Replacement of Bovine Mitochondrial DNA by a Sequence Variant Within One Generation

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ABSTRACT

Inheritance of mitochondrial DNA (mtDNA) in Holstein cattle was characterized by pedigree analysis of nucleotide sequence variation. mtDNA was purified from leukocytes of 174 individuals representing 35 independent maternal lineages, and analyzed for nucleotide sequence variation by characterization of restriction fragment length polymorphism and direct sequence determination. These data revealed 11 maternal lineages in which leukocytes from some individuals seemingly were homoplasmic for the reference mtDNA sequence at nucleotide 364, whereas those from other individuals were homoplasmic for a sequence variant at this position. Both alternative alleles were detected in all branches of these 11 lineages, suggesting that mutation at nucleotide 364 and fixation of the variant sequence occurred frequently in independent events. Thirteen instances were detected of mother-daughter pairs in which leukocytes of each of the two animals seemingly were homoplasmic for a different allele at nucleotide 364, demonstrating the bovine mitochondrial genome can be replaced completely by a nucleotide sequence variant within a single generation. The two alternative sequences seemingly arose de novo at similar frequency, ruling out replicative advantage or other selective bias as the explanation for rapid fixation of mutations at nucleotide 364. Another instance of intralineage sequence variation was detected at nucleotide 5602. This variation was detected in only one of the lineages examined, and evidently arose within three generations.

The mechanism by which mitochondrial DNA (mtDNA) is inherited in mammalian cells must account for the fact that nucleotide substitutions accumulate approximately five to ten times faster than similar mutations in nuclear DNA (BROWN et al. 1982; BROWN, GEORGE and WILSON 1979), even though hundreds or thousands of copies of the mitochondrial genome are present in each cell (BOGENHAGEN and CLAYTON 1974; MICHAELS, HAUSWIRTH and LAIPIS 1982). Several means have been suggested by which mtDNA could be subjected to greater rates of nucleotide substitution than is nuclear DNA [see BROWN (1981) for review]. Regardless of the specific mechanism, a mutation would first arise in a homoplasmic individual in which all mtDNA molecules originally were identical. Immediately after the mutagenic event, a single variant genome would be contained in a mitochondrion along with a majority of mtDNA molecules of the parental nucleotide sequence. Such a mixture of mtDNA sequence variants within a single mitochondrion, a single cell, or a single animal is termed heteroplasmic. In order for the mutation to be fixed in the animal population, a sequence variant must segregate from the majority of parental mtDNA molecules to become the sole mitochondrial genome in the germ cells of some successive generation.

Segregation of variant mitochondrial genomes in successive generations has been analyzed by pedigree analysis of mtDNA sequence variation in one lineage of Holstein cattle (lineage H15) in which heteroplasmic animals were identified (ASHLEY, LAIPIS and HAUSWIRTH 1989; LAIPIS, VAN DE WALLE and HAUSWIRTH 1988). Each of these animals contained two different mtDNA sequences in liver mitochondria, which were distinguished by the presence or absence of a HpaII recognition site at nucleotide (nt) 364 (nt numbering according to ANDERSON et al. 1982). The heteroplasmic individuals gave rise within two generations to homoplasmic progeny containing only one of the two sequence variants in liver mitochondria. Such rapid segregation of the nucleotide sequence variants cannot be explained stochastically by the random assortment of mtDNA molecules during somatic cell division. Instead, the data support a proposed mechanism of maternal inheritance of mammalian mtDNA in which the differential amplification of a few specific germ-line mtDNA molecules produces the mitochondrial genome in the succeeding generation (HAUSWIRTH and LAIPIS 1982; ASHLEY, LAIPIS and HAUSWIRTH 1989). This proposed hereditary bottleneck may allow for rapid fixation of a variant
mtDNA molecule present as a minority species in a heteroplasmic somatic cell.

