the ethanol/acetic acid stop solution overnight. The tissues were then briefly soaked in isopropyl alcohol and mounted in a mixture of methyl salicylate and Canada balsam (Struhl, 1981). For aldehyde oxidase staining, 50 mM acetaldehyde was substituted for the secondary butanol of the ADH stain.

In Situ Hybridization to Chromosomes

Polytene chromosomes of larval salivary glands were prepared for in situ hybridization according to Pardue and Gall (1975), with the modification of Hayashi et al. (1978). The probe consisted of the plasmid p13E3, a pUC13 (J. Messing, personal communication) subclone of a 4.7 kb Eco RI fragment containing the Adh^{F} gene. Plasmid DNA was nick translated in the presence of biotinylated dUTP (Enzo Biochem, Inc., New York, NY; Langer et al., 1981). Hybridization and detection of hybridization sites was carried out according to an unpublished protocol (EMBO Course on Chromosomal Localization of Genes, April 13-20, 1982, D. Ward, personal communication), except that no antibodies were employed; rather, ABC reagent (Vector Laboratories, Burlingame, CA) was used directly (D. Ward, personal communication).

Isolation of Drosophila DNA and RNA

DNA was isolated from adult flies by the procedure of Bender et al. (Bender et al., submitted). Total RNA was isolated from larvae and adults by Dounce homogenization in the urea lysis buffer of Holmes and Bonner (1973), following by pelleting of the RNA through a cushion of 5.7 M CsCl (Glisin et al., 1974). Larvae used for RNA preparation were mainly in the third instar, but included some first and second instar animals as well.

DNA Probes for Blot Hybridization

All DNA probes for blot hybridization were labeled by repair synthesis with Klenow polymerase in the presence of the appropriate α-^{32}P dXTPs, and purified by electrophoresis on nondenaturing agarose or polyacrylamide gels. The following plasmids served as a source of probes: sAF2 (Goldberg, 1980); p13X3, a pUC13 (J. Messing, personal communication) subclone of a 3.2 kb Xba I fragment containing the Adh^{F} gene; p70a (constructed and kindly provided by N. Spoerel), a subclone into the Bam HI site of pUC8 (Vieira and Messing, 1982) of a 70 nucleotide Sau3A I fragment containing sequences specific to the 5' end of "larval" *Adh* mRNA (Benyajati et al., 1983b); p221a (constructed and kindly provided by N. Spoerel), a subclone into the Bam HI site of pUC8 of a 221 nucleotide Sau3A I fragment containing sequences specific to the 5' end of "adult" *Adh* mRNA (Benyajati et al., 1983b).

Filter Blot Hybridization Conditions

DNA blots were prehybridized and hybridized at 68°C in 5× SSC, 1× Denhardt's (Denhardt, 1966), 20 mM sodium phosphate (pH 6.8), 50 μg/ml salmon sperm DNA. They were washed in 2× SSC, 0.1% SDS at 68°C. RNA blots were preincubated at 42°C in 5× SSC, 10× Denhardt's, 50 mM sodium phosphate (pH 6.8). Prehybridization (42°C) and hybridization were carried out in 5× SSC, 50% formamide, 1× Denhardt's, 20 mM sodium phosphate buffer (pH 6.8), 50 μg/ml salmon sperm DNA. Because of the short length of homology between the DNA probes and the RNA, hybridization was at 30°C. RNA blots were washed in 2× SSC, 0.1% SDS at 60°C.

Primer Extension Analysis

RNA was prepared from flies and larvae by homogenization in ice cold 10 mM Tris HCl (pH 7.5), 0.25 M sodium acetate, 1% sodium dodecyl sulfate, 5 mM ethylene diamine tetraacetate (EDTA), 10 mM vanadyl ribonucleoside complex (Berger and Birkenmeier, 1979). The homogenates were immediately extracted with phenol equilibrated with 0.1 M Tris HCl (pH 7.5) and 0.5% 8-hydroxyquinoline. Total nucleic acid was precipitated from the aqueous phase by addition of 2.5 volumes of ethanol. The nucleic acid was resuspended in 1 mM vanadyl ribonucleoside complex.

