Analysis of Nucleotide Substitutions of Mitochondrial DNAs in *Drosophila melanogaster* and Its Sibling Species

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To study the rate and pattern of nucleotide substitution in mitochondrial DNA (mtDNA), we cloned and sequenced a 975-bp segment of mtDNA from *Drosophila melanogaster*, *D. simulans*, and *D. mauritiana* containing the genes for three transfer RNAs and parts of two protein-coding genes, ND2 and COI. Statistical analysis of synonymous substitutions revealed a predominance of transitions over transversions among the three species, a finding differing from previous results obtained from a comparison of *D. melanogaster* and *D. yakuba*. The number of transitions observed was nearly the same for each species comparison, including *D. yakuba*, despite the differences in divergence times. However, transversions seemed to increase steadily with increasing divergence time. By contrast, nonsynonymous substitutions in the ND2 gene showed a predominance of transversions over transitions. Most transversions were between A and T and seemed to be due to some kind of mutational bias to which the A+T-rich mtDNA of *Drosophila* species may be subject. The overall rate of nucleotide substitution in *Drosophila* mtDNA appears to be slightly faster (~1.4 times) than that of the *Adh* gene. This contrasts with the result obtained for mammals, in which the mtDNA evolves ~10 times faster than single-copy nuclear DNA. We have also shown that the start codon of the COI gene is GTGA in *D. simulans* and GTAA in *D. mauritiana*. These codons are different from that of *D. melanogaster* (ATAA).

Introduction

On the basis of studies of restriction-site maps and DNA hybridization, it has been suggested that mammalian mitochondrial DNA (mtDNA) accumulates nucleotide substitutions 5-10 times faster than does single-copy nuclear DNA (Brown et al. 1979). Direct nucleotide sequence comparisons among primate mtDNAs have verified the high rate of substitution and revealed a predominance of transitions over transversions in the protein-coding genes (Brown et al. 1982). To explain this high rate of substitution in a genome that is packed so tightly with functional genes, it has been proposed that animal mtDNA has an unusually high mutation rate (Avise and Lansman 1983; Brown 1983, 1985).

This conclusion, however, may not be generally applicable. Powell et al. (1986) compared the extent of mtDNA divergence with that of single-copy nuclear DNA (as measured by DNA hybridization) between *Drosophila melanogaster* and *D. yakuba* and concluded that the rate of nucleotide substitution is nearly the same for the two types of DNA.

1. Key words: evolutionary rate, transitions, transversions, synonymous substitutions, mitochondrial DNA, *Drosophila.*

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Recently the complete nucleotide sequence of *D. yakuba* mtDNA and a portion of *D. melanogaster* mtDNA (4,869 bp) has been determined (de Bruijn 1983; Clary and Wolstenholme 1985). Wolstenholme and Clary (1985) compared the nucleotide sequences of a continuous 5-kb homologous piece of the two types of mtDNA. In contrast to the pattern of substitutions found in mammals, a predominance of transversions over transitions was found. However, the divergence time between *D. melanogaster* and *D. yakuba* is too large to allow one to obtain knowledge about the actual pattern of nucleotide substitution.

To study the rate and pattern of nucleotide substitution in mtDNA in more detail, we have cloned and sequenced homologous pieces of mtDNA from three species of the *melanogaster* subgroup, *D. melanogaster*, *D. simulans*, and *D. mauritiana* (fig. 1). Since these species probably diverged <5 Myr ago (Cohn et al. 1984; Stephens and Nei 1985), DNA sequence analysis can provide more accurate information about the nature and rate of nucleotide substitution. Furthermore, the DNA sequence of the alcohol dehydrogenase (*Adh*)-coding region of each of these three species has already been determined (Kreitman 1983; Bodmer and Ashburner 1984; Cohn et al. 1984). By using this information, we have made a detailed comparison with respect to rate and pattern of nucleotide substitution in mitochondrial and nuclear genes in *Drosophila*.

**Materials and Methods**

**Species and Strains**

For each of the three species of the *melanogaster* subgroup, the nucleotide sequence of several isofemale strains were examined. These isofemale strains have been maintained for >2 years in our laboratory. In our comparisons of evolutionary rates, we chose one representative (with respect to the nucleotide sequence) strain from each species. The strains used in the comparison are as follows:

**Drosophila melanogaster.**—The strain was from Sapporo, Japan, and collected in 1982.

**Drosophila simulans.**—The strain was from Nairobi, Kenya, and collected by O. Kitagawa in 1979.

**Drosophila mauritiana.**—This species is endemic to Mauritius Island. The strain used was collected by O. Kitagawa in 1979.

