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Molecular Analysis of Mutant and Wild Type Alcohol Dehydrogenase Alleles From Drosophila.

Mark Andrew Batzer

Louisiana State University and Agricultural & Mechanical College

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Molecular analysis of mutant and wild type alcohol dehydrogenase alleles from Drosophila

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The Louisiana State University and Agricultural and Mechanical Col., 1988
MOLECULAR ANALYSIS OF MUTANT AND WILD TYPE
ALCOHOL DEHYDROGENASE ALLELES FROM DROSOPHILA

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
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in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in
The Department of Zoology and Physiology
(Interdepartmental Studies in Genetics)

by
Mark Andrew Batzer
B.S., Michigan State University, 1983
M.S., Michigan State University, 1985
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ABSTRACT

Wild type alcohol dehydrogenase polypeptides (ADH) from *Drosophila melanogaster* transformants were examined using western blots and polyclonal antiserum specific for *Drosophila melanogaster* ADH. These data indicate that ADH from Hawaiian drosophilids was similar in molecular weight to *Drosophila melanogaster* ADH, but that the isoelectric points of the Hawaiian electromorphs were distinct. Therefore it was evident that western blots could be used to characterize ADH from different species of Drosophila.

Mutants induced in *Drosophila* spermatozoa at the alcohol dehydrogenase (*Adh*) locus using X-rays, 1-ethyl-1-nitrosourea (ENU) or ethyl methanesulfonate (EMS) were characterized using genetic complementation tests, western blots, Southern blots, northern blots and enzymatic amplification of the *Adh* locus. Genetic complementation tests showed that 22/30 X-ray induced mutants, and 3/13 ENU and EMS induced mutants were multi-locus deficiencies. Western blot analysis of the intragenic mutations showed that 4/7 X-ray induced mutants produced detectable polypeptides, one of which was normal in molecular weight and charge. In contrast 8/10 intragenic ENU and EMS induced mutants produced normal polypeptides. Southern blot analysis showed that 5/7 intragenic X-ray induced mutants and all 10 of the intragenic ENU and EMS induced mutants were normal with respect to the alleles they were derived from. These data indicated that X-rays primarily induced large deletions, while the alkylating agents ENU and EMS predominantly induced missense mutations. Therefore the *Adh* forward mutation assay recovered a
broad spectrum of mutations ranging from missense to multi-locus deficiencies.

Enzymatic amplification of the Adh locus using the polymerase chain reaction (PCR) procedure provided further evidence for classification of mutants, as missense, deletions or intragenic chromosomal rearrangements. Enzymatic amplification of Adh alleles represents a specific, rapid method to analyze newly induced mutations.

Only 3 of 43 mutations produced polypeptides detectable as electrophoretic variants. Therefore it is evident that screening heterozygotes for electrophoretic variant polypeptides is inefficient. Most mutants that alter phenotype would either make a polypeptide that comigrates with the normal allele or not produce a detectable polypeptide.
CHAPTER I: INTRODUCTION
The study of mutagenesis began in 1927 and 1928 when Muller discovered that X-rays induced mutations in D. melanogaster, and Stadler showed X-rays induced mutations in maize (1928a) and barley (1928b). Twenty years later Auerbach and Robson (1946) demonstrated that chemicals (nitrogen mustards) also induced mutations in D. melanogaster. Mutations may be divided into several classes which include deletions, point mutations and chromosomal rearrangements (Drake, 1970). Point mutations are single base pair changes which result in amino acid substitutions (missense) or stop codons (nonsense). Deletions in Drosophila are divided into large multi-locus deletions of more than one gene (deficiencies) and intragenic deletions of one or more base pairs. Rearrangements involve complex chromosome damage and include duplications and inversions. More recently molecular biology used in conjunction with genetics has altered mutation research, creating the field of molecular mutagenesis.

The study of molecular mechanisms of mutagenesis begins with the selection of a test organism. The insect D. melanogaster was chosen as our animal model system for several reasons. One, these animals are eukaryotes and permit the analysis of germ line mutations. Two, the generation time for D. melanogaster is only 14 days. Three, D. melanogaster is small, only 2.5 mm in length, and may be cultured in large numbers in a small area. Four, D. melanogaster is well defined genetically (Lindsley and Grell, 1968). Thus this insect provides an ideal model system for the study of germline mutations.

The next critical factor in molecular mutagenesis is the
selection of a specific locus. We have chosen the alcohol dehydrogenase (Adh) locus for this purpose. Adh is a single copy gene located on the left arm of chromosome two in D. melanogaster (Lindsley and Grell, 1968). The entire gene including the cis-acting regulatory sequences is contained within a 3.2 kilo basepair Xba I restriction fragment (Goldberg et al., 1983). The Adh gene contains two promoters, one for larvae and one used in adults which allow differential expression patterns throughout the life cycle of D. melanogaster (Savakis et al., 1986). The protein encoding portion of the gene is 900 basepairs (bp) in length, including two small (65 and 70 bp) introns (Benyajati et al. 1983, Kreitman, 1983). The Adh gene encodes a homodimeric alcohol dehydrogenase enzyme (ADH), 265 amino acids in length, with a subunit molecular weight of 27,000 daltons (Thatcher, 1980). The subunit exists as a number of differently charged electromorphs with subunit isoelectric points that vary from 8.2 to 6.5 as the result of several different single amino acid substitutions (Batzer et al., 1988a). The primary function of alcohol dehydrogenase is to reduce primary and secondary alcohols, allowing the insects to survive on decaying fruits in nature.

Adh null mutations were selected using a specific locus test according to the procedures of Sofer and Hatkoff (1972). This procedure involves exposure of flies to a gas (1-penten-3-ol) which ADH positive flies metabolize to a toxic ketone and die thus permitting the survival of Adh null lines. In this case a null mutation may be defined as any inheritable change resulting in the loss of ADH activity. ADH null lines survive in the laboratory in the absence of alcohol, but would be conditional lethals in nature.
Two different classes of mutagens were used in this study. X-rays which are photons produced from orbital electron shifting, and may produce free radicals and ions (Wang et al., 1975). Mutations induced by X-rays largely consist of rearrangements and deletions which are characteristic of multiple lesions (Aaron, 1979; Cote et al., 1986; Grimwade et al., 1985; Kelley et al., 1985; Pastink et al., 1987). 1-ethyl-1-nitrosourea (ENU) and ethyl methanesulfonate (EMS) are alkylating agents which preferentially ethylate DNA (Beranek et al., 1980). The N-7 of guanine and O-6 of guanine are the most reactive sites for ENU and EMS ethylation (Beranek, et al., 1980; Singer, 1983). If the O-6 lesion is not repaired then it may mispair during DNA replication resulting in a G:C to A:T transition (Johnson and Lewis, 1981; Russel et al., 1979; Vogel and Natarajan, 1979; Vogel et al., 1982; Vogel et al., 1985). The presence of N-7 ethylguanine weakens glycosidic bonds, which may result in DNA chain scission (Kammermeyer and Zimmering, 1985; Lewis et al., 1985; Popp et al., 1983). ENU serves as a model mutagen for nitrosourea-containing compounds such as cigarette smoke, automobile exhaust and some common foods. EMS has a higher Swain-Scott (s) value (0.67) than ENU (0.26) (Singer, 1976). Alkylating agents with higher s values have a preference for alkylating centers of higher nucleophilicity such as the N-7 of guanine rather than the O-6 of guanine (Vogel and Natarajan, 1979). EMS was used to compare the mutation spectra generated with a different distribution of ethyl adducts. X-rays, ENU and EMS are direct acting mutagens, and therefore do not require metabolic activation.
In this study Adh null mutations were induced by exposing *D. melanogaster* males to X-rays (100 keV at 5, 10 or 30 Gy), 5mM ENU, or 5mM EMS. This procedure mutagenizes DNA from spermatozoa, a specific post replication (and repair) germ cell stage allowing the accumulation of lesions which do not become fixed as mutations until after fertilization of the oocyte, at which time DNA replication occurs (Wurgler et al., 1972; Vogel et al., 1985). Exposing this germ cell stage facilitates the recovery of mutations which occur as a result of single as well as multiple lesions. Treated males are crossed to females homozygous for an Adh null allele (Adh\textsuperscript{na}) which can be maintained opposite large deficiencies (Batzer et al., 1988b). The filial 1 heterozygotes are then screened with l-penten-3-ol, facilitating the recovery of a wide variety of mutations ranging from missense to deletions and multi-locus deficiencies (Aaron, 1980; Kelley et al., 1985; Batzer et al., 1988b). Spermatozoa in *D. melanogaster* represent a germ cell stage which is comparable to spermatozoa in plants and animals. Thus the goal of this study is to determine the spectra of mutational events from *D. melanogaster* and relate this information to risk estimation in plants and animals, including humans.

The analysis of Adh null mutations consists of a number of informative tests. The first is a genetic complementation test. This test allows, through individual matings with various multiply marked fly stocks (adjacent loci), classification of mutants as intragenic (within the Adh locus) or intergenic (involving more than one locus) (Aaron et al., 1979; Ashburner et al., 1982; Kelley et al., 1985; Pastink et al., 1987; Batzer et al., 1988b). This test is very
useful, but may be subject to misclassification of inversions with break points in two or more loci, as well as multiple events which occur in two or more loci.

Molecular analyses consist of a number of nucleic acid hybridizations (Southern and northern blots of DNA and RNA respectively) as well as protein (western) blotting. These techniques require probes specific for the Adh locus such as $^3$P radiolabeled XbaI (Adh) DNA from the plasmid clone pSACL of Adh$^S$ for the nucleic acids (Goldberg, 1980) or a polyclonal antiserum directed against Drosophila ADH for the proteins (Batzer et al., 1988a,b; Hollocher and Place, 1987; LoMonaco et al., 1988). Mutants are analyzed by first attempting to identify the protein product from mutant alleles using western blots then, nucleic acids from the mutants are examined with Southern and northern blots to classify the mutational lesion. The nucleic acid hybridization techniques suffer from a lack of resolution. Southern blots can only resolve changes of 50 bp or larger unless a restriction site is created or lost, and northern blots resolve changes of 10% or more in length or intensity relative to a standard 50 (Kelley et al., 1985). Western blots also suffer from a lack of resolution, related to several factors including in vivo degradation, synthesis rates, and the presence of antigenic determinants (Batzer et al., 1988a,b). Therefore, these techniques by themselves are not enough to determine the exact location and nature of the newly induced mutations.

Molecular analysis at the DNA level utilizes gene cloning and DNA sequence analysis of the Adh null mutants. Until recently this type of analysis was far too time consuming and costly for eukaryotic
genes. The development of homologous recombination cloning (Seed, 1983) methodology has decreased the labor intensive portion of cloning mutations as well as eliminating the requirement for radioactive probes. Further new procedures such as the polymerase chain reaction (PCR) using a thermostable DNA polymerase should eliminate most DNA cloning and facilitate rapid analysis of a large number of mutants (Saiki et al., 1988). These methodologies, used in conjunction with DNA sequencing (and locus-specific primers) will allow the efficient detection and classification of mutations as point mutations (single base pair changes), intragenic deletions, inversions and duplications. Application of these tools to a wide range of mutagen induced mutations will enable establishment of risk estimates for man.


Sofer, W., and M.A. Hatkoff (1972) Chemical selection of alcohol dehydrogenase negative mutants in Drosophila, Genetics, 72, 545-549.


Stadler, L.J. (1928b) Mutations in barley induced by X-rays and radium, Science, 68, 186.


CHAPTER II: MOLECULAR ANALYSIS OF ALCOHOL DEHYDROGENASE ELECTROMORPHS IN WILD TYPE AND TRANSFORMED DROSOPHILA MELANOGASTER

Biochemical and Biophysical Research Communications
MOLECULAR ANALYSIS OF ALCOHOL DEHYDROGENASE ELECTROMORPHS IN WILD TYPE AND TRANSFORMED DROSOPHILA MELANOGASTER

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The protein expressed by the alcohol dehydrogenase locus (Adh) in D. melanogaster comprises a small group of electromorphs. We are able to study the expression of these electromorphs by electrophoretic separation and subsequent probing of blots of the separated polypeptides with antiserum for alcohol dehydrogenase (ADH). In the present study we have utilized this technique to study and compare the ADH electromorphs in wild type D. melanogaster with D. melanogaster transformants which carry an Adh gene from D. grimshawi, D. hawaiiensis or D. affinisdisjuncta and produce functional ADH (10,19). We have determined that polypeptides are produced by the donor loci in the transformed flies and further show that although the molecular weight of the expressed polypeptides is similar to D. melanogaster electromorphs, the isoelectric points are not similar. Thus this methodology offers the potential to study naturally occurring ADH electromorphs and null alleles independent of enzymatic activity assays.

The alcohol dehydrogenase locus (Adh) of Drosophila melanogaster is a single copy gene which has been sequenced (1). The gene contains four exons and a protein encoding region of 765 base pairs (1). The gene has two alternate promoters (larval and adult) separated by a 654 base pair intron (2). The alcohol dehydrogenase (ADH) polypeptide forms a homodimeric enzyme

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ABBREVIATIONS
AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate;
IEF-PAGE, isoelectric focusing polyacrylamide gel electrophoresis; kDa,
kilodaltons; NBT, nitro blue tetrazolium; PBS, phosphate buffered saline;
PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; TEMED,
N,N,N',N'-tetramethylethylene diamine.
which does not require any metallic cofactors and has a polypeptide subunit molecular weight of approximately 27kDa (3). The primary function of ADH is to metabolize primary and secondary alcohols thus serving as a detoxification mechanism in D. melanogaster (4). ADH from D. melanogaster is not homologous to horse or yeast ADH (3).