The primer for the primer extension assay was prepared by digesting plasmids pRB1 or pRX8 with Bst EII. These plasmids contain single Bst EII sites located in the middle exon of the ADH coding region. The resultant sticky ends were repaired with α-^{32}P labeled dXTPs and the Klenow fragment of DNA polymerase I, and the product was digested with Sau3a and separated on a denaturing polyacrylamide gel. The 106 nucleotide single-stranded fragment was eluted from the gel.

Approximately 20-30 μg of total nucleic acid was incubated with 1 ng of the primer fragment at 37°C for >6 hr in 5 μl of 10 mM Tris HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA. An equal volume of 10 mM DTT, 16 mM $MgCl_2$, 1 mM each dXTP, 1 mM vanadyl ribonucleoside complex, and 100 mM Tris HCl (pH 8.0) was added, and the primer was extended by the addition of reverse transcriptase to 500 U/ml and incubation at 40°C for 1 hr. The reaction was terminated by the addition of EDTA to 25 mM, and the RNA was degraded by the addition of NaOH to 0.5 M and incubation at room temperature for 15 min. An equal volume of 0.5 M Tris HCl (pH 7.5), 0.5 M acetic acid was added, and the cDNA was recovered by ethanol precipitation. The cDNA was analyzed on thin 5% urea sequencing gels.

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Appendix

Initial results of a rapid assay for expression of cloned Adh DNA

Introduction

Preliminary results of a rapid assay for the expression of cloned Adh genes are presented. In this assay, cloned DNA is micro-injected into Adh null pre-blastoderm embryos, and Adh activity is detected in larvae and adults derived from the injected embryos. Individual cells expressing near wild-type levels of Adh activity can be detected, and furthermore, large numbers of these cells can be observed from injection of a single embryo. While injection with certain plasmid constructions appears to retain wild-type expression of the Adh gene, with another DNA, expression has been observed in cells which do not normally produce detectable levels of Adh.

Results

The plasmids sAF2 and pAP5 contain the 11.8kb SacI fragment from an Adh^F strain, and differ primarily in that in the pAP5 plasmid, Adh sequences were inserted into hybrid dysgenesis P element DNA (see Chapter3, Figure 1). sAF2 and pAP6 DNA at 300 μg/ml were separately injected into $\underline{Adh}^{fn23}$ pr cn ; ry embryos. In the first trial, pre-blastoderm embryos were injected laterally at approximately 25-50% from the anterior pole. As much DNA was injected as the embryo could subjectively retain without bursting. The injected embryos were grown at 18 ^{o}C until hatching, and were then transfered to standard food vials at 25 ^{o}C. Four to five days later, the larvae were dissected and stained by a standard histochemical stain specific for Adh (Ursprung et al, 1970). Larvae derived from both sAF2 and pAP5 plasmid injections showed specific staining. Table 1 presents the number of cells in the intestines and the fat body staining in each larva dissected. While there was considerable variation in the number of cells staining per larva, the majority of larva showed some staining. One larva in particular

showed staining in approximately 200 of the normal 2100-2400 fat body cells (Rizki, 1980). Additionally, while stained cells seemed to be clustered in defined regions of the larva, only in some instances did adjacent cells stain. In Figure 1, of approximately 40 anterior midgut cells in the plane of focus, four are stained. While two of the stained cells are adjacent, the other two are surrounded entirely by unstained cells. This does not immediately suggest a clonal origin of the staining cells.

These results indicate that potentially unintegrated Adh plasmid DNA (see Discussion) retains the capacity for high efficiency transcription with the correct developmental specificity. In the experiment in which sAF2 was injected and larvae stained, wild-type *Adh*^{S} and *Adh*^{F} larvae were dissected and stained alongside the experimental larvae. The *Adh*^{S} strain has an Adh specific activity approximately 3-4 times lower than that of the *Adh*^{F} strain, and the injected DNA was derived from *Adh*^{F} flies (and shows *Adh*^{F} electrophoretic mobility in the transformation experiments described in chapter 3). Individual cells in the injected larvae showed staining approximately as intense as that of the *Adh*^{F} larvae, and stronger than that of *Adh*^{S} larvae, although there was some variation that involved overlap among the different phenotypes.