The polymorphisms within each species will be reported in a separate paper.

**mtDNA Preparation and Sequencing**

Mitochondrial fractions were prepared from adult flies by means of differential centrifugation and purified through discontinuous sucrose gradients (1.0 M, 1.5 M,
and 1.75 M) (Clayton and Vinograd 1969). Mitochondria were collected from the boundary between the 1.0-M and 1.5-M layers. The DNA was isolated from this fraction by means of sodium laurylsulfate (SDS)–phenol extraction. Purification by means of CsCl density-gradient centrifugation was not necessary. mtDNA was sequentially digested with BglII and XhoI as shown in figure 2. The 1,500-bp fragment was purified from low-melting-point agarose and ligated into the BglII and SalI sites in the polylinker region of pHY300PLK (Ishiwa and Shibahara 1985). After amplification and purification of the recombinant plasmid DNAs (Maniatis et al. 1982), the HindIII–EcoRV (~500-bp) and EcoRV–BamHI (~1,000-bp) fragments were cloned into M13 sequencing vectors (Messing et al. 1981). The nucleotide sequences of the resulting ssDNAs were determined by means of the dideoxy chain-termination method (Sanger et al. 1977).

In addition, we used a new assay system to facilitate the detection of different types of nucleotide substitutions. Instead of charging the sequencing gel in the order ACGT for each species, as is usually done, the ddATP reactions of each species were charged into adjacent wells. The other ddNTP reactions were loaded similarly on the same gel (fig. 3). This procedure aided the detection of substitutions, since sequence differences could be easily seen as gaps in the normal continuous bands.

For confirmatory purposes, the sequence of the same mtDNA fragment derived from Oregon-R and contained in recombinant plasmid M2/8 (provided by W. J. Gehring) was determined; it was found to be exactly as described by de Bruijn (1983).

Calculation of Divergences

Nucleotide sequence divergences of mtDNA (975 bp) and Adh (831 bp) were calculated in two different ways. For protein-coding regions, synonymous (causing no amino acid changes) and nonsynonymous (resulting in amino acid replacements) substitutions were obtained by using the method of Miyata and Yasunaga (1980). For

![Fig. 2. Cleavage map, sequencing strategy, and gene content of the cloned segment of Drosophila mtDNA. The circular map indicates HindIII recognition sites. The arrows in the diagram indicate the direction and length of the fragments sequenced. B = BglII; E = EcoRV; and X = XhoI. There are three tRNA coding regions and a 19-bp intergenic sequence between ND2 and COI.](https://academic.oup.com/mbe/article-abstract/4/6/638/977799/64663897779)
the entire sequenced region, which includes either tRNA and intergenic sequences (for mtDNA) or the 5'-untranslated region and introns (for the Adh gene), the divergences were calculated using the method of Jukes and Cantor (1969).

**Results**

**Gene Contents and Comparisons of the Sequenced Regions**

On the basis of the results of direct sequence analyses (de Bruijn 1983; Clary and Wolstenholme 1985), the sequenced region (975 bp) in the present experiment contains
three complete tRNA genes (tRNA$^{\text{trp}}$, tRNA$^{\text{cys}}$, and tRNA$^{\text{ tyr}}$), as well as portions of two protein-coding genes, 461 bp from the 3' end (including the stop codon) of NADH dehydrogenase subunit 2 (ND2) (Chomyn et al. 1985) and 309 bp from the 5' end (including the start codon) of cytochrome oxidase subunit 1 (COI). The gene organization is identical for the three species used.

The nucleotide sequences of the mtDNA segments for the three species are shown in figure 4. Differences among these species were observed at 45 positions. Nucleotide substitutions account for the differences at 44 of the 45 positions. Besides substitutions, there was an addition of an A-T pair in Drosophila mauritiana mtDNA within the 19-bp noncoding region between the tRNA$^{\text{cys}}$ and tRNA$^{\text{ tyr}}$ genes. Since this noncoding sequence is highly A+T rich, the exact position of the addition is uncertain (fig. 4).