Natural populations of D. melanogaster are largely polymorphic for two Adh alleles (Adh$^F$ and Adh$^S$) (1). Both enzymatic and electrophoretic mobility differences exist for electromorphs coded for by these alleles (5). Two other alleles, Adh$^{LS}$ and Adh$^{LF}$, also exist in low frequencies within a small number of natural populations and code for two different electromorphs (3). Finally another functional allele (Adh$^D$), the result of an Adh$^F$ strain mutagenized by ethyl methane sulfonate, also exists solely in laboratory populations (6). These electromorphs differ from each other by single amino acid substitutions which produce charge changes in the ADH protein (7). The protein coded by Adh$^F$ differs from the Adh$^S$ protein by a Lys$\rightarrow$Thr substitution at amino acid 192 (1). Adh$^D$ coded protein differs from Adh$^F$ coded protein by a gly$\rightarrow$glu substitution at amino acid 232 (7). Thus the Adh locus in D. melanogaster codes for a number of differently charged electromorphs.

ADH electromorphs have generally been characterized using separation by non-denaturing electrophoresis and subsequent detection of ADH enzymatic activity (8,9). In the present study, we have compared ADH electromorphs using a specific ADH immunological probe rather than enzymatic activity for the detection of electromorphs. We demonstrate the specificity of the antiserum to D. melanogaster ADH, and use it as a probe to identify the charge (pI) and size (Mr) of ADH polypeptides which are separated by either IEF-PAGE or 1-D SDS PAGE (respectively) and then blotted onto nylon membranes.

MATERIALS AND METHODS

Materials

Ampholines were obtained from LKB. Acrylamide, leupeptin pepstatin, benzamidine HCl, PMSF, phenanthroline, Goat anti-rabbit AP conjugate, NBT.
Sephadex C50-80, BCIP, trizma base, and Nonident P-40 were obtained from Sigma. Alpha 2 macroglobulin was obtained from Cal BioTech. Ultra pure urea was obtained from Schwartz-Mann Bio. TEMED was obtained from IBI. Biotrans 0.2 μM nylon membrane was from ICN. Ammonium persulfate, precasted SDS-PAGE Mr markers and Affi-Gel Blue were obtained from Bio-Rad.

Drosophila Stocks
The following stocks were maintained homozygous for the Adh locus AdhS, Adhpr cn, Adhpr, and Adhpr cn. Drosophila embryos containing the null allele Adh were transformed using P element transformation with Adh genes from related drosophilids as previously described (10). Stocks AA2-3, J1-2 and G1-17 were created through transformation of the entire Adh gene from Drosophila affinitis and Drosophila hawaiensis and Drosophila grimshawi respectively into homozygous Adh D. melanogaster embryos. These stocks were maintained homozygous for a single P element insertion (10). Mutant Df(2L)BR41 was balanced with Df(2L)BR41b cn bw making it a homozygous deficiency for the Adh locus, and will be designated as DfBR41 throughout the paper (11). All Drosophila stocks were maintained as described previously (11).

Preparation of Immunological Probe
A rabbit antibody to ADH was prepared and used as a probe for protein (western) blot analysis. ADH was isolated from an Oregon-R strain of Adh (both fresh and frozen flies were used). This antigen was isolated in homogenization buffer (125 mM Tris-HCl pH 7.5) with aprotonin protease inhibitor (10% v/v) added, ammonium sulfate (40% than 60%) precipitated, and isolated on an Affi-Gel Blue affinity column (size 100-200 mesh). The column was washed with homogenization buffer and eluted with 3 mM NADH. Specific activity of ADH was determined using UV spectrophotometry according to the method of Sofer and Ursprung (4). Rabbit* were injected intramuscularly with 250 μg of the antigen at 14 day intervals by Bethyl Laboratories, Montgomery, Texas. The antiserum, received from Bethyl Laboratories was screened by (1) inhibition of ADH activity and (2) Enzyme-Linked Immunosorbent Assay (ELISA) [data not shown].

Preparation of Protein Samples
Five adult flies (12) were decapitated and homogenized in 70 μl of homogenization buffer for the wild type and transformed lines. The homogenization buffer consisted of 125 mM Tris-HCl pH 7.5 with the following protease inhibitors: 200 μM leupeptin; 165 μM pepstatin; 600 μM benzamidine-HCl; 1.4 μM alpha 2 macroglobulin; 574 μM PMSF; 50 μM 1,10-phananthroline. The homogenate was then centrifuged for 10 min at 16,000 x g.

2 D PAGE
5 μl of sample preparation (described above) was added to 5 μl of lysis buffer (13) and then added to each isoelectric focusing gel. Isoelectric focusing was done according to O'Farrell (13); however, the second dimension was a 12.5% SDS polyacrylamide separating gel with a 6% stacking gel.

1 D SDS-PAGE
40 μl of the sample preparations (above) were added to 160 μl of SDS-PAGE lysis buffer (14) for the wild type and transformed stocks. The samples were heated at 100°C for 2 to 3 min then, 40 μl or 120 μl for the normal and transformed lines respectively, was applied to each well of a 12.5% polyacrylamide separating gel with a 6% stacking gel. The samples were run for 4 hr at 10 mA (constant current) through the stacking gel and 8 hr at 25 mA (constant current) through the separating gel. The electrophoretic mobility relative to several standards was then used to determine polypeptide subunit Mr (15). The standards used were ovalbumin (50kDa), carbonic anhydrase (39kDa), soybean trypsin inhibitor (27kDa) and lysozyme (17kDa).
IEF-PAGE

40 μl of sample preparation (described above) was added to 160 μl of lysis buffer (13) for the normal and transformed Drosophila stocks. Samples (40 μl for normal and 120 μl for transformed stocks, respectively) were added to each well of a 4% IEF gel. The samples were run at 10 mA (constant current) for 5 hr and then 3 mA (constant current) for 12 hr. The ADH protein was detected in all three separation techniques described above by western blot analysis. Electrophoretic mobility was then measured relative to pi standards Adh^p (pI=6.91), Adh^P (pI=7.74) and Adh^D (pI=6.24) to determine denatured polypeptide subunit pi's (16).

Western Blots

Transfer was conducted according to the method of Towbin et al. (17) except that Bio-Trans blotting membrane and 20% EtOH replaced nitrocellulose and NaOH, respectively. Proteins were electrophoretically transferred for 12 hr at 30 V (constant voltage). After transfer, blots were incubated for 12 hr in Dulbecco's PBS/5% non-fat dry milk to prevent non-specific antibody binding, and then placed in 1:10 (antibody/blocking solution) dilution of primary antibody for 24 hr. Primary antibody was then removed and the blots were washed 3 hr in 6 changes of Dulbecco's PBS. The blots were placed in 1:500 dilution of secondary antibody (Goat anti-rabbit IgG-AP conjugate) for 24 hr at 12°C. The membranes were washed as before, and the bound antibody was visualized by reacting the AP conjugate with NBT and BCIP.

RESULTS

Specificity of the ADH antiserum

The specificity of the immunological probe is illustrated in Figure 1.

Figure 1A and B are western blots of D. melanogaster proteins separated by 2-D PAGE. The first dimension is isoelectric focusing, and the second is SDS-PAGE (molecular weight). Figure 1A is a blot of proteins from Adh^p/Adh^P  

![Figure 1. Specificity of the immunological probe. Western blot analysis of 2-D polyacrylamide gels of A) proteins from Adh^p/Adh^P heterozygotes, and B) DfBK41 heterozygotes. Isoelectric focusing is in the horizontal direction (cathode to the right) and SDS-PAGE in the vertical direction. Proteins were transferred to nylon membranes and probed with rabbit ADH antiserum followed by goat anti-rabbit IgG AP conjugate. Bound antibodies were visualized by reacting AP conjugate with NBT and BCIP. Molecular weights (kDa) were obtained from prestained Mr markers.](image-url)
heterozygous flies showing that ADH antiserum recognizes two polypeptides at 27kDa and one polypeptide at 39kDa. Figure 1B is a blot of separated proteins from DfBR41 heterozygous flies in which the Adh gene is completely deleted (11). This blot only shows reactivity of ADH antiserum to the 39kDa polypeptide indicating that this is not the product of the Adh gene, and that the 27kDa polypeptides in Figure 1A are in fact the ADH polypeptides. Thus, the polyclonal antiserum recognized the ADH polypeptide (Mr=27kDa) and also cross-reacted with a 39kDa polypeptide.

**ADH polypeptide molecular weight analysis**

One dimensional SDS-PAGE and western blotting was used to screen for any deviation in Mr in the normal and transformed *D. melanogaster* stocks. Figure 2 shows that all of the *D. melanogaster* electromorphs (Adh, Adh, Adh, and Adh) produced an ADH polypeptide with normal Mr (27kDa). Mutant Df F R-1 produced no detectable ADH polypeptide (Figure 2, lane E). Adh, the stock used for transformation, also produced no detectable ADH polypeptide (Figure 2, lane I). This result for Adh is in agreement with a previous study using different ADH antisera (18). All three transformed Drosophila stocks produced an ADH polypeptide of approximately the same molecular weight as the ADH electromorphs of *D. melanogaster* (Figure 2). Polypeptide subunit Mr was then determined for 3 Hawaiian electromorphs and one *D. melanogaster* (Adh) electromorph and is summarized in Table 1.

![Figure 2](https://example.com/figure2.png)

*Figure 2*: Molecular weight analysis of ADH electromorphs expressed by different Adh alleles. Western blot of an SDS polyacrylamide gel. Lane (A) Adh. (B) Adh. (C) Adh. (D) Adh. (E) Df BR-1. (F) D. affinis, (G) D. grimshawi, (H) D. hawaiiensis and (I) Adh. Lanes F, G and H were from transformed *D. melanogaster* stocks (see text). Proteins were electrophoretically transferred to nylon membranes and detected as described in Fig. 1. Molecular weights (kDa) were obtained from prealigned Mr markers.
Table 1. Molecular Properties of ADH Polypeptide Subunits

<table>
<thead>
<tr>
<th>Species</th>
<th>Molecular Weight</th>
<th>Isoelectric Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. grimesi</td>
<td>26.30 ± 0.70</td>
<td>8.54 ± 0.11</td>
</tr>
<tr>
<td>D. hawallensis</td>
<td>26.87 ± 0.33</td>
<td>8.00 ± 0.09</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>26.50 ± 0.09</td>
<td>8.26 ± 0.06</td>
</tr>
<tr>
<td>D. affinitis-lunada</td>
<td>26.75 ± 0.96</td>
<td>8.54 ± 0.11</td>
</tr>
</tbody>
</table>

1. Molecular weight in kilodaltons ± standard deviation (n=7)
2. Isoelectric point (pI) ± standard deviation (n=6)
3. Determined from transformed D. melanogaster stocks (see text)
4. Adh allele

ADH polypeptide charge analysis

Denaturing IEF-PAGE and western blotting was used to screen ADH polypeptides on the basis of pI. Figure 3 shows that each of the wild-type D. melanogaster stocks produced a detectable ADH polypeptide with a unique pI. These electromorphs differ from each other by single amino acid substitutions (1,3,7). All three of the D. melanogaster transformants produced an ADH polypeptide with a pI that was different than any of the wild-type D. melanogaster electromorphs (Figure 3). Polypeptide subunit isolectric points were determined for 3 Hawaiian Adh electromorphs and one D. melanogaster (Adh US) electromorph (Table 1). These results demonstrate that the ADH polypeptide found in the transformed flies must be the product of the transformed Adh genes from the Hawaiian drosophilids (D. grimesi).
DISCUSSION

Several important findings have been determined in this study. First, we have developed and determined the specificity of antisera to D. melanogaster ADH. Second, we have used IEF-PAGE and 1-D SDS-PAGE in conjunction with western blotting to analyze the electropherograms coded for by Adh alleles from different species of Drosophila. Finally, as discussed below, we have shown that this type of polypeptide subunit characterization (Mr and pi) correlates well with DNA sequence data.

The data presented here indicate that the ADH antisera is very specific and only cross-reacts with ADH (Mr=27kDa) and one other polypeptide (Mr=39kDa) which allows it to be distinguished from the product of the Adh locus. In addition the isoelectric point of the 39kDa protein (pi=9.2) is outside the region in which ADH electrophorograms focused (pi 8.54 - 6.24). Therefore this polypeptide does not interfere with characterization of ADH subunit Mr or pi. The hybridization intensity of the Hawaiian ADH's is not as intense as that of the D. melanogaster electrophorograms. There are two possible reasons to explain this. First, the regulatory sequences present in Hawaiian alleles are not identical to those of the D. melanogaster Adh gene, therefore expression of Hawaiian Adh genes is characteristic of those species; and the amount of detectable ADH is characteristic different between species (10). Second, changes in the polypeptide primary structure of Hawaiian ADH may lead to loss or partial loss of an epitope and decreased antibody recognition.

Previous studies demonstrated that functional ADH polypeptides are produced in D. melanogaster stocks transformed with a D. affinidisi juncta Adh gene, and that the production of Adh mRNA and protein is developmentally regulated and tissue specific (10). However, the tissue specific distribution of ADH in the transformed flies is not identical to that of the donor or host species (i.e. D. affinidisi juncta or D. melanogaster.
respectively) (10). Although these results indicate that there may be
imperfect interaction of cis- and trans-acting regulatory components in
transformed flies, they demonstrate both correct processing of mRNA
transcripts, and a lack of any species specific post translational
modifications of ADH required for enzymatic activity. Furthermore the
electrophoretic separation techniques reported here (IEF-PAGE and 1-D
SDS-PAGE) are denaturing, resulting in the loss of some types of
post-translational polypeptide modifications. Also, electrophoresis on agar
noble gels, followed by staining for ADH enzymatic activity, indicates that
each of the Hawaiian Drosophila isozymes migrates identically whether
obtained from the donor species or from transformants (19). Therefore, the
subunit Mr and pi presented here are similar to those of wild type Hawaiian
flies. This study provides further support for the usefulness of transformed
D. melanogaster stocks in studying the properties of Adh genes from related
drosophilids.