Following these initial results, a series of injections were made at high DNA concentrations into various regions along the embryo in order to optimize the number of cells stained per embryo. For these experiments, a smaller plasmid, pRX1, containing the Eco RI to Xho I 4.5 kb Adh fragment from an *Adh*^{F} strain inserted into the Eco RI /Sal I pBR322 vector fragment was constructed and injected at 1 mg/ml into *Adh*^{fn4} *pr cn* flies. This is approximately 6-fold higher molar concentration than the previous sAF2 and

pAP5 injections. Injections were made at various locations along the embryo anterior-posterior axis. Typically, a few hundred fat body and midgut cells were stained, as well as somatic musculature. However, unlike wild-type larvae, staining was routinely observed in foregut, brain, and hindgut. Staining in the brain was intense in certaine instances, (Figure 2), but was consistently weak in the foregut and hindgut.

When adult tissue was examined, many fewer cells were observed to stain. Typically, 5-50 cells were stained. Staining was observed in Malpighian tubules, hindgut, anterior midgut and seminal vesicles. Unlike wild-type flies, the testis sheath in one fly (Figure 3) and the ovaries in another were stained. In addition, in two flies, the region corresponding to the most anterior portion of the cardia (stomach), just posterior to the insertion of the crop, was intensely stained. Lastly, the brain showed small patches of stain in two flies. It is not known whether or not this corresponds to the region of the brain that is reported to stain in wild-type flies (A. Place, personal communication). However, as only seven adults have so far been examined, these results must be further extended.

The correlation between site of injection and the tissues which were stained was marked (Table 2). Injections performed near the anterior pole of the embryos stained structures in the anterior of the larva, such as brain, fat body, foregut, and anterior midgut more frequently than structures in the posterior region such as the hindgut, posterior midgut, and posterior somatic musculature. However, in one case, injection in the posterior region resulted in stained brain tissue, and injection in the anterior region often stained posterior fat body and in one case posterior midgut and hindgut were stained. It should be noted that while there is not

an exact relative spatial correspondence between the primordia in the embryo and the larval tissues to which they give rise, a loose correlation does exist.

At this juncture, four alternative explanations were posited to explain the difference between the first experiments, where tissue specificity of staining seemed to be normal, and the second set of experiments, where aberrant tissue expression was observed. First, the plasmid DNA concentration differences might indicate a dosage response of tissue specificity. The six-fold higher molar concentrations of the pRX1 than the sAF2 and pAP5 plasmids may have titrated a negative regulator of transcription, DNA replication, plasmid episome stability, etc. that allowed the aberrant DNA expression. Second, the larger Adh region of the pAP5 and sAF2 plasmids may have contained a negative regulator of transcription, DNA replication, plasmid stability, etc., that was not contained on the smaller pRX1 plasmid. Third, Adh may normally be expressed in low levels in the brain, foregut, testis sheath, etc., but at a level normally undetectable by the histochemical staining technique in wild-type flies. The results obtained would then represent a quantitative increase in Adh expression in these cell types, but may not directly affect the tissue specificity of Adh expression. Lastly, the original observations obtained with the sAF2 and pAP5 plasmid injections resulted from a small sample size, and if larger amounts of stained material was analyzed, aberrant expression would be obtained.

In order to distinguish between these possibilities, the following experiments have been or are being performed. First, high concentrations (2 mg/ml) of the sAF2 plasmid were injected. If pRX1 at 1 mg/ml was titrating a negative regulator, then equivalent molar concentrations of sAF2 should

show the same phenotype. Very low numbers of cells per larva were stained in this series of injections, and staining was found only in fat body, midgut and muscles. The low yield of stained cells may result from intrinsic properties of the sAF2 plasmid, from inhibitors in the plasmid preparation used, or from the viscosity of such high DNA concentrations. In order to test these possibilities, a series of pRX1 injections at lower concentrations is being injected. The injected DNA is known to give expression with high efficiency, and viscosity does not seem to be a difficulty. As an additional test of the possibility that a negative regulator is being titrated by high concentrations of the injected plasmid, a plasmid containing a region extending from the middle of the Adh structural gene to the Eco RI site 5' of the gene, and which is therefore lacking a functional Adh gene, is being injected at high concentration into wild-type Adh embryos. Titration of a negative regulator should result in activation of the endogenous Adh gene.