This report describes the results of additional pedigree analyses of mtDNA sequence variation in 35 maternal lineages of Holstein cattle. All these lineages are independent of lineage H15 analyzed by LAIPIS, WILCOX and HAUSWIRTH (1982), at least since Holstein cattle registry began in 1885 (CHENERY 1885). Pedigree analysis using the HpaII site at nt 364 as a marker showed fixation of mtDNA sequence variants in cattle can occur in one generation, without a detectable heteroplasmic intermediate. Mothers seemingly homoplasmic for a particular mtDNA sequence in their leukocytes were found to produce daughters homoplasmic for leukocyte mtDNA of a different sequence. Heteroplasm was detected in neither mother nor daughter. This phenomenon occurred frequently, having been detected in 13 mother-daughter pairs from six independent maternal lineages. These results suggest the number of genetic determinants segregating during germ-line transmission of mtDNA can be even fewer than previously estimated (HAUSWIRTH and LAIPIS 1982), and that a single mtDNA molecule may in fact transmit genetic information to the succeeding generation.

**MATERIALS AND METHODS**

Holstein cattle maternal lineages: The Holstein cattle used in this study were from the I-State breeding research herd established at Iowa State University in 1968. The foundation females were purchased from 38 different Holstein breeders throughout Iowa, providing a wide spectrum of genetic diversity to the herd. The cattle were registered with the Holstein Association, and thus pedigree records of founder animals according to the Holstein registry, were used in this study. These pedigrees were mapped for sequencing as described (VIEIRA and MESSING 1982). Approximately 25 to 50 ng of isolated mtDNA in a total volume of 10 μl was digested with 10 units of the appropriate restriction enzyme in the reaction buffer specified by the manufacturer. After 1.5 hr at 37°, 1 unit of polIK (1 unit/μl), 10 μCi of [α-35S]dCTP (10 μCi/μl; <1000 Ci/mmol), and unlabeled deoxyribonucleotides (0.1 mm) were added to the reaction mixtures, which then were incubated for an additional 0.5 hr at 37°. The reactions were terminated by addition of 0.10 volume of loading dye mixture and analyzed by autoradiography after gels were dried under vacuum.

Nucleotide sequence analysis was also used to detect variation in mtDNA. The 4.3 kilobase (kb) PstI-SacI fragment of mtDNA containing the displacement loop (D-loop) region, 12S and 16S rRNA genes, and several tRNA genes (ANDERSON et al. 1982) was purified from preparative agarose gels and cloned in the phagemid vectors pUC118 and pUC119 (VIEIRA and MESSING 1987). ssDNA was produced from the recombinant plasmids, and nucleotide sequence determination was determined using synthetic oligonucleotide primers. The sequences and locations of each primer in the bovine mtDNA molecule are shown in Table 1. These primers allow determination of the nucleotide sequence of the 910-base pair (bp) D-loop region, by reading both the H and L strands. This region is located between nt 15,792 and nt 364 (nt numbers according to ANDERSON et al. 1982). The nucleotide sequence surrounding nt 5602 was determined by subcloning the 9.0-kb StuI-KpnI fragment comprising nt 3,684–5,732 into pUC118. Universal sequencing primer (−40) was used to determine the nucleotide sequence from the KpnI site at nt 5,732 to 5,500.

**RESULTS**

Figure 1 shows that DNA isolated from the postnuclear extracts of leukocytes is essentially pure mtDNA. Undigested post-nuclear DNA runs as a single species in agarose gels (Figure 1, lane 1). Digestion of this DNA molecule with PstI and SacI yields three fragments of 4.3, 5.1 and 7.0 kb (Figure 1, lane 2), in agreement with the known nucleotide sequence (ref-
Rapid Segregation of Bovine mtDNA

TABLE 1

<table>
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<th>L-strand sequencing primers</th>
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FIGURE 1.—Characterization of mtDNA purified from leukocytes. Mitochondrial DNA isolated as described in MATERIALS AND METHODS was analyzed by electrophoresis in a 0.8% agarose gel. Lane designations: 1, undigested post-nuclear DNA; 2, post-nuclear DNA digested with PstI and Sael; 3, molecular weight standards (bacteriophage lambda DNA digested with HindIII and EcoRI).

