RESTRICTION ENDONUCLEASE MAP VARIATION AND GENE ACTIVITY IN THE *ADH* REGION IN A POPULATION OF *DROSOPHILA MELANOGASTER*

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SUMMARY

Variation within a 12 kb region containing the *Adh* gene was studied in 16 highly inbred lines of independent extraction from a population of *Drosophila melanogaster*, "Texas". Out of a selection of 8 hexanucleotide specific restriction endonucleases, four restriction site and five DNA insertion/deletion variations were observed and the heterozygosity per nucleotide was 0.004. Linkage disequilibrium between two restriction sites, considerably removed from and on either side of the *Adh* transcriptional unit, identified two major classes of chromosome or haplotype in the population. These two haplotypes were strongly associated with the capacity of lines to respond to the environment (genotype-environment interaction) in ADH activity. It was concluded that a control or regulatory polymorphism was present in the population in association with the *Adh* transcriptional unit.

1. INTRODUCTION

The cloning of eukaryote DNA which contains the coding sequences of known genes potentially permits both a molecular analysis of the mechanism of gene expression as well as an assessment of the relationship, if any, between variations in coding and non-coding DNA of the different individuals in a population. The mapping of variations in nucleotide sequences in cloned genomic or mitochondrial DNA with restriction endonucleases has now been used in a number of evolutionary studies of variation within populations and between species. Examples are provided by the β -globin cluster in man (Jeffreys, 1979; Antonarakis, Orkin, Kazazian, Goff, Boehm, Waber, Sexton, Ostrer, Fairbanks and Chakravarti, 1982; Orkin, Kazazian, Antonarakis, Goff, Boehm, Sexton, Waber and Giardina, 1982), the *Adh* region of *Drosophila* species (Langley, Montgomery and Quattlebaum, 1982), mitochondrial DNA of *Drosophila* species (Shah and Langley, 1979), and of the deer mouse, *Peromyscus* (Avisé, Lansman and Shade, 1979). Restriction endonuclease mapping allows a relatively quick survey of large segments of DNA in many strains or individuals. As well as identifying restriction site or individual DNA base variations, the technique also identifies large insertions and deletions of DNA, relative to a standard sequence, in a selected section of DNA.

This study is of variations between 16 highly inbred lines of *Drosophila melanogaster*, derived from a single population "Texas", in the restriction map of 12 kb region of DNA which contains the coding sequences for the enzyme Alcohol dehydrogenase (ADH). The set of lines had been previously characterised with respect to several aspects of *Adh* gene activity, namely ADH enzyme activity, the level of immunologically cross-reacting material

specific to the ADH enzyme, Michaelis constants, aspects of gene interaction in expression, dominance and genotype-environment interaction (Birley, Marson and Phillips, 1980; Birley and Marson, 1981; Birley, Couch and Marson, 1981). The study is the first to report an association, within a population, of variation in restriction map and gene expression.

2. MATERIALS AND METHODS

(i) *The inbred lines*

Details of the 16 lines, highly inbred by brother-sister mating for over 120 generations and extracted from a large laboratory population, "Texas" are given in Birley and Barnes (1975) and in Birley, Couch and Marson (1981). Adults were reared on standard food medium (Birley *et al.*, 1982) at 25°, harvested and stored at -70°.

(ii) *DNA extraction and purification*

DNA was extracted from 1-1.5 gm of frozen material using CsCl₂ gradient centrifugation at 55,000 rpm for 72 hours at 15° in a Beckman 63 Ti rotor. Preparation of DNA for centrifugation followed a path of nuclear isolation and nuclear lysis in 2 per cent sodium N-lauryl sarcosine. After centrifugation the DNA was subjected to exhaustive dialysis against Tris (10 mM)-HCl-EDTA (1 mM) pH 7.5. Further details of the technique are to be found in Bingham, Levis and Rubin (1981).

(iii) *Restriction endonuclease digestion*

DNA was digested with restriction endonucleases at 37° in conditions recommended by the supplier (Bethesda Research Laboratories). Eight restriction endonucleases all specific for different hexanucleotide sequences were used. The enzymes were *Bam*HI, *Eco*RI, *Hind*III, *Hpa*-I, *Pst*-I, *Sal*-I, *Xba*-I, *Xho*-I. Electrophoresis of single and double endonuclease digests was carried out in 1 per cent agarose gels in a BRL H4 gel apparatus. The continuously circulated buffer was standard 1×TBE; Tris (89 mM)-boric acid (89 mM)-EDTA (2.5 mM) and low voltage (1.5 volts cm⁻¹) gradients were used (McDonnell, Simon and Studier, 1977) to separate the DNA fragments.