In order to determine whether Adh is expressed in tissues not previously thought to express it normally, staining conditions are being systematically varied, hoping to increase the sensitivity of the assay either by increasing the 'signal' (by heat or cold shock to permeabilize cells) or by reducing the background (eliminating phenazine methosulfate from the staining reaction, varying substrate concentrations, and treating cells to removed other dehydrogenases).

Most importantly, it will be necessary to determine which, if either, of the two known Adh promoters are being used in this rapid assay both in tissues that normally express Adh and those in which aberrant expression is found. This information may tell whether or not the factors responsible for

stage specific expression are the same as or intimately linked with those used for tissue specific expression. It will also be an important test of the physiological correctness of the rapid assay system.

Discussion

The expression of cloned genes in injected Drosophila embryos was first reported by Rubin and Spradling (1982). Embryos injected with rosy/P element hybrid plasmids for the purposes of germ-line transformation developed into flies which were often phenotypically closer to wild-type than the rosy null recipient embryos. The tissue specificity of rosy expression in these embryos was not, however, investigated. In this appendix, the expression of Adh plasmids injected into pre-blastoderm embryos was characterized. High levels of Adh expression was observed in individual cells. Under certain conditions, Adh appeared to be expressed only in tissues in which it is usually observed, while under another condition, expression was obtained in tissues where it is not normally expressed. This result has immediate implications and raises a number of questions.

The Adh gene can be expressed in a variety of tissues. This result is suggestive that the tissue specificity of Adh staining in wild-type flies is not artifactual. One could imagine either that only certain tissues were permeable to the staining components, or that fat body and intestines, by virtue of the extreme size of their polyploid cells, stain more heavily than diploid cells. These results demonstrate that the staining reaction can identify Adh expression in a variety of tissues, and supports the claim of tissue specificity of Adh expression. A response to this argument could be that in these injection experiments, the extremely high levels of expression allow the Adh detection. This seems unlikely because Adh is normally an

abundant protein (a few percent of total soluble protein), and much higher levels of Adh synthesis seem unlikely.

Is Adh production continuously variable in the injected embryo, or does the variable staining reflect normal differences in Adh activity? The staining in both wild-type larvae and larvae derived from the rapid assay is somewhat variable. A small fraction of cells in the experimental larvae showed what seemed to be extremely low levels of Adh staining that seemed to be outside of the variation present in the wild-type larvae. Because the staining procedure is not precisely quantitative, it is hard to discern whether these cell are producing 10%, 1% or less of wild-type Adh activity. Also, this variation may reflect the different developmental stages at which the larvae are taken for staining. Although the larvae were allowed to develop for similar periods of time, the normal variation in developmental rates is very large in injected animals (D. Goldberg, unpublished observations). Thus, the low levels of Adh staining may have been observed in larvae that were less mature than the control larvae. One means of escaping this problem would be to take only crawling larvae or white prepupae; however, since only small numbers of larvae are studied, this would require constant monitoring of vials for animals at the appropriate stage. In order to test if the variability in staining may be explained by differences in larval maturity, staged _Adh_^{F} larvae were stained for Adh activity. Certain tissues showed an increased in stain intensity with age. For example, staining in distal gastric cecae was very weak in three day larvae, but was intense in crawling 5 day larvae. In addition, the posterior midgut at all stages shows a speckled pattern, with many cells variably stained surrounded by unstained cells. However, most tissues showed uniform staining of consistent intensity at all stages and does not explain the variability in

staining.