Comparison of the nucleotide sequences shows that D. simulans and D. mauritiana are more similar to each other than either is to D. melanogaster. As shown in table 1, D. simulans and D. mauritiana differ from each other in 2.1% of nucleotide sites, whereas they differ from D. melanogaster in 3.7% and 3.1% of nucleotide sites, respectively.

Comparison of tRNA Genes

In the tRNA$^{\text{trp}}$ gene, nucleotide substitutions were not observed among the three species. The tRNA$^{\text{cys}}$ and tRNA$^{\text{ tyr}}$ genes were identical between D. simulans and D. mauritiana, whereas the D. melanogaster genes showed one substitution in each gene, as shown in figure 4. All substitutions occurred in the loops; one was in the TYC loop of the tRNA$^{\text{cys}}$ gene and the other in the variable loop of the tRNA$^{\text{ tyr}}$ gene. According to Wolstenholme and Clary (1985), the tRNA$^{\text{cys}}$ and tRNA$^{\text{ tyr}}$ genes each show two substitutions between D. melanogaster and D. yakuba, and the tRNA$^{\text{trp}}$ gene, which is the most variable, shows nine substitutions. In the present study there was no significant difference in variability between tRNA$^{\text{trp}}$ and the other two tRNAs, i.e., tRNA$^{\text{cys}}$ and tRNA$^{\text{ tyr}}$.

Start Codon

An anomalous start codon, ATAA, has been reported for the COI gene in D. melanogaster mtDNA and D. yakuba mtDNA (de Bruijn 1983; Clary and Wolstenholme 1985). This ATAA start codon was also found in all seven D. melanogaster nucleotide sequences examined in the present study. In two other species, however, we observed two different potential start codons, which have not previously been reported. As shown in figure 4, the start codon for the COI gene is GTGA in D. simulans and GTAA in D. mauritiana. To confirm these observations we again sequenced the regions including these start codons, using two sets of synthetic primers (fig. 4). The same results were obtained. It is not known whether these anomalous codons specify the same amino acid.

Comparisons of Protein-coding Sequences

Among the three species, the nucleotide divergence of ND2 was approximately equal to that of COI. However, the amino acid sequence divergence was very different for the two genes (table 1). For example, the nucleotide divergence between D. melanogaster and D. simulans is 4.1% for ND2 and 4.3% for COI. In contrast, the amino acid sequence divergences for ND2 and COI are 5.9% and 1.0%, respectively.

An analysis of the effect of the nucleotide substitutions on amino acid replacement in ND2 and COI is given in table 2. Most of the nucleotide substitutions in COI are
FIG. 4.—Sequence of 975-bp fragments of Drosophila mtDNAs. The D. melanogaster sequence is shown in the uppermost line. Differences from this sequence in the other two species are indicated. A single base addition was found at position 586–588 in the D. mauritiana sequence. The anomalous start codons of COI are boxed. The position of the synthetic primer sequence is underlined. The five genes present have the following number of nucleotides: ND2, 461 bp; tRNA(cys), 66 bp; tRNA(trp), 550 bp; tRNA(tyr), 700 bp; and COI, 309 bp. The 5' end of the ND2 gene and the 3' end of the COI gene are outside the fragment. The sense of each gene is indicated by arrows.
Table 1
Nucleotide and Amino Acid Sequence Divergences of Two mtDNA Genes among *Drosophila melanogaster* (mel), *D. simulans* (sim), and *D. mauritiana* (mau)

<table>
<thead>
<tr>
<th>PARAMETER AND SPECIES COMPARED</th>
<th>COI</th>
<th>ND2</th>
<th>tRNA</th>
<th>Total*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of nucleotides</td>
<td>305b</td>
<td>462</td>
<td>186</td>
<td>971</td>
</tr>
<tr>
<td>% Nucleotide sequence divergence:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mau-sim</td>
<td>3.0</td>
<td>2.4</td>
<td>0</td>
<td>2.1</td>
</tr>
<tr>
<td>mel-sim</td>
<td>4.3</td>
<td>4.1</td>
<td>1.1</td>
<td>3.7</td>
</tr>
<tr>
<td>mel-mau</td>
<td>2.6</td>
<td>4.3</td>
<td>1.1</td>
<td>3.1</td>
</tr>
<tr>
<td>% Amino acid sequence divergence:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mau-sim</td>
<td>0</td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mel-sim</td>
<td>1.0</td>
<td>5.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mel-mau</td>
<td>1.0</td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Including the noncoding sequence and excluding the start codon.

b Excluding the start codon.

synonymous. For example, comparison of the nucleotide sequences of the COI genes from *D. melanogaster* and *D. simulans* indicates that only one of 11 substitutions is nonsynonymous. By contrast, the ND2 gene shows that the number of nonsynonymous substitutions is approximately equal to or slightly more than that of synonymous substitutions (table 2). The synonymous:nonsynonymous substitution ratio in the ND2 gene between *D. melanogaster* and *D. simulans* is \( \sim 1:1 \).