(iv) *Detection of specific DNA sequences*

DNA was transferred to nitrocellulose sheets (BRL) using the principles of Southern (1975). The pBR322 based on sAS-1 plasmid (Goldberg, 1980) was labelled with α ³²P dCTP, 3000Ci mM⁻¹ (Amersham International) by nick-translation (Rigby, Diekmann, Rhodes and Berg, 1977) and hybridisation carried out in the presence of dextran sulphate (Wahl, Stern and Stark, 1979). Autoradiography followed with Kodak X-Omat RP film for 24-48 hours at -70°C.

(v) *Molecular weight determination*

Molecular weights were determined using a Summagraphics digitiser interfaced with the University of Birmingham DEC-20 computer. The computer programme was developed and kindly made available by

W. Quattlebaum (Genetics Section, National Institute of Environmental Health Sciences, North Carolina). Computational details are given in Schaffer and Sederoff (1980).

3. RESULTS

The basic restriction map was, as in the study by Langley *et al.* (1982), not dissimilar from that of the sAF-2 clone of Goldberg (1980); this is the restriction map presented in Fig. 1. To facilitate comparison with the work of Langley *et al.* (1982), the species invariant *Bam*HI site in the second exon of the *Adh* transcriptional unit has been given the co-ordinate of 0.0. Restriction sites within the probed region will be referred to in relation to this co-ordinate.

Variation for both restriction sites and for large DNA insertions and deletions was present in the "Texas" inbred lines. Restriction site polymorphism was observed at four positions, *Pst*I (-6.2), *Hind*III (-3.0), *Xho*I (+1.2) and *Bam*HI (+1.5). Variants at or very close to the *Hind*III and *Xho*I sites have been described previously by Langley *et al.* (1982). *Pst*I and *Bam*HI sites were not present within the sAS-I probe and are characteristic of this population. Five types of DNA insertion or deletion were present. Two insertions were large, Δ 3700 kb and Δ 2300 kb, and one insert Δ 350 kb was polymorphic in some lines (lines 3 and 27) and fixed in three others (lines 7, 18 and 28). Line 19 presented three major insertion/deletion variants.

Estimates of genetic variability for the region were obtained using the estimators of Ewens, Spielman and Harris (1981). The proportion of nucleotide sites which are polymorphic is 0.012 and the estimate of heterozygosity per nucleotide is 0.004. When considered as a whole, the heterozygosity for restriction sites, in the entire probed region is 0.86, estimated according to Nei and Tajima (1981).

Tests of linkage disequilibrium were carried out on the six 2×2 combinations of polymorphic restriction sites with Fisher's exact test (table 1). A test for linkage disequilibrium in relation to *Adh*^F and *Adh*^S alleles was not carried out since the sample contained only two *Adh*^{F/F} lines and would in that case be insensitive. Strong linkage disequilibrium was shown amongst three of the four restriction site polymorphisms, *Hind*III (-3.0), *Xho*-I (+1.2) and *Bam*HI (+1.5).

Possible relationships between genetical architectures in the set of lines and gene activity were investigated by relating previously published estimates of mean ADH activity and ADH protein level (level of ADH cross-reacting material), (Birley, Couch and Marson, 1981) to restriction site polymorphism. This approach was also carried out for the comparative response of lines in genotype-environment interaction for ADH activity (Birley and Marson, 1981). There was no evidence of an association between the mean ADH activity or mean ADH protein level and any of the four restriction site polymorphisms. This is not surprising since ADH activity is controlled in this population (Barnes and Birley, 1978) as well as in other populations (McDonald and Ayala, 1978; Laurie-Ahlberg, Maroni, Bewley, Lucchesi and Weir, 1980), by loci which are located upon different chromosomes (I and III) from that upon which the *Adh* structural gene (chromosome II) is located.

TABLE 1

Probabilities, given by Fishers' exact test for a 2 × 2 contingency table, for linkage disequilibrium between restriction site polymorphisms

Restriction site combination	<i>P</i>
<i>Pst</i> -I/ <i>Hind</i> III	0.189
<i>Pst</i> -I/ <i>Bam</i> HI	0.330
<i>Pst</i> -I/ <i>Xho</i> -I	0.214
<i>Hind</i> III/ <i>Bam</i> HI	0.003
<i>Hind</i> III/ <i>Xho</i> -I	0.036
<i>Bam</i> HI/ <i>Xho</i> -I	0.100