Is the transforming DNA replicated or integrated? This question in part devolves from the almost wild-type efficiencies of Adh expression in transformed cells. Fat body and anterior midgut chromosomes are approximately 100- and 150-ploid, respectively, in four day old larvae, and are probably 1.5 times this ploidy in mature crawling larvae (Welch, 1957). If only one copy of the transforming DNA is present in a cell, it is transcribing 100-200 times as efficiently as the normal integrated copy. This seems unlikely considering that Adh in larval fat body is thought to comprise a few percent of total soluble protein.

Assuming that many copies of the Adh gene are present in the cells expressing Adh, one may then ask if the injected Adh DNA is replicated to a high copy number per cell or if this DNA is deposited directly by the injection procedure. As an indirect approach to this problem, I have calculated that if a cell engulfs its entire volume of 20 kb plasmid DNA at 300 μg/ml (conditions of the original pAP5 injection), without concentration or dilution of the DNA, approximately 1000 molecules will be incorporated in the cell. While this figure is well above the 150 copies of Adh DNA found in mature larval fat body or midgut cells, the simplification that a blastoderm cell can incorporate its own volume of DNA seems unrealistic, and some dilution of the injected DNA seems likely. Also, if cell division occurs between blastoderm formation and the development of mature larval tissue, further dilution of the DNA would result. This reasoning suggests, but does not prove, that some DNA replication of the injected DNA is occurring.

Southern blotting of transformants will be required to determine if the transforming DNA is integrated or episomal. If integration into the genome

occurs, however, it must be extremely efficient. In the preliminary experiments, approximately 4 x 10^5 molecules of DNA were injected per embryo. Presumably, not all of this DNA is deposited in such a way as to be available for encapsulation by cells. Nonetheless, in one case over 200 cells showed Adh expression. If the transforming DNA was distributed evenly over only the transformed cells, this would imply an integration frequency of one integration event per 2000 DNA molecules, an extremely high efficiency.

Do the adjacent cells which stain for Adh represent clonal descendants of a single 'transformed' cell? Do the dispersed staining cells represent descendants of a single cell rearranged by cell movements? The cell divisions leading to larval fat body and intestines from the cellular blastoderm are not known in detail. Previous information on the development of these tissues was derived mainly by direct observation of sectioned embryos (Poulson, 1950), and the resolution in this work is not sufficient to help us answer this question (for example, the number of cells in both the fat body and midgut primordia is not known). Clonal analysis of somatic mosaics made at blastoderm formation has been done for some of the adult, but not the larval, internal organs. Such studies on larval organs would provide information on the number of cell divisions that blastoderm cells make in forming larval organs, and secondly, whether descendants of a single cell are adjacent in the mature organ or are dispersed by cell migrations.

Is the difference in larval and adult staining significant? The pRX1 injected embryos yielded larvae with many thousand cells intensely stained, whereas the adults arising from the same injection series showed an estimated 100-1000 fold fewer cells staining on average. In addition, many of the stained adult cells were from tissues such as Malpighian tubules and

brain that are known to be constituted wholly or partially from larval cells not lost during pupal development. The paucity of adult Adh staining may result from inability of the adult promoter to function in the rapid assay. Alternatively, whereas imaginal cells that form the adult structures typically replicate many times during larval development or pupation, many larval cells divide infrequently following cellular blastoderm formation. Thus, injected DNA present in imaginal cells will be diluted by cell division many times compared with larval cells. This would not be unlikely if the injected DNA is not replicated or segregated efficiently. Lastly, adult cells are typically smaller than their larval counterparts, and detection of Adh in adult cells may be less sensitive. As a first step in understanding the difference in larval and adult expression, it must be determined which, if either, of the two Adh promoters is being used in the rapid assay at the different stages.

In any case, the aberrant expression of Adh under certain injection conditions provides a case in which the chromosomal environment or intrinsic DNA sequence leads to promiscuous expression. Hopefully, this incorrect expression will provide in itself a fruitful subject for study.