The subunit Mr determination for 3 Hawaiian and 1 D. melanogaster ADH
electromorphs (Mr=27kDa) were not significantly different from each other,
and compare favorably with previous determinations based on amino acid
compositions of other D. melanogaster electromorphs (AdhF, AdhS) (3). The
Adh gene from D. affinidisjuncta has been sequenced; translation of the
protein coding region indicates that an ADH polypeptide the same size as D.
melanogaster is produced (20). The loss or gain of 10 amino acids is the
minimum detectable Mr difference using 12.5% polyacrylamide gels and western
blotting (11). However, a loss or change as small as a single amino acid
which alters the polypeptide pi is detectable. The subunit pi's of the
Hawaiian drosophilids were different than those of any D. melanogaster
electromorph with D. affinidisjuncta and D. grimshawi being identical
(pi=8.54), and D. hawaiiensis being substantially different (pi=8.0). These
data indicate that the ADH polypeptide (and protein encoding region of the
gene) from D. grimshawi should show a high degree of homology with the D.
affinidisjuncta ADH polypeptide (and Adh gene), and a lower degree of
homology with the ADH polypeptides (or genes) from D. hawaiiensis or any D. melanogaster electromorph. The Adh genes from D. grimshawi and D. hawaiiensis have been sequenced, and show the similarities predicted by the subunit Mr and pI analysis presented in this report (21).

The picture winged Hawaiian drosophilids (subgenera Drosophila) are distantly related to D. melanogaster (subgenus Sophophora), with an estimated divergence time of 40 and 60 million years based on historical (22) and immunological (23) data respectively. A comparison of the predicted amino acid sequence of the D. affinitisuntra Adh gene shows a high degree of homology with a D. melanogaster Adh5 electromorph, with only 54 out of 254 amino acid substitutions (20). This sequence homology and time of divergence indicate the potential application of this ADH antiserum for alcohol dehydrogenase subunit characterization in other species of Drosophila.

Estimates of genetic heterozygosity are often made based on data from electromorph analyses using detection systems which require enzymatic activity (24). In these systems active/null heterozygotes are mistaken for homozygous individuals, which leads to an apparent deficiency in number of heterozygotes as compared to Hardy-Weinberg expectations (25). In organisms such as oysters, models involving segregation of null alleles have been used as a possible explanation of heterozygote deficiencies and have been substantiated by pairwise crosses and sequential electrophoresis of offspring from pairwise crosses (26). Adh null alleles have also been found in a number of natural Drosophila populations (27,28,29,30). IEF-PAGE and 1-D SDS PAGE used in conjunction with western blotting offer the advantage of electromorph detection based on immunological recognition rather than functional (enzymatic) activity, and should prove useful for the detection of naturally occurring Adh null alleles (that produce non-functional ADH polypeptides). This methodology has already proven useful in the analysis of polypeptides expressed by experimentally induced Adh null alleles (11,16,31).

ACKNOWLEDGEMENTS
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REFERENCES

CHAPTER III: SPECTRA OF MOLECULAR CHANGES INDUCED IN DNA OF DROSOPHILA SPERMATOZOA BY 1-ETHYL-1-NITROSOUREA AND X-RAYS

Mutation Research
Spectra of molecular changes induced in DNA of Drosophila spermatozoa by 1-ethyl-1-nitrosourea and X-rays

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Summary

Mutations induced in Drosophila spermatozoa at the alcohol dehydrogenase Adh locus by 1-ethyl-1-nitrosourea (ENU) were compared to X-ray-induced mutations using genetic tests for complementation, southern blotting, western blotting and northern blotting. 8 of 10 ENU-induced mutations complemented all known adjacent loci and were presumed to be intragenic. In contrast, 8 of 30 X-ray-induced mutations were intragenic. Southern blot analysis showed that 2 of 7 intragenic mutations induced by X-rays were altered at the Adh locus, whereas all 8 intragenic ENU mutants appeared normal. Western blot analysis showed 4 of 7 intragenic mutants induced by X-rays produced a detectable polypeptide; 1 of the 4 had normal molecular weight and charge. In contrast, 7 of the 8 intragenic mutants induced by ENU produced a polypeptide of normal molecular weight and charge. One ENU and two X-ray-induced mutants, which had normal southern blots and no detectable polypeptide, produced normal molecular weight mRNA by northern blots. The interpretation of these results is that in spermatozoa X-rays induce primarily deletions that either produce deficiencies of the Adh locus or nonsense mutations within the locus, whereas ENU induces primarily missense mutations. This forward mutation assay based on loss of enzymatic activity efficiently recovered a broad spectrum of mutations ranging from missense to intragenic deletions and multi-locus deficiencies. Only 3 of these 40 mutations produced a polypeptide detectable as an electrophoretic variant.

The mechanisms of mutagenesis can be studied by comparing the changes in mutational spectra of different mutagenic agents. The detection of unique mutational spectra will be of great value when methods for monitoring DNA from humans are developed. In order to compare spectra of mutations induced by different mutagens in germ cells, comparisons must be made within a single germ cell stage because of great differences in mutagenic responses between germ cell stages. We have chosen to compare mutations induced by X-ray with those induced by 1-ethyl-1-nitrosourea (ENU) at the Adh locus in spermatozoa of Drosophila melanogaster.

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Spermatozoa represent a unique stage in germ-cell development in Drosophila and probably in man because mutational lesions accumulate but are not subject to repair (Würgler et al., 1972; Vogel et al., 1985) or fixation as a mutation through DNA replication until fertilization. The post-repair stage of human spermatogenesis is the only stage in human biology where mutational lesions may accumulate without being subject to repair; therefore, mechanisms of mutagenesis requiring multiple lesions may have greater importance in this stage in man.

A series of mutations at the Adh locus were induced by X-rays in Drosophila spermatozoa by Aaron (1979). We have used the same mating and selection systems as Aaron, so that our mutations induced by ENU may be compared to his X-ray-induced mutations. In previous studies, X-ray-induced mutations were analyzed by classical genetic analysis [using complementation mapping with known deletions (Aaron, 1979; Ashburner et al., 1982)] and molecular analysis [using southern blots and silver staining of proteins (Kelley et al., 1983) and in vitro synthesis of peptides (Chia et al., 1985)]. We have extended these analyses to include improved resolution of southern blots and the use of western and northern blots. The data were analyzed with the same genetic and molecular tests to permit the comparison of the mutational spectra from the 2 mutagens. From classical genetic analysis, it was suspected that there would be a wide range of mutational events detected by this mutational assay. Our finding is that the spectra of mutational events are even broader when viewed at the molecular level than when analyzed only by classical genetics.

The method of detection of mutation is a forward mutation assay (Sofer and Hatkoff, 1972) where one detects the loss of enzymatic activity of the alcohol dehydrogenase enzyme (DADH) in Drosophila melanogaster. Adh<sup>−</sup> mutations under natural conditions would be a conditional lethal (lethal at greater than 5% ethanol concentration), whereas under laboratory conditions, in the absence of alcohol, normal viability is observed. The gene which codes for DADH is a single copy gene with 2 introns in the 765 bp coding region (Benyajati et al., 1983a; Kreitman, 1983). The Adh locus has 2 promoters, one for larvae and one primarily used in adults with a 654-bp intron between the adult promoter and the larval promoter (Benyajati et al., 1983a). Kreitman (1983) has shown the Adh locus to be highly conserved in D. melanogaster, an insect which has world-wide distribution. He cloned and sequenced 11 Adh genes from 5 different populations in Europe, America, and Asia and found 43 single nucleotide polymorphisms with only one that resulted in an amino acid change. The single amino acid change observed by Kreitman (1983) is the common A to C polymorphism that results in a lysine to threonine polymorphism associated with Adh<sup>1</sup> and Adh<sup>3</sup> phenotypes, respectively. The polypeptide forms a homodimer that does not require a metal cofactor for activity and reduces both secondary and primary alcohols (Sofer and Ursprung, 1986). DADH is not homologous with horse or yeast alcohol dehydrogenase (Thatcher, 1980).

Here we report the contrast in mechanisms of mutagenesis between X-rays and ENU as observed by both molecular and classical genetic analysis of their induced mutations. Since the X-ray-induced mutants have also been analyzed by other molecular tests, we have an opportunity for comparing the information obtained by different tests at the molecular level.

Materials and methods

Chemicals and enzymes

Ampholines were obtained from LKB. Acrylamide, leupeptin, pepstatin, benzamidine HCl, phenylmethylsulfonyl fluoride, phenanthroline, goat anti-rabbit alkaline phosphatase conjugate, nitro blue tetrazolium, Sephadex G50-80, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), agarose, trizma-base, and nonident P-40 were obtained from Sigma. α-Macroglobulin was obtained from Cal Biotech. Urea was obtained from Schwartz-Mann Biotech. TEMED was obtained from IBI. Biotrans 0.2 μM nylon membrane was obtained from ICN. Ammonium persulfate, prestained SDS-PAGE molecular weight standards, and Affi-Gel Blue were obtained from Bio-Rad. GeneScreen Plus hybridization membrane and 32P was obtained from New England Nuclear. X-Omat XAR-2 film. GBX developer and rapid fixer were obtained from Eastman Kodak. Lightning Plus
intensifying screens were obtained from DuPont. Restriction endonucleases and nick translation reagent kit were obtained from Bethesda Research Laboratories Ltd.

Induction of mutations with 1-ethyl-1-nitrosourea (ENU)

1-Ethyl-1-nitrosourea (ENU), CAS No. 759-73-9, was dissolved in 0.01 M sodium acetate buffer pH 4.5 (NaOAc buffer) injected into a Sigma isopac bottle. The concentrated stock solution was diluted 1000-fold and the UV spectrum of the ENU solution was checked (238 nm maximum). The ENU concentration was determined at 238 nm using a reference standard provided by Gary Sega. Oak Ridge National Laboratory. The ENU was diluted using NaOAc buffer to a final concentration of 5 mM, and 0.75 ml of the ENU solution was then fed to 100 3-5-day-old net cn males for 24 h in a glove box. The procedure for feeding on a glass support has been previously described (Aaron et al., 1977). These mutants were selected in the F1 over the mutant Adh+ which is a mutant that is homozygously viable and hemizygously viable over a large deficiency Df(2L)64j. Selection of the Adh mutants over a mutant that was viable with a large deficiency permitted the recovery of large deficiencies that included the Adh locus. The mutants were selected using a screen based on the procedures of Sofer and Hatkoff (1972) and Aaron (1979) that exposed adults to 1-penten-3-ol, a secondary alcohol which Adh positive flies convert to a toxic ketone and die, thus permitting survival of Adh null lines. A diagram of the isolation scheme is shown in Fig. 1.

Drosophila stocks

Wild-type (control) stocks AdhS, AdhL5, net AdhF cn, b AdhF cn bw and AdhD pr cn were maintained homozygous for the second chromosome. All stocks including the null mutants described below were maintained at 25°C on standard corn meal, corn sugar, agar, brewer's yeast medium containing propionic acid as a mold inhibitor.

Null-mutations recovered at the Adh locus are given a unique number following our laboratory designation, BR#. If the mutation is a multi-locus deficiency, it is designated Df(2L)BR#. (Lindsley and Grell, 1968): if the mutation is intragenic as shown by complementation of adjacent loci, it is designated AdhBR# even though the mutation may be an intragenic deletion. The ENU-induced mutations reported in this paper are numbered 28-56 with some of the intervening numbers assigned to phenocopies that were later discarded. For simplicity, we will designate in this paper AdhBR28 as nBR28 and Df(2L)BR41 as DfBR41. The 10 ENU mutants reported in this paper were induced at Baton Rouge in the stock net AdhF cn; this stock was used as a control for the ENU-induced mutants. Mutants nBR28 and nBR43 were homozygous viable. Mutant DfBR41 was balanced with Df(2L)A48,b cn bw. Mutants nBR50, nBR51 and nBR54 were hemizygous over Df(2L)A47,b cn bw. Mutant DfBR55 was balanced with Int(2LR)O.Cy dp1AdhBR# pr cn. Mutant nBR56 was balanced with chromosome Df(2L)64j, b.

The X-ray-induced mutants were induced by
C.S. Aaron at the University of Leiden, the Netherlands (Aaron, 1979) and deposited with M. Ashburner, Cambridge, England and our laboratory when Dr. Aaron returned to the United States. The mutants classified as deficiencies were designated \( Df(2L)A^{*} \), whereas the intragenic mutants were designated \( Adh^{nL} \). In this paper we will designate these as \( DfA \) and \( nL \) respectively.

The following X-ray mutants were obtained from C.S. Aaron (1979): \( nLA2, nLA73, nLA74, nLA80, DfA178, nLA248, nLA249, nLA252, DfA379 \) and \( DfA445. \) The spontaneous mutant, \( nLA319. \) was also obtained from C.S. Aaron (1979). Mutants \( nLA2, nLA73, nLA248, nLA249 \) and \( nLA252 \) were homozygous viable. Mutants \( nLA74, nLA80 \) and \( nLA319 \) were placed over chromosome \( Df(2L)A^{4j}, \) a very large second-chromosome deficiency which included the \( Adh \) region (Woodruff and Ashburner, 1979a,b). Mutants \( DfA178, DfA379 \) and \( DfA445 \) were maintained over the second-chromosome balancer \( \text{In}(2LR)0, \) \( Cy \) (Lindsey and Grell, 1968). The stock, \( h Adh \) \( cn \) \( bw \), which Aaron (1979) used to induce his mutants was obtained from Dr. Aaron and used as a control for all of the X-ray-induced mutants except the spontaneous mutant, \( nLA319, \) which arose on the chromosome \( Adh^{Dpr} cn \) (Aaron, 1979). Mutant \( nLA378 \) was lost in transfer to our laboratory and also was not transferred to Cambridge University. We were not able to locate \( nLA378 \) in any laboratory.

