Restriction map variation in the Adh region of Drosophila
(population genetics/evolution/noncoding/insertion/deletion)

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Communicated by C. Clark Cockerham, June 16, 1982

ABSTRACT Restriction maps of a 12,000-nucleotide region containing the alcohol dehydrogenase (Adh) locus of the second chromosome of Drosophila were determined for (i) 18 independent second chromosome stocks of D. melanogaster established from several natural populations and (ii) five closely related Drosophila species. All of the detected map differences appear to lie outside the Adh coding block. Four polymorphic restriction sites and four insertion/deletion variants were identified among D. melanogaster chromosomes. The estimated heterozygosity per nucleotide site was 0.006. The relative distributions of restriction sites and insertion/deletion variations among subpopulations and species suggest that the insertion/deletion variation may be mildly deleterious.

In recent years, it has become possible to survey organisms for DNA sequence variation detected as differences in distributions of restriction endonuclease sites along specific regions of DNA (1). Both nuclear and cytoplasmic DNAs show variation in laboratory strains. These differences are important in localizing mutational lesions and functional regions. Restriction map variation also has been systematically surveyed in natural populations of several species, including Drosophila melanogaster, simulans, and viridis (2); the deer mouse (3); and man (4). These studies have allowed the estimation of sequence diversity among individuals, populations, and species DNA sequences that have been inaccessible (e.g., noncoding regions and mitochondrial genomes).

Goldberg reported the cloning of the DNA in and around the alcohol dehydrogenase locus (Adh) of D. melanogaster (5). A single transcript was found from this region—that of Adh (5). The availability of this cloned sequence afforded us the opportunity to survey restriction map variation in this region. We report here the restriction maps from 18 independent second chromosome stocks of D. melanogaster established from several natural populations. We also present restriction maps for five other species closely related to D. melanogaster. From these maps, estimates of sequence diversity over a 12,000-nucleotide region can be made. Insertion/deletion variation was found both within and between species. The distribution over individuals, subpopulations, and species suggests some selective effect of insertions/deletions outside the transcriptional unit.

MATERIALS AND METHODS

Drosophila Stocks. Eighteen stocks of D. melanogaster homozygous for independent second chromosomes isolated from several natural populations were received from C. C. Laurie-Ahlberg (6). Ten chromosomes were derived independently from individual Drosophila caught in Rhode Island (R1 through R10), four from Kansas (K1 through K4), two from Minnesota (M1 and M2), and two from North Carolina (N1 and N2). The stocks of other Drosophila species are lines available at the Bowling Green Stock Center. These were reared under standard laboratory conditions, frozen in liquid nitrogen, and stored at −70°C until DNA preparation.

Probes. Two recombinant plasmids received from D. Goldberg (5) were used as probe DNAs in this work: sAS1 and sAC1. sAC1 is a plasmid bearing the D. melanogaster DNA sequence between the EcoRI sites at positions −2.9 and +1.9 in Fig. 1; sAS1 contains the sequence between SacI sites (not shown) at −6.8 and +4.6 in Fig. 1.

Preparation of Drosophila DNA for Southern Gel Analysis. The method described by Bingham et al. (7) was used to prepare DNA from adult Drosophila. The Drosophila DNAs (1–2.5 μg per lane) were digested under conditions recommended by suppliers (Bethesda Research Laboratories, New England BioLabs, and Boehringer Mannheim), and the DNA digests were electrophoresed as described by McDonnell et al. (8). The eight restriction enzymes used in the survey were BamHI, EcoRI, HindIII, HpaI, KpnI, SalI, XhoI, and XorII, all of which have unique hexanucleotide recognition sequences.

Southern Transfer. Preparation of the gel, transfer of the DNA, preparation of the nitrocellulose filter, hybridization of the probe, washing of the filter, and detection of the bands autoradiographically were as described by Wahl et al. (9). To detect several of the smaller fragments, the technique of Thomas (10) was used. Labeling of probe DNA with 32P was performed as described by Rigby et al. (11). In situ hybridization of sAS1 DNA to D. yakuba, D. teissieri, and D. erecta was performed as described by Bingham et al. (7). Algorithms for sizing of restriction fragments by computer were provided by Schaffer and Sederoff (12).