References

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Table 1 - Quantitation of Adh histochemical staining

Drosophila.ft 1

Embryo	Midgut cells Staining	Fat body cells Staining
pAP5 DNA		
1	10	200
2	0	0
3	0	60
4	11	0
5	0	12
6	25	20
sAF2 DNA		
1	0	11
2	9	6
3	0	0
4	0	18

Notes - Injections were made at 25-50% from anterior of embryos at a plasmid DNA concentration of 300 μg/ml. Individual cells could be easily counted in midgut, but counts were difficult and unreliable for fat body. pAP5 embryo 1 and 3 counts are only approximate.

Table 2 - Position of Adh expression in larvae derived from injected embryos

	Foregut	Brain	Imaginal Discs	Ant. Fat Body	Anterior Midgut	Muscles	Post. Fat Body	Posterior Midgut	Malpighian tubules	Hindgut
	+	+		+	+	+	+			
Anterior		+		+	+	+	+	+		+
Injections	+	+		+	+	+				
	+	+		+	+					

									+	+
Posterior		+				+	+			
Injections						+	+	+	+	+

'+' indicates the presence of any stained material in the organs or tissues indicated. The tissues and organs are listed in order of their general location in a mature larvae, which does not necessarily indicate the relative positions of their primordia in the embryo (Poulson, 1950). Anterior injections were made at approximately 10-25% of the embryo length from the anterior pole, and posterior injection were made at similar distances from the posterior pole. Other injections made at various intermediate regions showed intermediate distributions of stained material.

<u>Figure 1</u> - <u>Distribution of stained cells in the midgut</u>

Approximately 40 larval midgut cells derived from pAP5 injection are in the plane of focus, of which four are stained. Note that the Adh stain is seen as crystals of precipitated tetrazolium dye, and that the crystals are excluded from the nucleus.