The first instance of intralineage sequence variation is a HinfI recognition site near nt 5602 that is not present in the reference sequence, but is present in several animals of lineage 60. Figure 2A shows examples of the reference allele L-5602 and of the variant allele S-5602, as they are defined by RFLP analysis. The reference sequence predicts a particular HinfI fragment of 481 bp to be present in the majority of animals analyzed (Figure 2A, lanes L-5602). However, in certain animals, this fragment is missing, and a new fragment estimated to contain 453 bp is present.

The size of each fragment in base pairs was estimated by comparing its mobility to that of known molecular weight standards (not shown) and was assigned a precise value from the known reference sequence of bovine mtDNA (Anderson et al. 1982). Samples yielding the digestion pattern predicted by the reference sequence are defined as containing allele L-5602. The sample shown in the lane marked S-5602 contains a variant mtDNA sequence characterized by loss of the 481-bp HinfI fragment and appearance of a new fragment of approximately 453 bp.

FIGURE 2.—Polymorphism in mtDNA sequence at nt 5602. (A) Mitochondrial DNA was digested with HinfI and end-labeled as described in MATERIALS AND METHODS. The digestion products were separated by electrophoresis in 4% polyacrylamide gels and visualized by autoradiography. The size of each fragment in base pairs was estimated by comparing its mobility to that of known molecular weight standards (not shown) and was assigned a precise value from the known reference sequence of bovine mtDNA (Anderson et al. 1982). Samples yielding the digestion pattern predicted by the reference sequence are defined as containing allele L-5602. The sample shown in the lane marked S-5602 contains a variant mtDNA sequence characterized by loss of the 481-bp HinfI fragment and appearance of a new fragment of approximately 453 bp. (B) H-strand nucleotide sequence surrounding nt 5602. The C residue at nt 5602 (marked by the asterisk) is a T residue in the reference sequence characterized by loss of the 481-bp HinfI fragment and appearance of a new fragment of approximately 453 bp. The T to C mutation forms the HinfI recognition site 5'-GAATC-3' (underlined).
(Figure 2A, lane S-5602). Thus, allele S-5602 is likely to be caused by a mutation within the 481-bp HinfI fragment of the reference sequence, which creates a new HinfI recognition site. Nucleotide sequence analysis confirmed this prediction (Figure 2B). S-5602 mtDNA contains the HinfI recognition site 5′-GAATC-3′ at nt 5602, where the reference sequence of the L-5602 genome, 5′-GAATT-3′, does not contain this site. The mutation at nt 5602 is located within the coding region of the mitochondrial tRNA^{Glu} gene, corresponding to a T to A transversion in the D-loop of the tRNA molecule.

Figure 3 shows that lineage 60 is polymorphic for the L-5602/S-5602 allele pair. Allele S-5602 is present exclusively in the branch of the lineage descended from animal 758, whereas allele L-5602 is present exclusively in the opposite branch of the lineage, descended from animal 401. Misidentification of maternal relatedness in this group of animals is ruled out by nucleotide sequence analysis of the D-loop region in several individuals from both lineal branches (Figure 3). All seven animals examined exhibited at nt 169, nt 16,085, and nt 16,121 a specific combination of nucleotide substitutions not seen in any of the other 34 maternal lineages analyzed (data not shown). Thus, despite nucleotide sequence variation between alleles L-5602 and S-5602, all the animals examined are related maternally.