Survey. The strategy for surveying the variability in the restriction maps around the Adh transcriptional unit (Adh region) was to digest each DNA with each restriction enzyme separately. There was obvious variation among the lines of D. melanogaster in the sizes of fragments identified by the sAS1 probe in digests with seven of the restriction enzymes (digests with Kpn1 appeared monomorphic). Shown by subsequent double digestions, all of the variation among these stocks was mapped outside the probed region except for the four polymorphic restriction sites and the four insertion/deletion differences indicated in Fig. 1. The most common arrangement was that of the sAF2 clone of Goldberg (5); this is the standard for D. melanogaster in Fig. 1. Two chromosomes derived from the 10 D. melanogaster caught in Rhode Island appear to be similar to clone sAS1 in restriction sites and arrangement (i.e., Δex).

One line, R7, was scored ambiguously. Between the HindIII sites at positions −2.6 and −3.4 on the map in Fig. 1 is another HindIII site. Estimates of variability calculated by assuming R7 had a third site at either positions −2.7 or −3.0 were not sig-

Abbreviation: Adh, alcohol dehydrogenase genetic locus.
RESULTS

Fig. 1 shows the positions of various restriction sites, insertions, deletions, and the Adh transcriptional unit (5, 19) in the second chromosome of Drosophila. The BamHI site in the coding sequence (apparently present in all stocks and species) was arbitrarily given a coordinate of 0.0. The positions of insertions/deletions in the D. melanogaster lines were within the indicated fragments. \( \Delta t \) is known to fall between the Sal I site at position +2.1 and a Pvu I site at +3.0 (5). Table 1 shows the distribution of the polymorphic restriction sites and insertion/deletion differences among the 18 second chromosome lines. Also indicated are the Adh electrophoretic phenotypes (Adh' or Adh*) and gene arrangements of the chromosomes (C. C. Laurie-Ahlberg, personal communication; unpublished data).

There were 11 distinct restriction maps among the 18 D. melanogaster second chromosomes surveyed. The unbiased estimate of restriction map heterozygosity is 0.95. Because the variation is of two distinct types, it is appropriate to estimate the contributions of restriction sites and insertions/deletions separately. The proportion of polymorphic nucleotide positions and heterozygosity can be estimated by several means (13-17). These give similar results. The proportion of polymorphic nucleotide sites in the sampled chromosomes is estimated to be between 0.012 and 0.015 (13, 15, 17). The estimated heterozygosity per nucleotide is 0.006 (13, 17).

Insertion/deletion variation is less easily quantified because there is no familiar theory of its origin and evolution and because the insertions and deletions vary in size. The estimated heterozygosity for insertions/deletions was 0.69 for this region. The size of the insertions/deletions ranged from 20 to 900 nucleotides (mean, 336 nucleotides).

![Restriction maps of the Adh region of D. melanogaster and other members of the melanogaster subgroup. Sites below the line for D. melanogaster were found in all eighteen second chromosomes. Upper sites, insertions (\( I \)), and deletions (\( D \)), were polymorphic (see Table 1). Insertions and deletions are roughly to scale; see Table 1 for estimated sizes. ■, Coding blocks of the Adh transcriptional unit, 5' left to 3' right (5, 18); |, approximated borders of the transcriptional unit (ref. 5; C. Benyajati, personal communication); \( \bullet \), fragments probed with sAS1; \( \circ \), fragments probed with sAC1. Scale is 1,000 nucleotides.](image)
Table 1. Restriction map variants among *D. melanogaster* second chromosome lines*

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* See text and Fig. 1 for explanation.
† St, standard gene arrangement; In2L(t), a cosmopolitan inversion (20); nt, nucleotides.

As expected from population genetics theory, there is linkage disequilibrium among some of these closely linked polymorphic elements. There are significant nonrandom associations among the three highly polymorphic elements (BamHI site at position −7.1, Adh<sup>65</sup> electrophoretic phenotypes, and Xho I site at position +1.2). In fact, only four of the expected eight combinations of these three elements were found in the sample.

One line each of *D. simulans*, *D. mauritiana*, *D. yakuba*, *D. teissieri*, and *D. erecta* was analyzed in a fashion similar to the analysis of the *D. melanogaster* lines. The DNA maps are presented also in Fig. 1. Table 2 summarizes the restriction site differences in the *Adh* region among the three sibling species. The *D. simulans* DNA shows the restriction map of the *D. melanogaster* standard (Fig. 1). The *D. mauritiana* DNA map shows one deletion (100 nucleotides) relative to the map of the *D. melanogaster* standard. It is difficult to align the restriction maps of *D. yakuba*, *D. teissieri*, and *D. erecta* DNAs because of the large number of divergent sites. Notice that the basic amount of DNA and its sequence arrangement appears to have been conserved. The central restriction sites of BamHI at position 0.0 and Hpa I at position −1.1 and flanking Sal I sites suggest conservation of a fundamental arrangement. This is further confirmed by the locations and amounts of sAC1- and sAS1-probed regions in all species.