Genotype-environment interaction for ADH activity has been previously assessed in the set of inbred lines (Birley and Marson, 1981) by the methods of biometrical genetics (Perkins and Jinks, 1968; Pooni and Jinks, 1980). In summary, the methods compare the performance of the set of inbred lines in a set of different environments, the measure of various environments being in this case purely biological and provided by the average performance of all lines. Two distinct forms of genotype-environment interaction can be distinguished from the survey of the "Texas" inbred lines (Birley and Marson, 1981); linear and non-linear. Lines classified as linear show a simple response to the environment which is largely linear. Lines classified as non-linear show a complex or inconsistent response to the environment. The classification of the set of inbred lines according to their response to the environment is shown in fig. 1 and the relationship between restriction site polymorphism and genotype-environment interaction is shown in table 3 as probabilities for Fishers' exact test for association in a 2 × 2 contingency table. Two restriction sites (*Hind*III (−3.0) and *Bam*HI (+1.5)) are associated with the type of response to the environment. Interestingly, it is these two restriction sites which show the most marked linkage disequilibrium (table 2), one (*Hind*III (−3.0)) on the 5' side and the other (*Bam*HI (+1.5)) on the 3' side of the *Adh* transcriptional unit (fig. 1). This particular association of restriction sites defines two major classes of haplotype in the population (table 3). The association between the two types of chromosome and type of genotype-environment interaction is strong ($P=0.016$; Fishers' exact test). Hence the population of inbred

TABLE 2

Restriction site polymorphism and genotype-environment interaction for ADH activity. The observed numbers of lines falling into the four categories of restriction site polymorphism (+ or −) and genotype-environment interaction response, depicted here as linear (+) and non linear (−), are given together with probabilities from Fishers' exact test for association in a 2 × 2 contingency table

Restriction site	++'	+−'	−+'	−−'	<i>P</i>
<i>Pst</i> -I	1	5	6	4	0.110
<i>Hind</i> III	5	1	2	8	0.024
<i>Xho</i> -I	4	9	3	0	0.062
<i>Bam</i> HI	1	7	6	2	0.020

TABLE 3

Linkage disequilibrium between the polymorphic HindIII and BamHI restriction sites and type of genotype-environment interaction

Restriction site		Genotype-environment interaction	
<i>Hind</i> III	<i>Bam</i> HI	Linear	Non-Linear
+	+	0	0
+	-	5	1
-	+	1	7
-	-	1	1

lines and presumably the population, "Texas", contain an apparently simple polymorphism for the control of genotype-environment interaction in ADH activity.

Associations between gene activity and the insertional and deletion variation are less easy to quantify because of the small sample of lines and because the different insertional/deletion variations are in all but one case, Δ_{350} , at low frequency, line specific, and vary in their position on the restriction map. The simplest test for association of this class of DNA polymorphism and genotype-environment interaction is to compare presence or absence of insertion with enzyme activity or response in genotype-environment interaction. No association was detected. In order to test for associations of gene action and insertional/deletion variation large samples of variation may be needed.

4. DISCUSSION

If gene frequencies in the set of "Texas" inbred lines have not been changed by natural selection during the period of inbreeding, the set of inbred lines can be considered a randomly chosen sample of variation within the "Texas" population. Observations of the variation for several metrical characters and for *Adh* allozyme frequencies (Birley and Barnes, 1975; Caligari and Mather, 1980) support this assumption. The variation between the lines reported here was detected as restriction endonuclease recognition sites and DNA insertions and deletions. Estimates of variation attributable to restriction sites and insertional/deletion variation are similar to those given by Langley *et al.* (1982) in a survey of 18 second chromosomes derived directly from four natural populations, though in general values are slightly lower in "Texas". Heterozygosity per nucleotide is here estimated as 0.004 and is similar to estimates of 0.006 and 0.004 obtained for the same DNA segment (Langley *et al.*, 1982) and from nucleotide sequence comparisons of the *Adh* exons with a sample of 11 independently extracted chromosomes (Kreitman, 1980). For the entire probed region, including both restriction site and insertion/deletion variation, heterozygosity is here estimated as 0.93 (Nei and Tajima, 1981), Langley *et al.*'s value being 0.95. That these estimates and the estimates of heterozygosity for insertion/deletion variation (see below) are only slightly lower than those found by the

latter authors for a natural population(s) is not surprising for reasons of sample size and on the expectations of neutral gene theory. Whilst the population size of "Texas" (3000) is lower than that of a natural population by perhaps a factor of 10^3 , the population has been in the laboratory for only 60 generations prior to the extraction of the inbred lines. The number of generations is thence much less than the effective size of "Texas"; even if N_e/N is 0.5, $N_e = 15 \times 10^2$. Thus the time for which random genetic drift can act is small compared to N_e and as shown by Malécot (1948) we can expect only a small loss of variation in "Texas" in the early generations of evolution.

The estimate of heterozygosity per nucleotide obtained by Kreitman (1983) was for the *Adh* exons and hence reflects not only silent site variation but also the extreme constraint of replacement site polymorphism which was limited solely to the amino acid difference corresponding to the *Adh*^F and *Adh*^S alleles. Whilst it is not yet known whether other coding sequences are present in the region of DNA homologous with the sAS-I probe the estimates of heterozygosity presented here strengthen Kreitman's observation of evolutionary constraint, since it can be presumed that the probed region of DNA must also contain a degree of non-coding DNA.