Evidence that \( nLA405 \) is \( Adh^{n23} \) and was recovered from a crossover was obtained from sequencing the mutant (Lomonaco et al., 1988). Therefore, \( nLA405 \) will not be considered an X-ray-induced mutant. The remaining \( 7 Adh^{nL} \) mutants could not be a crossover with \( Adh^{n23} \) because mutant \( Adh^{n23} \) is a 34-bp deletion at the 3' end of the \( Adh \) gene that results in the loss of an \( Avall \) restriction site (at bp 2317) (Benyajati et al., 1983b). The loss of this restriction site results in the formation of a 1236 bp fragment in an \( Avall-Xbal \) double digest. Southern blot analysis, using nick translated \( Xbal \) fragment as a probe, showed the presence of only 2 bands (1240 bp and 649 bp) for \( Adh^{n23} \). None of the 7 intragenic X-ray mutants presented here displayed similar restriction patterns; therefore, we conclude they do not represent crossovers with \( Adh^{n23} \).

### Genetic test for complementation

The mutants were first balanced over \( Int2LrIO, \) \( Cy \) \( dp^{11}, Adh^{nB} \) \( pr \) \( cn^{*} \) \( (Cy \) \( O \) \) and then the genetic test for complementation was conducted by using the following deficiencies: \( Df(2L)A^{4j}, Df(2L)A^{48}, Df(2L)A^{47}, Df(2L)A^{63} \) and \( Df(2L)A^{379}. \) These deficiencies were balanced over the second-chromosome balancer \( Cy \) \( O. \) A cross between the new mutant balanced over \( Cy \) \( O \) and a known deficiency balanced over \( Cy \) \( O \) will give a 2:1 ratio of curly to non-curly flies if the mutation is viable over the deficiency.

### Isolation of Drosophila DNA for southern analysis

The DNA used for southern blot analysis was isolated by a procedure adapted from Bingham (1981). Adult Drosophila (approximately equal to 1 gram), frozen in liquid nitrogen, were homogenized in 30 ml nuclear isolation buffer at pH 7.5 and 4°C containing 100 mM Tris-HCl, 100 mM NaCl, and 20 mM EDTA. Homogenization was done using a Tekmar tissumizer. Large particles were removed by filtering through sterile gauze under aspiration. Nuclei were collected by centrifugation for 10 min at 1500 \( \times g \) at 4°C. The nuclear pellet was resuspended in 20 ml isolation buffer using a 10-sec agitation, and centrifuged as before. The nuclear pellet was then resuspended in 3 ml lysis buffer containing 50 mM Tris-HCl, 25 mM sodium lauroyl sarcosine, and 20 mM EDTA. The lysate from this step was placed at 50-55°C for 30-60 min. 1.1 g of CsCl per gram of lysate was added and this solution was placed at 50-55°C for 30 min. This solution was centrifuged for 16 h at 55K rpm in a Sorvall TV865 rotor at 20°C. The viscous fractions were collected and dialyzed against 10 mM Tris and 1 mM EDTA for at least 24 h. The sample was scanned for purity with a UV spectrophotometer from 320 to 230 nm and stored at 4°C.

### Transfer to GeneScreen Plus

5 \( \mu \)g of genomic DNA was digested with the designated enzymes and electrophoresed on a 1.5-2.0% agarose gel. Agarose gels were treated with 0.2 N NaOH and 0.5 M NaCl for 30 min at room temperature. The gels were then treated with 200 mM Tris, 100 mM NaOAc.
and 10 mM EDTA at pH 8.3 for 15 min. This was repeated twice. The denatured gels were then placed in an IBI Horizontal Blotting System and the DNA was transferred electrophoretically at 2 AMPS for 1.5 h onto GeneScreen Plus hybridization membrane.

**Hybridization**

To minimize background, the blots were soaked in 15 mM NaCl, 1.5 mM Na Citrate, 1% SDS at 65°C for approximately 1 h. The blots were prehybridized for 15–30 min at 42°C (Reed and Mann, 1985). This was followed by hybridization with the heat denatured 32P labeled probe in the same solution for 16–20 h at 42°C. The membranes were washed according to Reed and Mann (1985). Membranes were exposed to XAR-2 film with Lightning Plus Intensifying Screens for 24 h at −70°C.

**Isolation of XbaI probe**

The 3.2-Kb XbaI fragment from the pSAC1 Adh clone (Goldberg, 1980) was isolated by digesting the clone to completion at 37°C with Xbal. The sample was run on a 1.5% agarose mini-gel at 100 V for 1–2 h. The 3.2-Kb fragment was electroeluted from the gel, purified by direct extraction, and quantified by ethidium bromide fluorescence according to Maniatis et al. (1982).

DNA fragments (XbaI) were radiolabeled with [15P]dATP (3000 Ci/mnmole) using a nick translation kit according to the manufacturers directions. Labeled DNA was separated from unincorporated nucleotides by filtration through a Sephadex G-50-80 column in 10 mM Tris, 1 mM EDTA (pH 8.0). The typical yield was 2.7 x 10^6 dpm/μg of DNA.

**Preparation of immunological probe**

A rabbit antibody to DADH was prepared and used as a probe for western blot analysis. DADH was isolated from an Oregon-R strain of Adh7 (both fresh and frozen flies were used). This antigen was isolated in homogenization buffer (125 mM Tris–HCl pH 7.5) with apronin protease inhibitor added, ammonium sulfate (40% then 60%) precipitated, and isolated on an Affi-Gel Blue affinity column (size 100–200 mesh). The column was washed with homogenization buffer and eluted with 3 mM NADH. Specific activity of DADH was determined using UV spectrophotometry according to the method of Sofer and Ursprung (1968). Rabbits were injected intramuscularly with 250 mg of the antigen at 14-day intervals by Bethyl Laboratories, Montgomery, TX. The antisera, received from Bethyl Laboratories, was screened by (1) inhibition of DADH activity and (2) Enzyme-Linked Immunosorbent Assay (ELISA) [data not shown].

**Preparation of protein samples**

5 adult flies (Kelley et al., 1985) were decapitated and homogenized in 70 μl of homogenization buffer. The homogenization buffer (above) also contained the following protease inhibitors: 200 μM leupeptin; 145 μM pepstatin; 600 μM benzamidine–HCl; 1.4 μM α-macroglobulin; 574 μM phenylmethylsulfonyl fluoride; 50 μM 1,10-phenanthroline. The homogenate was centrifuged for 10 min at 14000 r.p.m. using an Eppendorf 5415 microfuge.

**1-D SDS-PAGE**

10 μl of the sample preparations (above) were added to 40 μl of SDS–Page lysis buffer (Laemmli, 1970). The samples were heated at 100°C for 2–3 min and then applied to each well of a 12.5% polyacrylamide separating gel with a 6% stacking gel. The samples were run for 4 h at 18 mA (constant current) through the stacking gel and 8 h at 25 mA (constant current) through the separating gel.

**IEF-PAGE**

5 μl of sample preparation (described above) were added to 45 μl of lysis buffer (O’Farrell, 1975). 25 μl of this mixture were added to each well. The samples were run at 10 mA (constant current) for 5 h and then 5 mA (constant current) for 12 h.

**Two-dimensional gel electrophoresis**

5 μl of sample preparation (described above) were added to 5 μl of lysis buffer (O’Farrell, 1975) and then added to each isoelectric focusing gel. Isoelectric focusing was done according to O’Farrell (1975); however, the second dimension was a 12.5% SDS polyacrylamide separating gel with a
6% stacking gel. The DADH protein was detected in all 3 separation techniques (described above) by western blot analysis.

Western blots

Transfer was conducted according to the method of Towbin et al. (1979) except that BioTrans blotting membrane and 20% EtOH replaced nitrocellulose and MeOH, respectively. Proteins were electrophoretically transferred for 12 h at 30 V (constant voltage). After transfer, blots were incubated for 12 h in Dulbecco’s PBS/5% non-fat dry milk to prevent non-specific binding, the membranes were placed in 1:10 (antibody/blocking solution) dilution of primary antibody for 24 h. Primary antibody was then removed and the blots were washed 3 h in 6 changes of Dulbecco’s PBS. The blots were placed in 1:500 dilution of secondary antibody (goat anti-rabbit IgG-alkaline phosphatase conjugate) for 24 h at 12 °C. The membranes were washed as before, and the bound antibody was visualized by reacting the alkaline phosphatase conjugate with nitro blue tetrazolium and 5-brom-4-chloro-3-indolyl phosphate (BCIP).

Results

Induction of mutations

7 ENU-induced Adh null mutants, numbered nBR50 and above, were recovered from an examination of 25118 F1 test progeny. All 7 of these mutants were induced by treating spermatozoa. The level of ENU exposure (5 mM) led to a mutation frequency of $2.7 \times 10^{-4}$ for the Adh locus. The same treatment level also gave a sex-linked recessive lethal response of 35% that is 270 times the spontaneous frequency (data not shown). Mutants nBR28 and DfBR41 were induced in females, and nBR43 was induced by 200 mM ENU treatment of spermatozoa; therefore, they are not included in the mutation frequency estimate. The X-ray-induced mutants were also induced in sperm or late spermatids by Aaron (1979) using 100 kV X-rays at 5 Gy, 10 Gy and 30 Gy. This exposure level induced 10–100 times the spontaneous mutation frequency. For the doses used in this study, it is probable that the recovered mutants are induced and not of spontaneous origin.

Genetic complementation analysis

10 null mutants were recovered at the Adh locus following treatment with ENU and subsequent selection of mutants with exposure of adults to 1-penten-3-ol (Sofer and Hatkoff, 1972). The mutants were first balanced over In(2LR+o. $C_{i}^{1/2}$ $Adh_{n}$ $pr cn^{2}$ and then the genetic test for complementation was conducted by using the following deficiencies: Df(2L)64, Df(2L)A44, Df(2L)A47, Df(2L)A63, and Df(2L)A79. One Adh null-mutant (DfBR41) failed to complement the adjacent loci outspread (osp) and no-ocelli (noc), and at least 1 lethal distal to noc. Complementation data show that DfBR41/Df(2L)A79 is noc osp Adh, while DfBR41/Df(2L)A47 and DfBR41/Df(2L)A64 are lethal. DfBR41/Df(2L)A44 and DfBR41/Df(2L)A63 are viable osp Adh. Mutant DfBR55 is a deficiency that is lethal in combination with Df(2L)A64 and Df(2L)A48. Only 2 flies that were noc osp and Adh were produced from a cross with Df(2L)A48 in a population of 131 flies, which should have contained 44 noc osp Adh flies (expected ratio 2:1). All 8 other ENU-induced mutants comple-
TABLE 1
GENETIC COMPLEMENTATION DATA FOR ENU-IN-
DUCED Adh NULL MUTANTS

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Genetic loci</th>
<th>nor</th>
<th>esp</th>
<th>1(2)35Bb</th>
</tr>
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<tr>
<td>nBR28</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D(2L)BR41</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>nBR43</td>
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<td>nBR54</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Df(2L)BR55</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>nBR56</td>
<td>-</td>
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</table>

+ : Complements locus
- : Does not complement locus

Complemented all adjacent loci. These data are summarized in Table 1. Complementation data for X-ray-induced mutants were reported by Aaron (1979) and Ashburner et al. (1982).

Southern blot analysis
DNA from the 8 ENU-induced and 7 X-ray-induced (1 X-ray mutant had been lost) intragenic Adh null mutants, from flies either homozygous or heterozygous over a deficiency for the Adh locus, was digested with XbaI and EcoRI restriction endonucleases. DNA from flies homozygous for deficiency of the Adh gene (DfBR41/DfA48) was used as a negative control. These digests were examined by southern blots using a 3.2-kb XbaI restriction fragment from a genomic Adh clone as a probe. The XbaI fragment has been shown to include the structural gene for Adh and all essential cis-acting regulatory elements necessary to complement an Adh null allele (Goldberg et al., 1983). A diagram and restriction map of the Adh locus is shown in Fig. 2. Our results show that DNA from 2 of the 7 intragenic X-ray mutants (nLA2 and nLA248) contained alterations (Fig. 3a), whereas DNA from 5 intragenic ENU mutants (nBR28, nBR43, nBR50, nBR51 and nBR53) produced normal southern blots (Fig. 3b). Mutants nBR53, nBR54 and nBR56 are not shown, but were also normal. Therefore all 8 intragenic ENU mutants appeared normal. Mutant DfBR41 produced no detectable DNA (Fig. 3b). Finally nLA319, a spontaneous mutant of Adh70, contained no detectable alterations (Fig. 3a). The data on individual mutants are summarized in Table 2.

Western blot analysis
Since the majority of intragenic Adh mutants (8 of 8 intragenic ENU mutants and 5 of 7 intragenic X-ray mutants) contained normal DNA as detected by southern blots, we attempted to ascertain whether any of these mutants produced a detectable and/or modified alcohol dehydrogenase protein. Western blots of electrophoretically separated proteins from flies homozygous or heterozygous over a deficiency for the Adh locus were probed with polyclonal rabbit DADH antiserum.