In *D. yakuba* and *D. teissieri*, we found a second region with homology to sAC1. sAS1 probed no additional material at these sites, suggesting the homology of this secondary region is limited to the sAC1 region. The restriction maps of these secondary sites were unique. These results were consistent with in situ hybridization results (data not presented) that showed additional and relatively lightly labeling sites on the third chromosomes at 96D 1-2 in these two species (*Adh* is at 35A-B on the second chromosome).

**DISCUSSION**

The advent of recombinant DNA technology allows (in principle) the investigation of genetic variation in any element at the fundamental level, the nucleotide sequence. Studies such as this are early glimpses into that large portion of the genome that was previously hidden: noncoding regions. Despite their limited sensitivity to nucleotide substitution, restriction maps are nevertheless revealing unexpected variation. We report here levels of restriction site and insertion/deletion variability in (i) 18 independent second chromosome stocks of *D. melanogaster* established from several natural populations and (ii) five other species of the *melanogaster* subgroup of *Drosophila*. Perhaps the most novel aspect of the results reported here is the high incidence of insertions/deletions. The published observations of insertional variation among *Drosophila* stocks foreshadow these results (21, 22), as do recent reports on insertion/deletion polymorphism in the human hemoglobin and insulin regions (23–25). There are no data yet on the possible involvement of transposable elements in the insertion/deletion variation reported here, but their role in laboratory stock differences (21, 22) make them appealing suspects.

The *D. melanogaster* chromosomes in this study were isolated and established in stock lines by crosses that were presumably dysgenic (26). However, two lines of evidence weigh against a dysgenic origin in the laboratory for most if not all of the insertions/deletions detected here. First, insertions/deletions were recovered at a much higher frequency (0.44) than was expected from published mutation rates attributable to hybrid dysgenesis (27). Second, multiple recovery of apparently identical insertions/deletions indicates that they were present in the natural populations.

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Can anything be deduced about the rate of occurrence and consequences of these insertions/deletions based on their distributions? The following interpretation of our results is consistent with an associated deleterious effect. It is obvious that other hypotheses involving as yet unknown mechanisms of mutation and reversion might also be constructed to fit these data. The sample reported here, although small, is composed of subsamples from four North American localities. Note that the rare DNA arrangement at each site occurs in only one subsample. The insertions/deletions appear to be restricted to local populations, whereas the restriction site polymorphisms are more widely distributed.

The second line of evidence comes from comparisons of the restriction maps of the Adh region of D. melanogaster and its relatives. No insertion/deletion differences were observed between maps of D. simulans DNA and the most common arrangement of D. melanogaster DNA. One deletion distinguished the D. mauritiana DNA arrangement from the common D. melanogaster arrangement. The average number of insertion/deletion differences between the Adh regions of D. melanogaster and D. simulans and between those of D. melanogaster and D. mauritiana were 0.44 and 1.44, respectively. This can be compared to a value of 0.69 for the unbiased estimate of the expected insertion/deletion heterozygosity in this region among the D. melanogaster chromosomes. Thus, insertion/deletion variation was no greater between these species than within D. melanogaster, whereas restriction site variation was 10 times greater among these species (see below). Comparisons with D. yakuba, teissieri, and erecta extended this impression.

Crude estimates of the proportion of substituted nucleotides (13-17, 19, 28) were all between 0.05 and 0.20, depending on how the maps were aligned. But it appears that, in these more divergent comparisons, the amount and arrangement of sequences were conserved. There is evidence for several insertion/deletion substitutions, but the data were inconsistent with the accumulation of any large (>1,000 nucleotides) insertion/deletion differences.