The distributions of the different kinds of nucleotide substitutions (transitions and transversions) among the protein genes are given in table 3. A predominance of transitions is observed only in the case of the comparison made between the most closely related species. When *D. simulans* and *D. mauritiana* are compared, 84% of all nucleotide differences are transitions. A predominance of transitions between two *D. melanogaster* sequences has been reported elsewhere (Wolstenholme and Clary)

Table 2
Frequencies of Nucleotide Substitutions in the Protein Genes (ND2 and COI) of mtDNA

<table>
<thead>
<tr>
<th>SUBSTITUTION TYPE AND SPECIES COMPARED</th>
<th>COI</th>
<th>ND2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synonymous:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mau-sim</td>
<td>8</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>mel-sim</td>
<td>10</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>mel-mau</td>
<td>6</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>Nonsynonymous:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mau-sim</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>mel-sim</td>
<td>1</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>mel-mau</td>
<td>1</td>
<td>13</td>
<td>14</td>
</tr>
</tbody>
</table>

NOTE.—Abbreviations are as given in table 1.
Evolution of *Drosophila* mtDNA

Table 3  
Frequency Distributions of Transitions and Transversions in Synonymous and Nonsynonymous Substitutions in the Two Protein Genes of mtDNA

<table>
<thead>
<tr>
<th>SUBSTITUTION TYPE</th>
<th>SPECIES COMPARED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mau-sim</td>
</tr>
<tr>
<td>Synonymous:</td>
<td></td>
</tr>
<tr>
<td>Transition . .</td>
<td>14 (6)</td>
</tr>
<tr>
<td>Transversion . .</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Total . . .</td>
<td>15</td>
</tr>
<tr>
<td>Nonsynonymous:</td>
<td></td>
</tr>
<tr>
<td>Transition . .</td>
<td>2</td>
</tr>
<tr>
<td>Transversion . .</td>
<td>2</td>
</tr>
<tr>
<td>Total . . .</td>
<td>4</td>
</tr>
<tr>
<td>Total . . .</td>
<td>16</td>
</tr>
</tbody>
</table>

**NOTE.**—Numbers in parentheses denote the numbers of substitutions at the fourfold-degenerate sites in the respective pairs. *yak* = *Drosophila yakuba*; all other abbreviations are as given in table 1.

Comparison of *D. melanogaster* with *D. simulans* and *D. mauritiana* shows that the frequencies of transitions and transversions are approximately equal. This finding is similar to the results obtained from the comparison between *D. melanogaster* and *D. yakuba* (Wolstenholme and Clary 1985). Such time-dependent decreases in the relative frequencies of transitional substitutions are well known in mammalian mtDNA, but the percent of transitions in hominoids, which had diverged ~8–10 Myr ago, is ~70% (Brown et al. 1982). However, the percent of transitions between *D. melanogaster* and the other two species was ~55%, even though the divergence time of these species is estimated as being <5 Myr (Cohn et al. 1984; Stephens and Nei 1985).

The distribution of transitions and transversions is clearly different between synonymous and nonsynonymous substitutions (table 3). Among the three species a predominance of transitions is evident in synonymous substitutions. On the other hand, in the comparison of synonymous substitutions between *D. melanogaster* and *D. yakuba*, the frequency of transversions was equal to that of transitions. The observed number of transitions is nearly the same for all species comparisons irrespective of the divergence time involved. That is, the number of transitions observed between *D. simulans* and *D. mauritiana*, the most closely related species, is 14, whereas the number is 16 between *D. melanogaster* and *D. simulans* and 16 between *D. melanogaster* and *D. yakuba*, which are more distantly related. However, transversions seem to accumulate steadily with increasing divergence time. Therefore, it is likely that multiple hits of transitional substitutions occurred during the time of divergence between *D. melanogaster* and the other three species.