Fig. 4(A and B) shows western blots of proteins separated by two-dimensional polyacrylamide gel electrophoresis (2-D gels) (O'Farrell, 1975) and probed with DADH antiserum in order to show the specificity of the immunological probe. In Fig. 4A, 2 polypeptides (27 kD and 39 kD) were visualized in blots of separated proteins from flies which expressed the Adh° allele. In Fig. 4B, only the 39-kD polypeptide was visualized in blots of separated proteins from Df(2L)BR41/Df(2L)A48 flies. Both deficiencies have the Adh gene completely deleted. Thus, the polyclonal DADH antiserum recognized the DADH polypeptide (Mr = 27 kD) and also cross-reacted with a 39-kD polv-
Fig 3: Southern blot analysis of mutant DNA. (a, left) Autoradiograph of X-ray-induced mutant genomic DNA digested with XbaI and XhoI. Lane (A) wild-type, (B) nLA2, (C) nLA73, (D) nLA74, (E) nLA80, (F) nLA80, (G) nLA248, (H) nLA249, (I) nLA252, (J) nLA319. (b, right) Autoradiograph of ENU-induced mutant genomic DNA digested with AatII and XbaI. Lane (A) wild-type, (B) DfBR41. (C) nBR28, (D) nBR43, (E) nBR50, (F) nBR51, (G) nBR52. Mutant DNA was transferred to GeneScreen Plus via electroblot and probed with 3P nick translated XhoI fragment. Molecular weights (kD) were determined from HaeIII digest of φX174 RF DNA.

### TABLE 2

**SUMMARY OF THE MOLECULAR PROPERTIES OF INTRAGENIC Adh NULL MUTANTS**

<table>
<thead>
<tr>
<th>Mutagen</th>
<th>Adh mutant</th>
<th>Peptide analysis</th>
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<th>Northern *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Production</td>
<td>Molecular weight</td>
<td>Isoelectric point</td>
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<td>Ac</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>nLA80</td>
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<tr>
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<td></td>
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</tr>
<tr>
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<td></td>
<td>nBR56</td>
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</tr>
</tbody>
</table>

* Northerns were run only when no peptide was produced and Southern was normal, except for Df(2L)BR41

N. normal with respect to control.
A. altered with respect to control.
L. lower molecular weight.
Ac. more acidic p.f.
- +. produced a detectable polypeptide
- -. did not produce a polypeptide
Ba. more basic p.f.
* probe binds
\* no binding of probe.
Fig. 4. Specificity of antisera to DADH. Western blot analysis of two-dimensional polyacrylamide gels of (A) Adh² and (B) DfBR41. Isoelectric focusing is in the horizontal direction (cathode to the right) and SDS-polyacrylamide gel electrophoresis in the vertical direction. Separated proteins were transferred to nylon membranes and probed with rabbit DADH antisera followed by goat anti-rabbit IgG-alkaline phosphatase conjugate. The bound antibody was visualized by reacting the alkaline phosphatase conjugate with nitro blue tetrazolium and BCIP. Molecular weights (kD) were determined from pre-stained SDS-PAGE markers.

The antibody used by Chia et al. (1985) also recognized this polypeptide. The antibody used by Chia et al. (1985) also recognized this polypeptide. The antibody used by Chia et al. (1985) also recognized this polypeptide.

Proteins from X-ray- and ENU-induced mutants were separated by one-dimensional polyacrylamide gel electrophoresis (1D-gels) (Laemmli, 1970), blotted, and probed with DADH antisera to detect any change in molecular weight of DADH polypeptide (Fig. 5a and b). The following results were obtained for the X-ray-induced mutants: (1) nLA80, nLA249, and nLA252 were shown to produce a DADH polypeptide of normal molecular weight; (2) nLA2 produced a slightly smaller (less than 10%) DADH polypeptide; (3) nLA73, nLA248 and nLA74 produced no detectable DADH polypeptide (Fig. 5a). 7 of the 8 intragenic ENU-induced mutants produced a DADH polypeptide of normal molecular weight (Fig. 5b). Two other ENU-induced mutants, DfBR41 (intergenic) and nBR51 (intragenic), produced no detectable DADH polypeptide (Fig. 5b). Finally, nLA319, a spontaneous mutant of AdhD, also produced a DADH polypeptide of normal molecular weight (not shown). These data are summarized in Table 2.

Proteins from X-ray- and ENU-induced mutants were separated by isoelectric focusing, blotted and probed with DADH antisera to detect any change in the charge of the DADH polypeptide (Figs. 6a, b and c). X-ray-induced mutant nLA249 produced a polypeptide with a normal charge, whereas nLA2 and nLA252 produced polypeptides which had a more negative charge, and nLA80 produced a polypeptide with a more positive charge, all with respect to Adh¹cn hwo., the stock from which they were derived (Figs. 6a and c). ENU mutants nBR43, nBR50 and nBR52 produced ADH polypeptides of normal charge while DfBR41 (intergenic) and nBR51 (intragenic) produced no detectable DADH polypeptide (Fig. 6b). Mutants nBR28, nBR53, nBR54 and nBR56 were also normal, but were run on different blots (not shown) with the same controls. Therefore the polypeptides produced by all the ENU-induced mutants have a normal charge as compared to net Adh¹cn, the parental stock. In addition, the polypeptide from the spontaneous mutant of AdhD, nLA319, had a normal charge when compared to AdhDpr cn (not shown).

AdhD is a functional allele recovered from an EMS exposed Adh¹ strain (Grell et al., 1968) that differs from Adh¹ by a single amino acid substitution of glutamic acid for glycine at position 232 (Schwartz and Jornvall, 1976). These data are also summarized in Table 2.

The X-ray-induced mutants have previously been analyzed by Kelley et al. (1985), who used silver staining of proteins separated by 2-D electrophoresis (O'Farrell, 1975), and Chia et al. (1985), who used in vitro translation and immunoprecipitation of DADH. An interesting comparison can be made between these 2 studies and our results. The western blot technique, using a polyclonal antibody produced against DADH, detected an altered protein from mutant nLA2 that the silver staining technique failed to detect, pre-
Fig. 5. Molecular weight analysis of mutant polypeptides. (a, left) Western blot of an SDS-polyacrylamide gel of 10 X-ray-induced mutants. Lane (A) nLA2, (B) nLA73, (C) nLA74, (D) nLA80, (E) DfA178, (F) nLA248, (G) nLA249, (H) nLA252, (I) DfA379, (J) DfA445, (K) Adh'. (b, right) Western blot of an SDS-polyacrylamide gel of 9 ENU-induced mutants. Lane (A) Adh', (B) nBR56, (C) nBR54, (D) nBR53, (E) nBR52, (F) nBR51, (G) nBR50, (H) nBR43, (I) DfBR41, and (J) nBR28. Separated proteins were transferred to nylon membranes and probed with rabbit DADH antiserum followed by goat anti-rabbit IgG-alkaline phosphatase conjugate. The bound antibody was visualized by reacting the alkaline phosphatase conjugate with nitrilotriazolium and BCIP. Molecular weights (kD) were determined from prestained SDS-PAGE markers.

sumably because of the altered migration of nLA2. Mutant nLA252 was not available to Kelley et al. (1985). Following in vitro translation, Chia et al. (1985) detected one polypeptide, nLA74, of low molecular weight that was not detected by western blot analysis, presumably because of in vivo degradation of the mutant polypeptide or the use of a different antibody.

Northern blot analysis

Three mutants which had no detectable DNA alterations (southern blot analysis), and did not produce a cross reacting DADH polypeptide were analyzed using northern blots to see if they produced a normal Adh transcript. RNA from the negative control, DfBR41, did not hybridize with the Xba1 fragment, whereas RNA from the wild-type produced strong hybridization. In northern blots, RNA from the 3 mutants nLA73, nLA74 and nBR51 all hybridized with the Xba1 probe with the same molecular weight as wild-type (data not shown). The results of these experiments are summarized in Table 2.

Statistical analysis

22 of 30 X-ray-induced mutants are multi-locus deficiencies (Aaron, 1979; Ashburner et al., 1982) in contrast to 2 of 10 ENU induced multi-locus deficiencies. A chi square contingency table shows that X-rays induced a significantly (P < 0.01) greater number of deficiencies than ENU. A similar contingency table, comparing the mutants with polypeptides of normal molecular weight and charge to the remainder of the mutants, shows that ENU induced a significantly (P < 0.001) greater number of mutants with normal polypeptides than X-rays. The difference between X-rays and ENU is significant (P < 0.01) even if the comparison is limited to intragenic mutants only. From Hollocher and Place (1987), the expected probability of a GC = AT transition in the Adh gene producing a mutant polypeptide with no change in charge is 67%. The observed data for the ENU-induced mutants do not significantly differ from that expected if all the ENU mutants are transitions. However, a similar comparison for the X-ray-induced mutants shows a significant (P <
0.01) deviation from results predicted by GC = AT transitions. Only 1 (out of 30) X-ray-induced mutant has a DADH polypeptide of normal molecular weight and charge.

Discussion

This study shows substantial differences between the mutagenic action of X-rays and ENU by analyzing mutations induced in the same germ cell stage (i.e. spermatozoa) using southern blots, northern blots, western blots and classical genetic complementation tests. Following treatment of spermatozoa, the process of mutation fixation, mutagenesis, has been shown to occur in the egg because a change in genotype of the maternal female with respect to DNA-repair-deficient mutants may substantially alter the mutation frequency following treatment of sperm or late spermatids in the paternal male (Würgler et al., 1972; Vogel et al., 1985). This delay of repair allows the primary DNA lesions from X-ray or alkylating agents to accumulate in the sperm or late spermatids. It is evident that X-rays are primarily mutagenic through a mechanism of chain breaking (only 8 of 30 Adh mutations are intragenic by complementation test (Aaron, 1979; Ashburner et al., 1982; Chua et al., 1985). 5 of 7 intragenic mutations show normal southern blots. however, nLA252 has been shown to be a 9-bp deletion by DNA sequencing (LoMonaco et al., 1987) leaving only 4 possible mutations that may not be the result of chain breakage. Therefore, the predominant mechanism of X-ray-induced mutation is chain breaks leading to intragenic deletions or larger multi-locus deficiencies. Our results compare favorably with the results of X-ray mutagenesis at other loci in D. melanogaster (Grimmwa et al., 1985; Cote et al., 1986; Pastink et al., 1987). In contrast, we found that most mutations induced by ENU (8 of 10) are intragenic with normal southern blots. Therefore, there is a significant difference in the mutation spectra of the alkylat-
ing agent ENU and ionizing radiation at this level of analysis.

One advantage of analyzing germ-line mutations is that they may be readily placed in a different genetic background. By genetic test we can determine that the contrast in mutation spectra would not have been so large if we had selected mutants over a deficiency. In this study, mutants were analyzed over the large deficiency Df(2L)64j that included the Adh locus (Woodruff and Ashburner, 1979a, b). Only 9 of the 30 mutations induced by X-rays were viable (Aaron, 1979), whereas 8 of 10 mutations, induced with ENU, were viable over this deficiency. Therefore, if the selection system had attempted to select the mutant opposite a large deficiency, only a small fraction of all the deficiencies would have been recovered; however, all of the intragenic mutants would have been recovered since the Adh locus, in the absence of alcohol, was not essential for viability. Selecting mutants at a specific locus gives a greater proportion of large deficiencies than small deficiencies because of the higher probability of a large deficiency removing the locus that is the basis of selection. X-rays actually induce a larger proportion of deficiencies affecting only a few loci as seen in the work by Lefevre (1981).

The analysis of peptides produced by mutant alleles presented in this paper provides considerably more information than would normally be available from a western blot analysis for the following 3 reasons: First, the mutations are induced in germ cells and, following complementation analysis, may be by use of conventional genetic crosses be placed over a deletion so that the only polypeptide from the Adh locus will be from the mutant allele. This genetic manipulation avoids the problem of co-migration of the mutant polypeptide with the polypeptide from the alternate allele. Second, a strong recognition site for polyclonal antibodies is in the 5' end of the gene (Hollocher and Place, 1987); therefore, chain termination mutations in the 3' end of the gene may be recognized by an antibody (Martin et al., 1985; Hollocher and Place, 1987). The limiting factor in recognition of nonsense mutation appears to be size of the polypeptide that can survive in vivo degradation. For example, mutant nL47A is not recognized by our polyclonal antibody from an in vivo preparation, but a low molecular weight polypeptide was precipitated by a polyclonal antibody from an in vitro translation (Chia et al., 1985). Third, the Adh gene has been sequenced and the consequence of specific changes in DNA may be used to predict the peptide. A computer analysis of the Adh locus with the 3 reading frames translated shows any frameshift corresponding to 1-base-pair insertion or a 2-base-pair deletion will usually produce a stop codon in the structural gene. A smaller than expected peptide is produced if the frame shift (1-base-pair deletion or a 2-base-pair insertion) is 5' to base pair 687 or if the frameshift (a 2-base-pair deletion or 1-base-pair insertion) is 5' to the base pair 615. Bases are numbered from the A at the beginning of translation and do not include introns. In vivo, these frameshift mutants produce either a polypeptide of significantly reduced molecular weight if in the 3' end of the gene or no detectable polypeptide if the mutant is within the 5' end of the gene because of in vivo degradation of abnormally short polypeptides. A mutational change that results in the equivalent frameshift of 1-base-pair addition or a 2-base-pair deletion at base pair 615 reads through the stop codon and produces a polypeptide with a 15% increase in molecular weight. A 1-base-pair deletion or a 2-base-pair insertion 3' to base pair 687 reads through the stop codon and produces a peptide with an altered charge by the addition of 2 lysine residues. This peptide can be distinguished from the normal peptide by western blot analysis of an IEF-polyacrylamide gel.