One interpretation of these data is that insertions/deletions do indeed become substituted but less frequently than restriction site differences. Perhaps the smaller insertions/deletions are less deleterious and, thus, more likely to be fixed. Similar conclusions appear from the analyses of the heat shock loci at 87A and 87C (ref. 29; A. J. Leigh Brown and D. Ish-Horowitz, personal communication) and the hemoglobin regions of primates (30). One can only speculate on the mechanisms or magnitude of the apparent deleterious effects of these rearrangements. They could have slight but significantly deleterious effects on fitness (a selection coefficient of perhaps $10^{-5}$) through alterations of the expression of Adh. Their effect might be even less direct such as on chromatin structure and chromosome segregation. If they involved sequences from transposable elements, the deleterious effects might arise from several possible interactions with other transposable elements—e.g., increased mutability, sterility, or meiotic drive (26). Considering their frequency and apparent selective effects, these small DNA rearrangements flanking the coding block are an important form of genetic variation that, with increasing molecular genetic information, must be incorporated into our understanding of the genetic basis of evolutionary processes.

The distribution of insertions/deletions along the Adh region in the D. melanogaster chromosomes may be nonrandom. The three largest insertions/deletions occurred within a 1,000-nucleotide region of the coding block. In fact, seven of the nine restriction map polymorphisms (see Fig. 1) occurred in two separate 1,000-nucleotide segments that are located 1,000–2,000 nucleotides on either side of the coding block. This apparent clustering of population genetic variation in intergenic regions is similar to that reported in the a-hemoglobin and insulin regions in man (23–25). The illumination of these observations must await further study.

The amount of variability of the nucleotide substitution type can be estimated from restriction maps by several techniques. These methods gave similar estimates of heterozygosity of 0.006 per nucleotide for D. melanogaster DNA. This can be compared to estimates of 0.007 for mitochondrial DNA in D. melanogaster (2, 13) and 0.001 for the human b-hemoglobin region (4, 15, 16). The restriction maps of the Adh region in D. simulans and D. mauritiana showed many restriction site differences (see Table 2). Average numbers of differences from the D. melanogaster chromosomes were 9.1 and 13.1, respectively, whereas the only differences between D. simulans and D. mauritiana chromosomes were the four additional sites in D. mauritiana. These differences corresponded to estimates of proportions of nucleotide substitutions of 0.039 and 0.054 between D. melanogaster and D. simulans and D. melanogaster and D. mauritiana, respectively; that between D. simulans and D. mauritiana was 0.013. These were calculated as suggested by Nei and Tajima (13) by using the D. melanogaster within-species estimate of 0.004 (excluding HindIII sites at positions −3.0 and −2.7). Estimates using other statistical techniques were very similar (15, 17). The Adh regions of D. simulans and D. mauritiana diverged twice as much as the most divergent pair of D. melanogaster chromosomes, whereas the divergence between the Adh regions of D. melanogaster and the other two species was greater than 3-fold.

We have found that both insertion/deletion and restriction site polymorphisms are common in the Adh region. Their distributions among populations and species suggest a relative selective disadvantage for insertions/deletions. Because all of the detected variation is outside the Adh coding block, alteration in sequences flanking the Adh locus may have some selective effects. Although the lack of detected transcripts, other than that of Adh, from this region does not preclude other coding blocks, it is likely that most of the region we have analyzed is intergenic (5, 31). The intergenic regions have been the object of study from both a genetic and a molecular direction (31). Genetically these regions appear to be recalcitrant to mutation that is phenotypically visible (32–34). Molecular studies indicate that independent transcriptional units are often separated by large intergenic regions (31). In the yeast mating-type loci (35) and mammalian hemoglobin loci (36, 37), sequences at least 800 nucleotides from the transcriptional unit have some effect on expression. The mechanism of these distant effects is not clear. The significance of this type of variation to our understanding of developmental and molecular genetics is not yet apparent, but the possible implications for population genetics are important because most of the DNA of higher organisms is of this intergenic type. The observations and theory of population genetics hold that most evolutionarily significant variation is attributable to small differences at many loci (38). It is possible that most of the mutations leading to small changes in phenotype will be in the intergenic regions. Thus, the target and numbers of mutations of small effect may be much larger than that for those of large effect. Most of the evolutionarily important variation may be in these intergenic sequences that we have only recently begun to study and whose functional mechanisms are so obscure that interpretation of specific differences in these regions is presently out of the question.

We thank D. Goldberg for the Adh clones. The many helpful suggestions of P. M. Bingham are greatly appreciated. Leslie Gardner's timely and careful preparation of the manuscript is appreciated. This paper was improved by the criticisms of many colleagues; we thank
them also. Henrik Gyurkovics kindly determined the cytogenetic location of the secondary sites of in situ hybridization.