The second instance of intralineage sequence variation is a HpaII recognition site at nt 364 (HpaII-364) that is not present in the reference sequence, but appears frequently in many of the maternal lineages analyzed in this study. When the HpaII site is present at nt 364 (allele S-364), the 2761-bp fragment of reference allele L-364 is cleaved into two smaller fragments, of 2510 bp and approximately 251 bp. The precise size of the smaller fragment depends on a run of G residues starting at nt 353 and ranging in length from 9 to 19 nucleotides (HAUSWIRTH et al. 1984). Figure 4 shows an example of this polymorphism. Allele L-364 contains only the 2761-bp fragment (Figure 4, lane 1), whereas allele S-364 exhibits only the 251-bp fragment (Figure 4, lane 2). The 2510-bp fragment of allele S-364 is not detected because it is obscured by other HpaII restriction fragments of similar size.

A subset of S-364 alleles also was examined by RFLP analysis using MspI, a HpaII isoschizomer insensitive to cytosine methylation. In all instances the RFLP patterns produced by both enzymes were identical (data not shown), indicating that cytosine methylation is not responsible for the evident sequence variation. Direct nucleotide sequence analysis of the mtDNA region containing the polymorphic site showed that allele S-364 contains two C residues 5′ to a run of G residues in the H strand, whereas allele L-364 contains a single C residue 5′ to the run of G residues (Figure 5).

Thus, the S-364 allele analyzed in this study is precisely the same mutation identified in the polymorphic lineage H15 (LAIPIS, VAN DE WALLE and HAUSWIRTH 1988). Owing to variability in the number of consecutive G residues beginning at nt 353 (HAUSWIRTH et al. 1984), the mutation causing the HpaII-364 polymorphism could be defined either as a G to C transversion on the H-strand, or as an insertion of a single C residue on the H-strand (Figure 5).

Polymorphism at HpaII-364 was by far the most common sequence variation observed in this study, with the reference allele L-364 found in leukocytes of 97 animals and the variant allele S-364 found in leukocytes of 77 animals. As far as could be determined from the limited number of animals examined, ten of the maternal lineages contained only allele S-364, 14 lineages contained only allele L-364, and the remaining 11 lineages were polymorphic, containing some animals with allele S-364 and others with allele L-364 (Table 2).

Figure 6 shows the distribution of animals with the S-364 and L-364 alleles in lineage 17. Both alleles are found in relatively distant branches of the lineage, for example, in the descendants of animal 1671 and animal 2009. Of the 13 animals examined in lineage 17, six contained allele L-364 and the remaining seven contained allele S-364. In certain other polymorphic lineages, one of the two alleles at HpaII-364 seemed to predominate. For example, in lineage 66, allele L-364 was present in 11 of the 13 animals analyzed (Figure 7).

Nucleotide sequence analysis again was used to pre-
Mitochondrial DNA was digested with HpaII. The digestion products were separated by electrophoresis in 8% polyacrylamide gels and visualized by autoradiography. The size of each fragment in base pairs was estimated by comparing its mobility to that of known molecular weight standards (not shown) and, where possible, assigned precise values from the known reference sequence of bovine mtDNA (Anderson et al. 1982). Lane 1 contains leukocyte mtDNA from a cow containing allele L-364. This digestion pattern matches that predicted from the reference sequence, with the exception of two predicted fragments of 1283 bp and 291 bp, neither of which was detected in the mtDNA molecules analyzed in this study. Instead, a fragment of approximately 1574 bp (marked by the square [■]) is present in all samples. Because the 1283-bp and 291-bp fragments are adjacent to each other on the mtDNA molecule, the most likely explanation for this discrepancy is that a HpaII site in the reference sequence is absent from all the animals analyzed in this study and that the two missing fragments are fused to form the 1574-bp fragment. Lane 2 contains mtDNA from a cow containing allele S-364. In this instance the 2761-bp fragment present in lane 1 is replaced by a 2510-bp fragment (not visible) and a fragment of approximately 251 bp (marked by the circle [●]) . The remaining lanes contain mtDNA isolated from different tissues of animal 2726 from lineage 75 (see Figure 8). Lane designations: 3, liver; 4, ovary; 5, kidney; 6, brain; 7, heart.

include misidentification of ancestry. The mtDNA sequences of two animals in lineage 17, one carrying allele S-364 and the second carrying allele L-364 (Figure 6), revealed variation from the reference sequence at nt 173 and nt 445, a combination of mutations found only in that lineage (data not shown). Thus, despite their variation at nt 364, these two animals are maternally related. Likewise, lineage 75 (Figure 8) exhibited variation at nt 16,231 and nt 216 that was consistent in both L-364 and S-364 animals and was unique among the 35 lineages examined in this study (data not shown).