There are 7 ENU-induced mutants (nBR28, nBR43, nBR50, nBR52, nBR53, nBR54 and nBR56) that produce polypeptides similar to the normal allele (Adh*) in molecular weight and charge. From the above computer analysis of the Adh gene, we conclude that these 7 mutations cannot be 1- or 2-base-pair deletions or insertions. If we assume that a deletion or insertion that does not produce a frameshift has a probability of 1/3 because the base-pair deletion or insertion is a multiple of 3, then the probability is less than 10^-1 that all 7 mutations are deletions or insertions. The majority of these 7 mutations are probably missense mutations that result in single amino acid substitutions. Many types of changes in DADH polypeptide primary structure could lead
to enzyme inactivation. Examples include the loss of substrate binding site, loss of NAD-binding site, or inability to form dimers due to improper folding and any alteration in structure that results in proteolytic degradation in vivo.

Although the ENU-induced mutants predominantly produce nonfunctional proteins of normal molecular weight and charge, 3 of 10 ENU-induced mutations are unique. Mutant \textit{Df(2L)BR41} is a deficiency of the \textit{Adh} locus that is not homozygously viable, but is hemizygoously viable with \textit{Df(2L)A48}. This mutant had no detectable \textit{Adh} DNA, mRNA or DADH polypeptide, thus confirming the classification of this mutant as a deficiency with all levels of analysis. Dr. M. Ashbumer (personal communication) has mapped the distal break point of \textit{Df(2L)BR41} to the same position as the distal break point of \textit{Df(2L)A263} (Ashbumer et al., 1982) and the proximal break point to between \textit{Adh} and \texttt{1(2)J5Bb}, therefore explaining the viability over \textit{Df(2L)A48} with osp\textsuperscript{n} and \textit{Adh}\textsuperscript{n} phenotypes. Mutant \textit{Df(2L)BR55} is a multi-locus deficiency which includes the \textit{Adh} locus and does not complement adjacent loci \texttt{noc}, osp and \texttt{1(2)J5Bb}. Mutant nBR51 is probably the result of a change early in the \textit{Adh} coding sequence resulting in a nonsense codon or missense codon that leads to loss of the DADH antigenic determinants. The basis for this conclusion is that the mutant appears normal in southern blots and has hybridizable \textit{Adh} mRNA but does not produce a detectable DADH-polypeptide.

X-rays induced a spectrum of mutations with at least 26 of 30 mutations resulting from DNA-chain breakage: in contrast, ENU induced 7 out of 10 mutations producing a polypeptide of normal molecular weight and charge. From analysis of these X-ray- and ENU-induced mutations, it is concluded that this forward mutation assay based on loss of enzymatic activity efficiently recovers a broad spectrum of mutations ranging from missense to intragenic deletions and multi-locus deficiencies.

Since only 3 of 30 X-ray-induced mutations and none of the 10 ENU-induced mutations produced detectable electrophoretic variant polypeptides, it is evident that observing electrophoretic variants of proteins is an inefficient method for screening human populations for mutations in the heterozygote because most mutants that alter phenotype would either make a protein that comigrates with the normal allele or not make a detectable protein. Furthermore, in man we expect an even greater proportion of variant proteins to be destroyed in vivo because altered proteins will probably be degraded by proteolytic enzymes. In tissue culture, mouse L cells selectively degrade proteins from missense mutations (Capocchi et al., 1974). The observation of electrophoretic variant polypeptides is an inefficient method for detection of new mutations unless the variant polypeptide can be analyzed in a genetic system that permits (1) detection of loss of protein function and (2) separation from the polypeptide of the normal allele. However, electrophoresis of polypeptides from previously selected mutants in a system that permits genetic analysis is useful in the classification of mutations.

Acknowledgements

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CHAPTER IV: MOLECULAR ANALYSIS OF AN ETHYL METHANESULFONATE

INDUCED DEFICIENCY IN DROSOPHILA MELANOGASTER
MOLECULAR ANALYSIS OF AN ETHYL METHANESULFONATE INDUCED DEFICIENCY IN DROSOPHILA MELANOGASTER


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SUMMARY

Mutations induced in *Drosophila melanogaster* spermatozoa at the alcohol dehydrogenase (*Adh*) locus by ethyl methanesulfonate were analyzed using genetic complementation tests, western blots and Southern blots. 2 of 3 EMS induced mutants complemented adjacent loci. Southern blot analysis showed that the 2 intragenic mutants were normal while the intergenic mutant did not hybridize to an *Adh* specific probe. Western blot analysis showed 1 of 2 intragenic mutants produced a detectable polypeptide of normal molecular weight and charge. These data indicate that the two intragenic mutants are probably missense mutations, whereas the third mutant is a large multi-locus deficiency. We have also mapped the breakpoint of X-ray induced *Adh* deficiency (*Df(2L)A63*) to a 900 bp *XbaI-EcoRI* fragment 3' to *Adh*. 
INTRODUCTION

Mechanisms of mutagenesis can be studied by characterizing mutations induced at a specific locus. *D. melanogaster* is an ideal animal model for eukaryotic germline mutagenesis. *D. melanogaster* is small, has short generation time and is genetically well defined (Lindsley and Grell, 1968). The alcohol dehydrogenase locus (*Adh*) of *D. melanogaster* is a single copy gene which has been sequenced (Kreitman, 1983). The gene contains four exons, and a protein encoding region of 765 bp, with separate promoters for larval and adult flies (Kreitman, 1983). The alcohol dehydrogenase polypeptide (ADH) forms a homodimeric enzyme with a subunit Mr of 27kDa (Thatcher, 1980).

We have induced *Adh* null mutations in Drosophila spermatozoa with ethyl methanesulfonate (EMS). EMS is a direct acting monofunctional alkylating agent which has previously been shown to induce mosaic mutations in Drosophila (Lee et al., 1970). Mutations induced by EMS are normally the result of G:C to A:T transitions in bacteria (Burns et al., 1986) and Drosophila (Martin et al., 1985; Hollocher and Place, 1987). *Adh* null mutations were selected using a forward mutation assay originally described by Sofer and Hatkoff (1972). The assay employs a gas 1-penten-3-ol which ADH positive flies metabolize to a toxic ketone resulting in the survival of *Adh* null flies. Here we report the characterization of a deletion which is at least 3.2 kb long, (much larger according to genetic complementation data) following EMS exposure, and selection of non-mosaic mutants.
MATERIALS AND METHODS

Chemicals and enzymes

Chemicals and enzymes used were previously described in Batzer et al. (1988b). EMS was purchased from Eastman Chemicals. Magnagraph membrane was from Micron Separating Incorporated.

Drosophila stocks

Wild type stocks Adh\(^F\), Adh\(^S\), Adh\(^US\) and Adh\(^D\) were maintained homozygous for the second chromosome. Newly induced mutations were assigned numbers and designated as multi-locus deficiencies or intragenic mutations as previously described (Batzer et al., 1988b). Mutant Df(2L)BR3 was not homozygously fertile or hemizygously fertile opposite any known second chromosome deficiencies, it was maintained as Df(2L)BR3/In(2LR)O,Cy dp\(^{1v1}\) Adh\(^RB\) pr cn\(^2\) and had to be mated in order to place it opposite a small X-ray induced Adh deficiency Df(2L)A63 (Aaron, 1979), making it hemizygous for molecular analyses. Adh\(^nBR4\) was homozygously viable. Adh\(^nBR18\) was heterozygous with Df(2L)64j,b a large second chromosome deficiency (Woodruff and Ashburner, 1979a,b). Mutant Df(2L)BR41 a large second chromosome deficiency (Batzer et al., 1988b), was balanced with Df(2L)A63. Further detailed description of the Drosophila stocks and chromosomes may be found elsewhere (Lindsley and Grell, 1968).

All stocks, both wild type and mutant were maintained at 25°C on standard Drosophila media consisting of corn meal, corn sugar, agar, brewer's yeast and propionic acid as a mold inhibitor.
Induction of mutations

Solutions of 5mM and 10mM EMS in 1% sucrose were fed to 460 wild type male flies as previously described by Aaron et al. (1977). Treated flies were then mated to homozygous Adh$^{n4}$ bw$^D$ virgin females for 3 days, selecting mutations induced in spermatozoa. Adh$^{n4}$ is an alcohol dehydrogenase null mutation that is homozygously viable, and viable over a large deficiency, Df(2L)64j,b. This procedure does not select mosaic mutations. The F$_1$ progeny were screened for the absence of ADH activity by exposure to 1-penten-3-ol as described by Sofer and Hatkoff (1972). Adh$^{nBR3}$ and Adh$^{nBR4}$ were induced using 5mM EMS. Adh$^{nBR18}$ was induced with 10mM EMS.

Genetic test for complementation

The mutants were first balanced over In(2LR)O,Cy dp$^{lv1}$Adh$^{nB}$ pr cn$^2$(CyO) and then the genetic test for complementation was conducted using the following deficiencies: Df(2L)64j,b, Df(2L)A48, Df(2L)A63, Df(2L)A379 with the addition of the following chromosomes 1(2)35Ba$^2$ Adh$^{n11}$ cn vg, 1(2)35Bb$^p$ pf, and b os$^{76e}$ Adh$^{n2}$ rds pr cn. The deficiencies were balanced over second chromosome balancer CyO. A cross between a new mutant balanced over CyO and a known deficiency balanced over CyO will give a 2:1 ratio of curly to non-curly flies if the mutation is viable over the deficiency.

ADH protein analysis

The specificity of the polyclonal antiserum and methods for 1-D PAGE, IEF PAGE and western blotting as well as polyclonal antiserum
are described elsewhere (Batzer et al., 1988a,b).

**Isolation of Drosophila genomic DNA and Southern blotting**

Drosophila genomic DNA was isolated from 200 adult flies as previously described by Chia et al. (1985). 10 μg of genomic DNA was digested to completion with XbaI and AvaII or EcoRI and XbaI and electrophoresed for 15 hr. at 15 volts on 1.0% agarose gels. The gel was then denatured and transferred onto Magnagraph membrane as previously described by Batzer et al. (1988b). The blots were baked 2 hr at 65°C, soaked in 0.1X SSC, 1% SDS at 65°C for 30 min., then prehybridized 45 min. at 42°C (Reed and Mann, 1985). This was followed by hybridization with heat denatured nick translated probe (2.0 x 10⁷ cpm) for 16-20 hr. at 42°C. The membranes were washed and exposed to Kodak XAR-2 film as previously described by Batzer et al. (1988b).

**Isolation of probes**

The pSAC1 and pSAF2 plasmid Adh clones (Goldberg, 1980) were isolated according to Davis et al. (1986). pSAC1 is a Drosophila Adh clone containing a 4.8 Kb EcoRI fragment (Goldberg, 1980). pSAF2 is a larger Adh clone containing an 11.8 Kb SalI fragment. Drosophila P-element transformation studies have shown that the entire Adh locus is contained within a 3.2 Kb XbaI restriction fragment (Goldberg et al., 1983). Both pSAC1 and pSAF2 contain the XbaI fragment. The 3.2 kb XbaI (Adh) fragment was isolated from pSAC1 as previously described (Batzer et al., 1988b). The 3 probes were nick translated with alpha [³²P]dATP (3000 Ci/m mole) as
previously described (Rigby et al., 1977). The typical yield was 2.7 x 10^8 dpm/μg of XbaI fragment, pSAC1 or pSAF2.
RESULTS

Genetic complementation analysis

3 null mutants were recovered at the Adh locus following treatment with EMS and selection of mutants by exposure of adults to 1-penten-3-ol (Sofer and Hatkoff, 1972). Mutant Df(2L)BR3 did not complement adjacent loci outspread (osp), no-o-cell (noc) cinnibar (cn) or adjacent lethals 1(2)35Bb' and 1(2)35Ba2. The mutant was not viable with deficiencies Df(2L)A48 and Df(2L)A47. Crosses to Df(2L)A379 resulted in 3 of 41 non curly offspring instead of 14 expected from a 2:1 ratio. The mutant was maintained hemizygous for the Adh locus opposite Df(2L)A63, a small X-ray induced Adh deficiency (Aaron, 1979). Mutant AdhBR18 was viable opposite Df(2L)64j,b, Df(2L)A47, Df(2L)A48 and Df(2L)A379 and complemented all adjacent loci. Mutant AdhBR4 was maintained homozygous and complemented the adjacent loci. These data are summarized in Table 1.

Western blot analysis

Proteins from EMS induced mutants were separated by 1-D PAGE (Laemmli, 1970), blotted and probed with ADH antiserum to detect any change in Mr of the ADH subunit polypeptide (Figure 1a). Mutants Df(2L)BR3 and AdhBR4 produced no detectable ADH (Figure 1a, lanes B and C respectively). Mutant AdhBR18 produced a polypeptide of normal Mr compared to AdhF, the positive control (Figure 1a, lanes D and A respectively). The subunit Mr for AdhBR18 was calculated as
Proteins from EMS induced mutants were separated by isoelectric focusing, blotted and probed with ADH antiserum to detect changes in the charge of the ADH subunit polypeptide (Figure 1b). ADH electromorphs Adh$^F$, Adh$^S$, Adh$^D$ and Adh$^US$ shown in Figure 1b, lanes A, B, C and D respectively, contain single amino acid substitutions and were used as pi markers (Batzer et al., 1988). Mutants Df(2L)BR3 and Adh$^{nBR4}$ produced no detectable ADH polypeptide (Figure 1b, lanes F and G respectively). Mutant Df(2L)BR41, a known deficiency for Adh locus, also produced no detectable ADH (Figure 1b, lane E). Mutant Adh$^{nBR18}$ produced a detectable ADH polypeptide with a subunit pi of 7.69 (Figure 1b lane H). These data are summarized in Table 2.