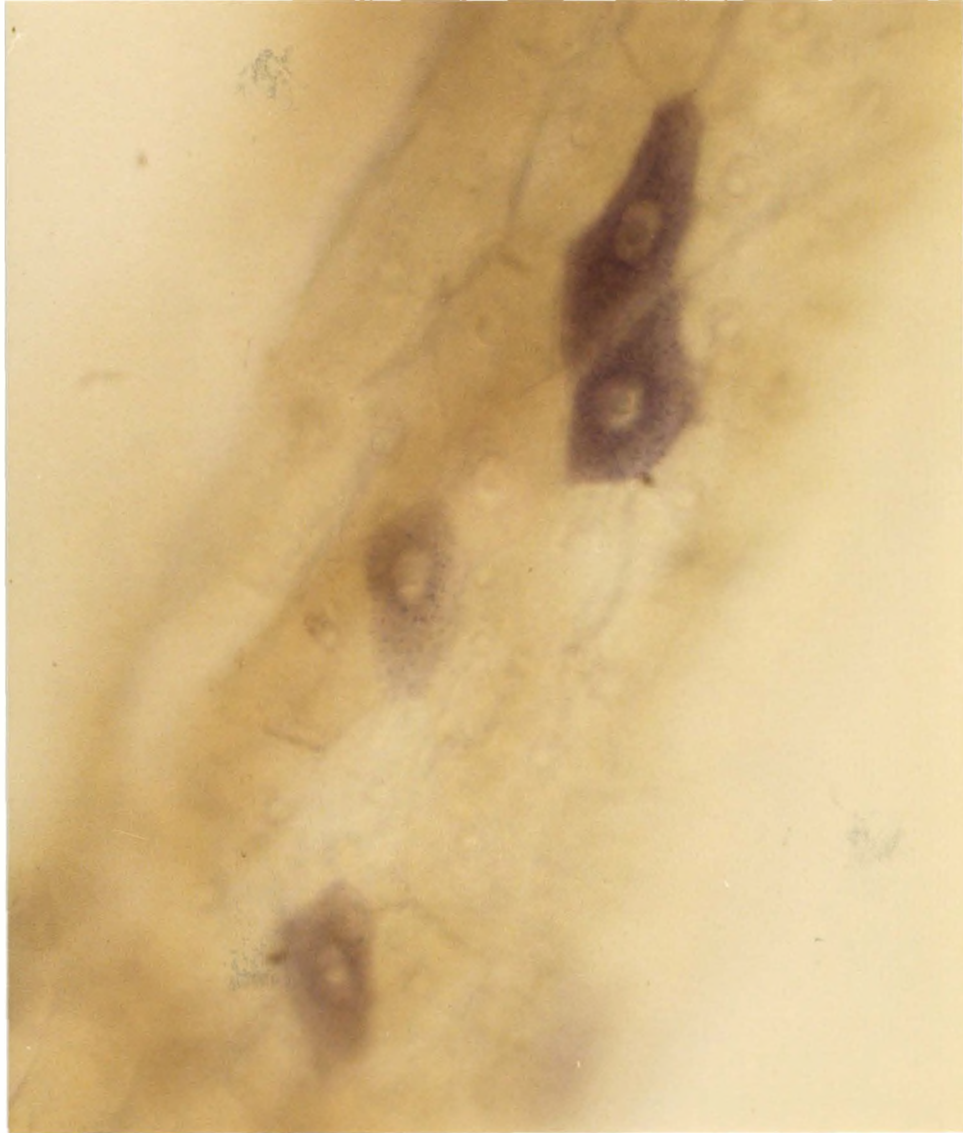
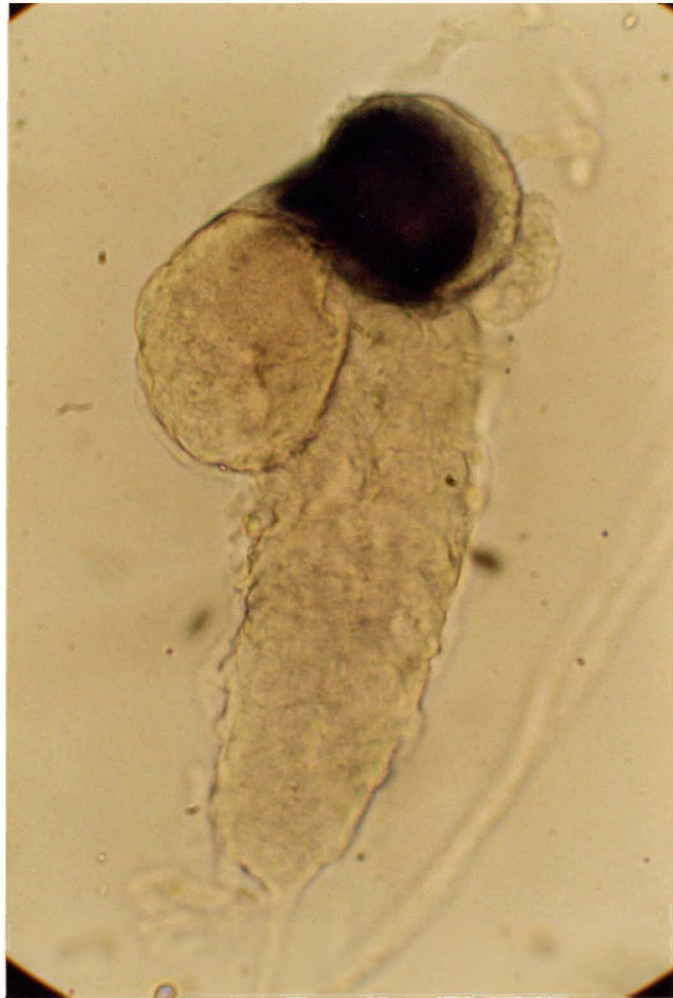


Figure 2 - Staining of brain tissue

A whole mount of larval brain and ventral ganglion is shown. One lobe of the brain is stained intensely, whereas the other lobe and the ventral ganglion are colorless.



<u>Figure 3</u> - <u>Staining of the testis sheath</u>

A whole mount of testis and associated accessory genital apparatus. Cells in both seminal vesicles are stained, and a testis sheath cell is seen to stain moderately. Two adjacent testis sheath cells, not easily seen in this photograph, are very lightly stained.