If the difference in nucleotide at fourfold-degenerate sites is not important for anticodon-codon recognition, then the relative frequency of nucleotide substitutions at these sites would reflect the frequency of the corresponding mutations in comparisons of closely related species (Li et al. 1985). The values in parentheses in table 3 stand
for the number of substitutions for fourfold-degenerate sites. They show that transitions occurred more frequently than transversions between *D. mauritiana* and *D. simulans* and between *D. melanogaster* and *D. simulans* but not for the other comparisons.

By contrast, nonsynonymous substitutions show a predominance of transversions over transitions. Nonsynonymous substitutions in mammalian mtDNA, however, have been reported to show a predominance of transitions (Brown et al. 1982). In the present study, 75% of nonsynonymous substitutions between *D. melanogaster* and *D. simulans* are transversions, 80% of which are substitutions between A and T. A bias toward A-T substitutions in nonsynonymous substitutions has elsewhere (Wolstenholme and Clary 1985) been observed in the comparison between *D. melanogaster* and *D. yakuba*. As described above, the point mutations have a tendency to be transitions rather than transversions. Thus, for a proper interpretation of the predominance of A-T transversions in nonsynonymous substitutions, we need to consider whether some factor(s) restricts the nonsynonymous substitutions in the ND2 gene to A-T transversions. Six of the nine amino acid replacements found in the ND2 gene were changes to chemically similar amino acids. However, as mentioned above, nonsynonymous substitutions in mammalian mtDNA show a predominance of transitions (Brown et al. 1982). Therefore, the bias toward A-T substitutions in *Drosophila* mtDNA cannot simply be due to a requirement for amino acid similarity; there may be something unique to *Drosophila* mtDNA that produces this substitution pattern.

**Rate of Nucleotide Substitutions**

Table 4 shows the nucleotide divergences estimated by means of Miyata and Yasunaga's method (1980) for the *Drosophila* mtDNA and *Adh* genes (Cohn et al. 1984, fig. 2). The values at nonsynonymous sites were calculated for each of the two genes of mtDNA and compared with those of the *Adh* gene. The overall divergence rate of the two genes appears to be approximately equal to that of *Adh* as given in table 4. On the other hand, the divergences at synonymous sites were three to five times as large as those of the *Adh* gene. In mammals, however, the divergences of mtDNAs have been reported to be ≥10 times as large as those of single-copy nuclear DNAs (Brown et al. 1982).

Figure 5 shows the relationships of the evolutionary rates between the mtDNA genes and the *Adh* gene for the three species of *Drosophila*. For this purpose, the

<table>
<thead>
<tr>
<th>Species Compared</th>
<th>mtDNA</th>
<th><em>Adh</em></th>
<th>mtDNA</th>
<th><em>Adh</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>mau-sim</td>
<td>0.111 ± 0.029</td>
<td>0.021 ± 0.012</td>
<td>0.007 ± 0.003</td>
<td>0.007 ± 0.004</td>
</tr>
<tr>
<td>mel-sim</td>
<td>0.128 ± 0.031</td>
<td>0.035 ± 0.016</td>
<td>0.021 ± 0.006</td>
<td>0.010 ± 0.005</td>
</tr>
<tr>
<td>mel-mau</td>
<td>0.097 ± 0.027</td>
<td>0.029 ± 0.014</td>
<td>0.020 ± 0.006</td>
<td>0.010 ± 0.005</td>
</tr>
</tbody>
</table>

NOTE.—Abbreviations are as given in table 1.

* Data are from Cohn et al. (1984).
FIG. 5.—Relationships between the nucleotide sequence divergences in mtDNA and Adh from three Drosophila species. The points represent estimates of nucleotide divergence (as determined by using the Jukes and Cantor method [1969]) from pairwise comparisons of the three species. The dashed line corresponds to the situation if both nucleotide sequences (mtDNA and Adh) have evolved at an equal rate. The regression coefficients of sequence divergence of mtDNA on that of Adh are 2.3 for the protein-coding sequence (circles) and 1.4 for the entire sequence determined (black dots).

Divergences were calculated by means of the method of Jukes and Cantor, making no distinction between synonymous and nonsynonymous substitutions. In the protein-coding region (except for introns and intergenic sequences), the evolutionary rate of mtDNA turns out to be only 2.3 times larger than that of the Adh gene. When the entire sequence of mtDNA examined is considered, the evolutionary rate is very similar for the mtDNA and Adh genes.