Southern blot analysis

DNA from the 3 EMS induced Adh null mutants, from flies homozygous or heterozygous over a deficiency for the Adh locus was digested with XbaI and AvaII or EcoRI and XbaI restriction endonucleases. These double digests were examined with Southern blots using pSAF2, pSAC1 or the 3.2 kb XbaI restriction fragment from a genomic Adh clone as a probe. (Figure 2a, b and c respectively).

Figure 2a shows that DNA from all of the EMS induced mutants hybridized to pSAF 2. Mutants Adh$^{nBR4}$ and Adh$^{nBR18}$ both appeared normal compared to Adh$^F$ when restricted with AvaII and XbaI (Figure 2a, lanes B, C and D respectively), or EcoRI and XbaI (Figure 2a, lanes G, H and I respectively). Mutants Df(2L)BR3 and Df(2L)BR41 also hybridized, but were missing 2 and 4 bands for the EcoRI-XbaI and XbaI-AvaII double digests respectively (Figure 2a, Lanes F, J, A and E respectively).
Figure 2b shows that DNA from all of the EMS induced mutants hybridized to pSAC1. Mutants Adh<sup>nBR4</sup> and Adh<sup>nBR18</sup> both appeared normal compared to Adh<sup>F</sup> when restricted with AvaiI and XbaI (Figure 2b, lanes B, C and D respectively), or EcoRI and XbaI (Figure 2b, lanes G, H and I respectively). Mutants Df(2L)BR3 and Df(2L)BR41 also hybridized, but were missing 2 and 4 bands for the EcoRI-XbaI and XbaI-AvaiI double digests respectively (Figure 2b, Lanes F, J, A and E respectively).

Figure 2c shows that mutants Df(2L)BR3 and Df(2L)BR41 did not hybridize to XbaI probe when restricted with XbaI-AvaiI or EcoRI-XbaI (lanes A, E, F and J respectively). However, mutants Adh<sup>nBR4</sup> and Adh<sup>nBR18</sup> both appeared normal compared to Adh<sup>F</sup> when restricted with EcoRI and XbaI (Figure 2c, lanes G, H and I respectively) or AvaiI and XbaI (Figure 2c, lanes B, C and D respectively).
DISCUSSION

We have characterized several Adh null mutations induced in Drosophila spermatozoa. The mutants were selected as heterozygotes with Adh$^{n4}$, a nonsense mutant (Chia et al., 1977). Selection opposite Adh$^{n4}$ facilitates the recovery of a broad range of mutations varying from missense to multi-locus deficiencies (Batzer et al., 1988b). Thus the set of mutations recovered in this manner represents the true mutagenic spectrum of EMS. These mutants were not selected as mosaics.

Mutant Df(2L)BR3 did not complement the adjacent loci. This mutant was also not fertile opposite any known Adh deficiencies. Southern blot analysis showed the mutant did not contain any hybridizeable Adh DNA (XbaI probe), but did hybridize to pSAC1 and pSAF2. Since a previously describe multi-locus deficiency (Df(2L)BR41) also hybridized to pSAC1 and pSAF2 the hybridizeable DNA represents the balancer allele Df(2L)A63. Western blot analysis of Df(2L)BR3 also showed no cross-reacting ADH polypeptide present. Thus the classification of mutant Df(2L)BR3 as a large multi-locus deficiency is verified by all levels of analysis.

The data from Southern blot hybridization of Df(2L)BR3 and Df(2L)BR41 may be used to locate the breakpoint of a small X-ray induced deficiency Df(2L)A63 (Aaron, 1979). Both of the mutants hybridized to pSAC1 and pSAF2, however neither contains the 660 bp 5' EcoRI-XbaI restriction fragment when compared to Adh$^F$. These data indicate that the breakpoint is located 3' to the Adh locus in the 900 bp XbaI-EcoRI fragment just outside the Adh locus. DNA sequence
analysis of the $Df(2L)A63$ breakpoint should yield insight into repair of larger deletions. Comparison with breakpoint sequences from intragenic mutants should demonstrate whether or not repair of large and small deletions is the same.

The production of chromosomal rearrangements by alkylating agents is not new. Bishop and Lee (1973) showed that EMS was capable of inducing chromosomal breakage. Another monofunctional alkylating agent (ENU) has also been shown to induce large deletions at the $Adh$ locus (Batzer et al., 1988b) and a smaller deletion at the $white$ locus (Pastink et al., 1988). These mutations are probably the result of $N\cdot7$ ethylguanine lesions, which weaken glycosidic bonds or are misrepaired, leading to the formation of apurinic sites and DNA chain scission. Many other studies with EMS mutagenesis have failed to uncover any large deletions. This is probably due to the system used eg. bacteria (Burns et al., 1986) or because the loci are X linked (hemizygous) (Vrielings et al., 1988a,b).

Mutants $Adh^{nBR4}$ and $Adh^{nBR18}$ both complemented the adjacent loci. These mutants also appeared normal in Southern blots using all three probes. Western blots showed that $Adh^{nBR4}$ did not produce a detectable polypeptide, while $Adh^{nBR18}$ produced a polypeptide of normal molecular weight and charge. From Hollocher and Place (1987) it was calculated that 67.26% of $GC \rightarrow AT$ transitions in the $Adh$ gene would result in mutant polypeptides with conservative (charge) amino acid substitutions while 7.64% would result in a termination codon. Thus we conclude that these two mutants are missense mutations which resulted from $GC \rightarrow AT$ transitions caused by $O\cdot6$ ethylguanine lesions. Thus the selection procedure presented here for the $Adh$
locus originally described by Aaron (1979) and later used by Batzer et al. (1988a) and this work allows the recovery of both deletions and single base changes to evaluate mutation spectra.
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ABBREVIATIONS

AP, alkaline phosphatase; BCIP, 5-bromo-4-chromo-3-indolyl phosphate; EMS, ethyl methanesulfonate; ENU, 1-ethyl-1-nitrosourea; IEF-PAGE, isoelectric focusing polyacrylamide gel electrophoresis; Kb, kilobases; kDa, kilodaltons; Mr, molecular weight; NBT, nitro blue tetrazolium; pI, isoelectric point; RF, replicative form; SDS, sodium dodecylsulfate; SSC, 0.15 M sodium chloride, 0.015M sodium citrate (pH 7.0).
ACKNOWLEDGEMENTS

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Figure 1. Analysis of mutant polypeptides. (a) Western blot of an SDS polyacrylamide gel. Lane (A) Adh^F, (B) Df(2L)BR3, (C) Adh^{nBR4}. (D) Adh^{nBR18}. (b) Western blot of an IEF polyacrylamide gel. Lane (A) Adh^S, (B) Adh^F, (C) Adh^{US}, (D) Adh^D, (E) Df(2L)BR41, (F) Df(2L)BR3, (G) Adh^{nBR4}, (H) Adh^{nBR18}. Proteins were electrophoretically transferred to nylon membranes and probed with rabbit ADH antisera followed by goat anti-rabbit IgG-AP conjugate. Bound antibodies were visualized by reacting the AP conjugate with NBT and BCIP. Molecular weights in Figure 1a were determined from prestained Mr markers.
Figure 2. Southern blot analysis of mutant genomic DNA. Lanes A-E were digested with Avall and XbaI, and lanes F-J were digested with XbaI and EcoRI for Figure 2a, b and c. (a) Autoradiograph of mutant genomic DNA probed with $^{32}$P nick translated pSAF2. Lane (A) $Df(2L)BR3$, (B) $Adh^{nBR4}$, (C) $Adh^{nBR18}$, (D) $Adh^F$, (E) $Df(2L)BR41$, (F) $Df(2L)BR3$, (G) $Adh^{nBR4}$, (H) $Adh^{nBR18}$, (I) $Adh^F$, (J) $Df(2L)BR41$. (b) Autoradiograph of mutant genomic DNA probed with $^{32}$P nick translated pSACL. Lanes are the same as Fig. 2a. (c) Autoradiograph of mutant genomic DNA probed with $^{32}$P nick translated XbaI fragment. Lanes are the same as Figure 2a. Mutant DNA was transferred to magnagraph via electroblot as described in text. Fragment lengths (Kb) were determined from HaeIII digests of RX 174 RF DNA.
Table 1. Genetic complementation data for EMS induced Adh null mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Genetic loci</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>noc</td>
<td>osp</td>
<td>1(2)35Ba²</td>
</tr>
<tr>
<td>1(2)35Bb'</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nBR3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>nBR4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>nBR18</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Complements locus
- Does not complement locus
Table 2. Molecular properties of EMS induced Adh null mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>polypeptide subunit</th>
<th>Southern blot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$p_1^*$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$pSAF2$ $pSACl$ $Xba$</td>
</tr>
<tr>
<td>Xba</td>
<td></td>
<td>$A$ $A$ $O$</td>
</tr>
<tr>
<td>nBR3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nBR4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nBR18</td>
<td>$7.69 \pm .06$</td>
<td>$25.64 \pm 1.49$</td>
</tr>
</tbody>
</table>

- Does not produce a protein
- Altered with respect to control
- Normal with respect to control
- * Isoelectric point $\pm$ standard deviation (n=5).
- ** Molecular weight in kDa $\pm$ standard deviation (n=5).
- O Did not hybridize to probe
CHAPTER V: FURTHER EVIDENCE THAT ALKYLATED AGENTS INDUCE LARGE DELETIONS USING ENZYMATIC AMPLIFICATION OF DNA
FURTHER EVIDENCE THAT ALKYLATING AGENTS INDUCE
LARGE DELETIONS USING ENZYMATIC AMPLIFICATION OF DNA

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SUMMARY

*Drosophila melanogaster* alcohol dehydrogenase null allele DNA sequences were amplified using the polymerase chain reaction (PCR) procedure. Mutants which resulted from single base pair changes (*Adh*<sup>nBR4</sup> and *Adh*<sup>nBR48</sup>) appeared normal when compared to the wild type allele. DNA from mutants with large deletions (*Df(2L)BR3* and *Df(2L)BR41*) was not amplified. Mutant *Adh*<sup>nLA248</sup>, a 250 bp tandem duplication, was amplified but the resultant fragment appeared 250 bp larger than the wild type allele. Thus, this technique is as sensitive as, and less labor intensive than, Southern blots for the analysis of mutations.
INTRODUCTION

Molecular mechanisms of mutagenesis may be evaluated by analyzing specific locus mutations. The mutation spectra of different mutagens may then be compared at different loci, as well as in different organisms. The alcohol dehydrogenase locus (Adh) of Drosophila melanogaster is a single copy gene located on the second chromosome (Lindsley and Grell, 1968). The gene has been sequenced, and has a protein encoding region of 900 bp separated by two small (65 and 70 bp) introns (Kreitman, 1983). The expression of the Adh locus is developmentally regulated using alternative splicing of mRNA facilitating the use of larval and adult promoters (Savakis et al., 1986). Drosophila alcohol dehydrogenase (ADH) is a homodimeric enzyme with a subunit Mr of 27 kDa that does not require any metallic cofactors (Thatcher, 1980).

We have previously induced Adh null mutations using monofunctional alkylating agents ethyl methanesulfonate (EMS) and 1-ethyl-1-nitrosourea (ENU) (Batzer et al., submitted; Batzer et al., 1988), as well as with X-rays (Aaron, 1979). Several of the mutants induced with alkylating agents were classified as point mutations, however 3 of 13 were multi-locus deficiencies. Previous studies using alkylating agents have failed to detect large deletions from EMS mutagenesis in bacteria (Burns et al., 1986) and at the White and Adh loci in Drosophila melanogaster (Vrielings et al., 1988a,b; Hollocher and Place, 1987 respectively). However Bishop and Lee (1973) showed that EMS was a clastogenic agent. These differences in mutation spectra for the alkylating agents are probably due to the
mutation selection/isolation procedure, or because the locus of interest was X-linked (hemizygous). Therefore we concluded that the mutation isolation/selection scheme originally reported by Aaron (1979) allowed a non-biased mutation spectrum (Batzer et al., 1988; Batzer et al., submitted).

The "polymerase chain reaction" (PCR) for amplifying specific gene sequences has recently been developed (Saiki et al., 1988). This methodology has been used in conjunction with Southern blotting and hybridization with sequence specific probes to study Sickle cell mutations (Impraim et al., 1987). This procedure also has been used to study beta globin gene mutations (Diaz-Chico et al., 1988). PCR also has been used to detect translocations characteristic of subclinical diseases (Lee et al., 1987). Here we report the characterization of Adh null mutations using PCR in conjunction with Southern blotting. We demonstrate that PCR alone is a rapid method for analyzing intragenic chromosomal rearrangements, point mutations and deletions at the Adh locus of Drosophila melanogaster.
MATERIALS AND METHODS

Chemicals and enzymes:

Chemicals and enzymes used were as previously described in Batzer et al. (1988) except for the following. Magnagraph membrane was from Micron Separation Incorporated. Taq DNA polymerase and reagents for PCR were from Perkin Elmer Cetus.

Drosophila stocks:

Mutant \textit{Df(2L)BR3} was not homozygously fertile or hemizyglyously fertile opposite any known second chromosome deficiencies, it was maintained as \textit{Df(2L)BR3/In 2(LR)O,Cy dy1vI Adh\textsuperscript{nB} pr cn\textsuperscript{2}}. These flies were then mated to place \textit{Df(2L)BR3} opposite \textit{Df(2L)A63} a small X-ray induced \textit{Adh} deficiency (Aaron, 1979). \textit{Adh\textsuperscript{nBR4}} and \textit{Adh\textsuperscript{nLA248}} were homozygously viable. Mutant \textit{Adh\textsuperscript{nBR18}} was viable opposite \textit{Df(2L)64J,b} a large second chromosome deficiency (Woodruff and Ashburner 1979a,b). Mutant \textit{Df(2L)BR41} was balanced with \textit{Df(2L)A63}. Stocks were maintained as previously described (Batzer et al., 1988a,b).