The restriction site polymorphisms are present in apparently intergenic regions of DNA. The endonuclease *Pst*-I, polymorphic for a restriction site in this study, was not used in the survey of Langley *et al.* (1982). Two restriction sites (*Hind*III (-3.0) and *Xho*-I (+1.2) of the remaining three restriction endonuclease polymorphisms observed here share, compared with the study of Langley *et al.*, the same restriction site. Indeed, as noted by Langley *et al.* (1982), it appears that the same restriction site polymorphisms are common to a number of populations. On the other hand, the variation for DNA insertions and deletions appear to be population specific and this may reflect different evolutionary mechanisms or dynamics associated with this latter class of variation. The high frequency of insertional/deletion variation for this region, estimated heterozygosity 0.69 (Langley *et al.*) and 0.57 ("Texas"), appears to be characteristic of populations in general. The positions of the insertions/deletions also appears to be localised into distinct regions some 2–3 kb on each side of the *Adh* transcriptional unit and is not associated with restriction site polymorphism. This class of variation has also been observed in other tracts of DNA, for example, human haemoglobin (Higgs, Goodbourn, Wainscoat, Clegg and Weatherall, 1981) and is well known in mutant laboratory stocks of *Drosophila*. Sometimes the insertional variation can be attributable to transposable elements (Green, 1980; Strobel, Dunsmuir and Rubin, 1979) and, in turn, these have been identified in association with the laboratory mutants (Bingham, 1981; Engels and Preston, 1981). At present the nature and origin of the insertions and deletions in the *Adh* region is unknown. They might even be in regions in which there is a high degree of genetic instability and dysgenic crosses in *Drosophila* have been associated with the production of DNA insertions through transposition (Green, 1977; Engels, 1981). In this context and as noted by Langley *et al.* (1982) chromosomes made isogenic by crosses to certain chromosome "balancer-stocks" are liable to induce dysgenic phenomena during their extraction in the laboratory. The recovery of insertional and deletion variations in this study with inbred lines which have not been outcrossed to mutant laboratory stocks suggests

that they are indeed a natural feature of populations and those insertions which do originate from hybrid dysgenesis do not occur at least in this region of the chromosomes at a sufficiently high frequency for their recovery.

One perhaps surprising feature of the data is that one insert, Δ_{350} , is polymorphic within the two lines 3 and 27. Although there is a restriction site difference (*Hind*III (-3.0)) between these two lines there is no further evidence of variation. Indeed these two lines are phenotypically distinct for a number of metrical characters (Birley and Barnes, 1973; Caligari and Mather, 1980) and it is unlikely that the observations are due to stock contamination.

The novel feature of this study was the relationship between DNA haplotype and the response of lines in ADH activity to environmental variance. The linkage disequilibrium between restriction sites on chromosomal markers was strongest for the *Bam*HI/*Hind*III combination. Moreover the gametic ditypes for this combination of restriction sites show a strong association with genotype-environment interaction. Of course the association may be a chance event, reflecting the ancestry of the chromosome. Indeed for such reasons, levels of linkage disequilibrium might be expected to be particularly high in such a relatively short DNA segment. Nonetheless, the DNA concerned bounds the transcriptional units and whilst any evolutionary, causal relationship between the DNA, marked by the polymorphic restriction sites and the control of *Adh* activity is not proven, the association by virtue of its relationship with the ADH activity phenotype is a potential target for any form of natural selection capable of acting on ADH activity. One further aspect of the observed linkage disequilibrium was that the associated disequilibria of the *Hind*III, *Bam*HI and *Xho*-I sites with the *Pst*-I site variation was weak, further strengthening the argument for association of genotype-environment interaction and the *Bam*HI/*Hind*III chromosome segment. Another view of the genotype-environment interaction is that it originates from variation of enzyme catalytic properties. However, a study of the Michaelis constants for ADH in the set of inbred lines failed to show any statistically significant differences within *Adh*^{S/S} or within *Adh*^{F/F} lines. The only difference was that attributable to the ADH-F and ADH-S enzyme difference (Sroczyński and Birley, unpublished data).

Although no clear relationship emerged between DNA inserts/deletions and genotype-environment interaction, as discussed earlier in this class of variation is highly heterogeneous. Whether this class of naturally occurring DNA variation affects the ADH phenotype is unknown, however, their often low frequency implies they could be very mildly deleterious in fitness. Obviously more detailed survey and *in vitro* experiment is required. However, this study has shown that a study of naturally occurring variation in DNA may be useful in the study of gene function.

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