Discussion
DNA Divergences of the Two Genes in mtDNA

We have seen that the frequency of nonsynonymous substitutions is much higher in the ND2 gene than in the COI gene (table 2). However, the total number of nucleotide substitutions is nearly the same for the two genes (table 1). Comparison of the six mitochondrially coded protein genes (ND2, COI, COII, COIII, ATPase6, and ATPase8) between Drosophila melanogaster and D. yakuba have also shown that nearly the same extent of nucleotide divergences exists for these genes but that the amino acid sequence divergence varies substantially (1.0% for COI vs. 6.3% for ND2) (Wolstenholme and Clary 1985). Among the closely related hominoids—human, gorilla, and chimpanzee—a similar tendency has been found in the nucleotide divergences of ND4 and ND5 (Brown et al. 1982). In the present study, however, only ~20% and ~50%, respectively, of the COI and ND2 nucleotide sequences were examined, so that the reliability of our results may not be so high. Furthermore, the Drosophila species used in the present study may not have diverged enough to make a reliable comparison of the number of nonsynonymous substitutions. If the number of nonsynonymous substitutions is small, a total DNA divergence is expected to consist largely of synonymous substitutions. However, this is not the case. As shown in table 2, nonsynonymous
substitutions in the ND2 gene seem to occur nearly as frequently as synonymous substitutions. These observations indicate that nucleotide substitutions in the COI gene, in contrast to those in the ND2 gene, have proceeded under very strong functional constraints (Kimura 1983) during the evolution of melanogaster-subgroup species. To discover the details of this problem, however, we need to accumulate more data. We are presently completing a sequence study of the remaining regions of COI and ND2 as well as those of some other genes.

Transitions and Transversions

Statistical analysis of synonymous substitutions revealed a predominance of transitions over transversions (table 3). The predominance of transitions is well known in mammalian mtDNAs (Brown et al. 1982; Brown and Simpson 1982). In the case of Drosophila mtDNA, however, the situation is somewhat different. In the case of the comparison of synonymous substitutions between D. melanogaster and D. yakuba, species that diverged $\sim 13-17$ Myr ago (Beverley and Wilson 1982; Bodmer and Ashburner 1984), the frequency of transitions was approximately equal to that of transversions. This observation can be explained simply in terms of multiple transitional substitutions. However, comparison of mammalian species having diverged $\sim 10$ Myr ago shows that 73% of all synonymous substitutions are transitions (Brown et al. 1982). The frequencies of different kinds of point mutations are expected to be a function of the base composition of the nucleotide sequence (Aquadro and Greenberg 1983). Since the protein-coding region sequenced in the present study shows only a 20% G+C content, it is probably this high A+T content in the genome that causes more frequent substitutions between A and T (transversions) in Drosophila mtDNA. Indeed, most of the transversions observed at the fourfold-degenerate sites are substitutions between A and T. On the other hand, in mammalian mtDNAs, transitional substitutions have occurred more frequently than transversions. Again the mutational bias toward A-T substitutions seems to be responsible for the transversion bias in nonsynonymous substitutions. Thus, the relative ratios of transitional and transversional substitutions may be substantially different between Drosophila and mammalian mtDNA.

Rate of Nucleotide Substitution

Although estimates of the divergence times of Drosophila species are difficult to make, those available for D. melanogaster and D. simulans are 2.0–5 Myr (Cohn et al. 1984; Stephens and Nei 1985). If we use these estimates, the evolutionary rate of synonymous substitutions in Drosophila mtDNA becomes $1 \times 10^{-8}-3 \times 10^{-8}$/site/year. If multiple hits of transitional substitutions had occurred during the course of divergence, these values would be underestimates. Since the rate for mammalian mtDNAs has been estimated to be $3.6 \times 10^{-8}-9.4 \times 10^{-8}$/site/year (Brown et al. 1982), the evolutionary rate of Drosophila mtDNA appears to be somewhat slower than that of mammalian mtDNA.

If we consider the entire region of DNA and use Jukes and Cantor's method, the rate of nucleotide substitution in mtDNA is $\sim 1.4$ times that for the Adh gene. This result is in rough agreement with the results obtained by Powell et al. (1986) in the comparison of mtDNA and single-copy nuclear DNA (DNA hybridization) in
D. melanogaster and D. yakuba and also with those obtained by Vawter and Brown (1986) in a similar study that used data from the sea urchin.

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