Isolation of Drosophila genomic DNA:

Drosophila genomic DNA was isolated from 200 adult flies as previously described by Chia et al. (1985).
PCR Amplification:

Oligonucleotide primers ADPCR 1 and ADPCR 6 (Figure 1) were synthesized by automatic phosphoramidite chemistry (Applied Biosystems Model 380A, Foster City, CA) and purified by gel electrophoresis and reverse phase chromatography (Matteucci and Caruthers, 1980). These primers flank the entire protein encoding region of the Drosophila Adh gene as well as two small introns as shown in Figure 1. The GC content of the primers was 52% and 48% for ADPCR 1 and ADPCR 6 respectively. Reaction mixtures were a modified version of the manufacturer's protocol containing 3 µg Drosophila genomic DNA, 25 pmol of each primer and 2.5 units of Taq DNA polymerase. Each cycle consisted of 1 min. at 94°C (denature), 2 min at 45°C (anneal) and 5 min. at 72°C (extension). Samples were subjected to 25 cycles of amplification.

Asymmetrical amplification was carried out on 300 ng (original) of amplified DNA using 0.25 pmol of ADPCR 1 and 25 pmol ADPCR 6. The samples were amplified with 25 cycles as described above.

Southern Blotting:

300 ng or 30 ng of original DNA for symmetric and asymmetric amplification (respectively) was electrophoresed 1.5 hr. at 50V on 1.0% Agarose gel which contained 0.5 µg/ml ethidium bromide. The gel was then denatured and transferred to Magnagraph membrane as previously described by Batzer et al. (submitted). The blots were heated 2 hr at 65°C then soaked in 0.1X SSC, 1% SDS at 65°C for 30 min., and prehybridized 45 min. at 42°C (Reed and Mann, 1985). This was followed by hybridization with heat-denatured nick-translated
probe (4.5 x 10^7 dpm) for 16-20 hr. at 42°C. The membranes were washed and exposed to Kodak XAR-2 film for 45 min. at 17°C.

Isolation of XbaI probe:

DNA from pSACl, a plasmid Drosophila Adh clone (Goldberg, 1980), was isolated according to Davis et al., 1986. The 3.2-Kb XbaI fragment from pSACl Adh clone was isolated by digesting the clone to completion at 37°C with XbaI. The sample was run on 1.5% agarose mini-gel at 50V for 2 hr. The 3.2-Kb fragment was electroeluted from the gel, purified by direct extraction, and quantified by ethidium bromide fluorescence according to Maniatis et al. (1982). The 3.2-Kb XbaI restriction fragment has previously been shown to carry all necessary information to complement an Adh null allele (Goldberg et al., 1983). DNA fragments (XbaI) were radiolabeled with alpha [32P]dATP (3000 Ci/mmole) by nick translation (Rigby et al., 1977). Labeled DNA was separated from unincorporated nucleotides by filtration through a Sephadex G 50-80 column in 1 X TE (pH 8.0). The typical yield was 2.7 x 10^8 dpm/µg of DNA.
RESULTS

Figure 2 shows the result of PCR amplification. This gel shows that mutants Adh$^{nBR4}$ and Adh$^{nBR18}$ as well as Adh$^F$ all amplified an 1155 bp fragment (Lanes B, C and D respectively). Amplification resulted in the production of 1.0-3.0 ug of the desired DNA fragment per reaction. Mutants Df(2L)BR3 and Df(2L)BR41 showed no apparent amplification of any size fragment (Lanes A and F respectively). Mutant Adh$^{nLA248}$ also amplified, but the amplified band was approximately 250 bp longer than Adh$^F$ (lane F).

Southern blot analysis of the amplified DNA is shown in Figure 3a. This Figure shows that DNA from mutants Adh$^{nBR4}$ and Adh$^{nBR18}$ as well as Adh$^F$ hybridized to $^{32}$P labeled XbaI fragment (Lanes B, C and D respectively). Mutant Adh$^{nLA248}$ also hybridized, but the band that hybridized was 250 bp longer than Adh$^F$ (lane E) Mutants Df(2L)BR41 and Df(2L)BR3 did not hybridize (Lanes F and A respectively). Each lane that hybridized contained a second smaller fragment that also hybridized, and represents ssDNA from Adh. This band was not clearly visible in agarose gels from regular or asymmetric amplification of the Adh locus, as a result of little or no intercalation of ethidium bromide (Maniatis et al., 1982).

Southern blot analysis of asymmetrically amplified DNA is shown in Figure 3b. Asymmetrical amplification enriches for ssDNA by varying the amount of each primer used, allowing the preferential amplification of a single strand. The results are similar to those above except that there is an increase in the amount of the faster migrating band that hybridized (ssDNA).
DISCUSSION

This study leads to two important findings. First, we have optimized the PCR amplification conditions for the Drosophila Adh locus. Second, we show the enzymatic amplification is a rapid means to screen for Adh deletions as well as other gross chromosomal changes.

Enzymatic amplification of Drosophila genomic DNA resulted in the production of an 1155 bp fragment from Adh<sup>nBR4</sup> and Adh<sup>nBR18</sup> as well as Adh<sup>F</sup>. These mutants were previously classified as point mutations using genetic complementation tests, Southern blots and western blots (Batzer et al., submitted). Enzymatic amplification of the Adh locus adds further evidence that these two mutations were not the result of any gross chromosomal rearrangements or deletions.

DNA from mutants Df(2L)BR3 and Df(2L)BR41 did not amplify using PCR. Both of these mutants were previously classified as deficiencies using genetic complementation tests, Southern and western blots (Batzer et al., submitted; Batzer et al., 1988). The fact that the DNA from these mutants did not amplify demonstrates that deletions which lack Adh genes will not be amplified. These data also show that the deoxyoligonucleotide primers are specific for the Drosophila Adh locus.

Mutant Adh<sup>nLA248</sup> also was amplified, but the amplified DNA was larger than the wild-type Adh<sup>F</sup>. This mutant was induced using X-rays by Aaron in 1979. Later the mutant was cloned and sequenced and found to be a 250 bp internal duplication (Chia et al., 1985).
Mutant Adh\textsuperscript{nLA248} also was shown to be abnormal using Southern blots, and did not produce a detectable ADH polypeptide (Batzer et al., 1988). Thus the fact that the amplified fragment (which contains the 250 bp duplication) is larger than the wildtype was expected.

Southern blot analysis of the PCR amplified DNA showed that the DNA fragments visualized in the agarose gels hybridized with a probe specific for \textit{Drosophila melanogaster} Adh (XbaI fragment). However both of the Adh deficiencies did not hybridize. Taken together these data provide convincing evidence that our Drosophila Adh deoxyoligonucleotide primers (ADPCR 1 and ADPCR 6) are Adh locus specific.

Although the analysis of new mutants using PCR is rapid the analysis of PCR generated data must be accompanied by other information. Deletion of either one of the two regions to which primers bind would also result in unsuccessful amplification, therefore the lack of amplification does not necessarily indicate that the mutant is a large deletion. It is possible that a deletion within a PCR primer which disrupts as little as 2 GC base pairs would also prevent amplification by significantly altering the temperature at which the primer anneals to target DNA. If both primers do not anneal properly at approximately the same temperature no amplification will occur. Thus the use of PCR with locus-specific primers in conjunction with Southern blotting of genomic DNA using locus-specific probe or in conjunction with genetic complementation data allows rapid characterization of newly induced deletions. Southern blot analysis of amplified mutant DNA should also enhance the resolution of these blots by allowing increased detection of
fragments smaller than 100 bp.

PCR of newly induced mutants is sufficient for detecting intragenic chromosomal rearrangements within the protein encoding portion of the Adh locus. The altered fragment size shown by Adh^{nLA248} clearly shows that 100 - 200 bp duplications or deletions will migrate abnormally. This study also shows that single base pair mutations do not display altered migration after amplification when compared to wild type alleles. Although this type of analysis is not a substitute for DNA sequencing of intragenic lesions it does provide a further basis for the classification of mutants as single base pair changes, large deletions, or intragenic chromosomal rearrangements.

The most beneficial use of PCR may be in the amplification of DNA for cloning, or asymmetrical amplification of DNA for chain-termination DNA sequencing (Sanger et al., 1977). The amplification of the DNA for cloning is however not without some inherent problems. These problems stem from the fact that Taq DNA polymerase is error prone (Ken Tindall NIEHS personal communication). Thus the DNA sequence analysis of mutants must be done from a number of clones pooled together to eliminate any errors introduced by Taq polymerase during amplification.
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ABBREVIATIONS

bp, base pairs; dsDNA, double stranded DNA; kDa, kilodalton; Mr, molecular weight; PCR, polymerase chain reaction; pI, isoelectric point; RF, replicative form; SDS, sodium dodecyl sulfate; SSC, 0.15M sodium chloride, 0.015M sodium citrate pH 7.0; ssDNA, single stranded DNA; TE, 10mm Tris, 1mm EDTA.
ACKNOWLEDGEMENTS

We would like to thank Dr. Simon H. Chang for synthesizing the deoxyoligonucleotide primers. We would also like to thank Ron Bouchard for photography and Sharon Kelley for typing this manuscript. (Supported by National Institute for Environmental Health Sciences P01-ES03347 and Department of Energy FG05-86ER60393 to WRL.)
Figure 1. Enzymatic amplification of the Drosophila Adh locus. Primers ADPCR 1 and ADPCR 6 were complementary to the non-coding and coding Adh strands respectively and amplified an 1155 bp fragment. The 1155 bp Adh fragment encompassed the entire protein encoding region of the Adh gene (protein encoding exons are shown as darkened boxes). The figure is drawn to scale with restriction sites, and primer locations as indicated.
ADPCR 1

1155 bp

ADPCR 6

XbaI  Avail  Avail  Avail  XbaI

ADPCR : 5'-GCTCTACGTAACCGAAGCTTC-3'

ADPCR 6  3'-CCGACTAAGCTACGTGTGAGCTTAACAGG-5'
Figure 2. Agarose gel electrophoresis of enzymatically amplified Drosophila genomic DNA. Lane (A) Df(2L)BR3, (B) Adh$^{nBR4}$, (C) Adh$^{nBR18}$, (D) Adh$^F$, (E) Adh$^{nLA248}$, (F) Df(2L)BR41. DNA was enzymatically amplified as described in materials and methods. 1/10th of the amplified DNA (300 ng of original DNA) was electrophoresed as described in the text. Fragment lengths (bp) were determined from HaeIII digests of QX 174 RF DNA.
Figure 3. Southern blot analysis of PCR amplified DNA. Figure 3a shows an autoradiograph of a Southern blot of amplified DNA probed with $^{32}$P nick translated XbaI fragment. Lane (A) Df(2L)BR3, (B) Adh$^{nBR4}$, (C) Adh$^{nBR18}$, (D) Adh$^{F}$, (E) Adh$^{nLA248}$, (F) Df(2L)BR41. PCR amplified DNA was electrophoresed as described in materials and methods. Figure 3b shows an autoradiograph of a Southern blot of DNA after asymmetric amplification. Lanes are the same as those for 3a. Fragment lengths (bp) were determined from HaeIII digests of QX 174 RF DNA.
CHAPTER VI: SUMMARY
Chapter I introduced the alcohol dehydrogenase locus (Adh) of Drosophila melanogaster and the forward mutation assay for Drosophila alcohol dehydrogenase (ADH) null mutations. Methods for the characterization of Adh alleles such as genetic complementation tests, Southern, northern, and western blots were described and compared.

The characterization of Adh from other species of Drosophila is described in Chapter II. This chapter dealt with the preliminary characterization of wild type ADH from Hawaiian drospholids using D. melanogaster transformants. These data showed that the ADH polypeptide subunits from the Hawaiian flies were the same molecular weight as D. melanogaster ADH, but that the isoelectric point of these peptides was unique as compared to the major D. melanogaster electromorphs. This section shows that the initial characterization of wild type or mutant Adh alleles may be conducted using protein blotting and antiserum specific for Drosophila ADH.

Mutation spectra of X-rays and 1-ethyl-1-nitrosourea (ENU) were compared in Chapter III. X-rays induced primarily deletions, whereas ENU induced predominantly missense mutations in Drosophila spermatozoa. However, two large deletions were isolated following ENU mutagenesis. The ADH polypeptides from the ENU-induced mutations were predominantly normal molecular weight and charge. Most of the X-ray-induced mutations did not produce a detectable polypeptide. Therefore, the observation of mutant polypeptides is an inefficient method for detecting new mutations in heterozygotes. Most mutants
would comigrate with the normal allele or not produce a detectable polypeptide. The forward mutation assay for the Adh locus efficiently recovered a spectrum of mutations that ranged from missense to intragenic chromosomal rearrangements and multilocus deficiencies.

Mutations induced with the monofunctional alkylating agent ethyl methanesulfonate (EMS) were analyzed in Chapter IV. These data confirmed the fact that monofunctional alkylating agents were capable of inducing large deficiencies, as well as point mutants. The fact that alkylating agents induce large deletions contradicted many previous studies. This was probably due to the selection systems and loci that other groups had used. This study also provided further evidence that the Adh locus represents a unique unbiased forward mutation assay for the evaluation of mutation spectra.

Rapid analysis of Drosophila melanogaster Adh null alleles using the polymerase chain reaction (PCR) was described in Chapter V. These data show that the amplification of Adh null alleles was specific and rapid. Furthermore this analysis provides additional evidence for the classification of newly induced Adh null alleles, and may eventually facilitate cloning and DNA sequence analysis of the alleles.
Dear Dr. Lennarz:

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