Examing the inheritance pattern of alleles L-364 and S-364 in the polymorphic lineages revealed numerous examples of mother-daughter pairs differing with respect to the nucleotide sequence at the HpaII site. For example, leukocyte mitochondria from animal 1966 in lineage 66 contained allele S-364; those from one of her daughters contained the same allele, whereas a second daughter contained allele L-364 in leukocyte mitochondria (Figure 7). The same phenomenon can be seen in lineage 17 for animals 2640 and 2286, two S-364 animals that each produced an L-364 daughter (Figure 6). Furthermore, in lineage 75, the alleles at position 364 seem to switch between

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* Polymorphic lineage.
FIGURE 6.—Mitochondrial DNA sequence polymorphism in maternal lineage 17. Mitochondrial DNA from 13 animals was analyzed by digestion with HpaII, as shown in Figure 4. Animals 2491, 2835, 2892, 3108, 3274 and 3280 contain allele L-364. Animals 2286, 2536, 2640, 2709, 2781, 3110 and 3203 contain allele S-364. Mitochondrial DNA from individuals marked with an asterisk (*) was characterized by nucleotide sequence analysis of the D-loop region, to confirm maternal relatedness. Nucleotide sequence polymorphisms detected in both animals at nt 173 and nt 445 were unique to lineage 17 (data not shown).

FIGURE 7.—mtDNA sequence polymorphism in maternal lineage 66. mtDNA from 13 animals was characterized by digestion with HpaII, as in Figure 6. Two animals, 1986 and 3221, contained allele S-364, whereas the remaining 11 animals analyzed contained allele L-364. Individuals marked with an asterisk (*) were characterized by nucleotide sequence analysis of the D-loop region to confirm that the animals were related maternally. All animals analyzed by nucleotide sequencing displayed divergences from the reference sequence (Anderson et al. 1982) at nt 216 and nt 16,231 (data not shown), a combination unique to lineage 75.

TABLE 3
mtDNA replacement in mother-daughter pairs

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<th>Genotype of mother</th>
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<tr>
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<td>S-364</td>
<td>L-364</td>
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mother-daughter pairs in three successive generations (Figure 8; animals 1743, 2473 and 3060). Different mtDNA sequences at nt 364 between mothers and daughters were observed in 7 of the 11 known polymorphic lineages (data not shown). Of 32 mother-daughter pairs analyzed, 15 showed different mtDNA sequences at nt 364 (Table 3). Selective bias was not detected for either of the two alleles, because five of the observed genotype switches were from L-364 to S-364, and the remaining eight were in the opposite direction (Table 3).

The observed shift in leukocyte mtDNA sequence within a single generation raised the possibility that many of the mothers were heteroplasmic, i.e., they contained a mixture of S-364 and L-364 alleles. However, the detection methods used in this study indicated that all the animals examined contained exclusively one mitochondrial genotype. To measure the effectiveness of the assay, a control experiment was performed in which two seemingly pure mtDNA samples were mixed in varying proportions and analyzed for the presence of sequence polymorphism at restriction endonuclease recognition sites (Figure 9). The results showed that a heteroplasmic mixture containing 10% or more of a minority variant could be detected clearly (Figure 9, lane 3), and a heteroplasmic population containing 5% variant molecules most likely would have been identified (Figure 9, lane 2). Therefore, in these analyses, mothers in which 95% or more of the mtDNA molecules in leukocytes are of a particular nucleotide sequence frequently produced daughters in which 95% or more of the leukocyte mtDNA molecules are of a different sequence.
allows extremely rapid fixation of nucleotide sequence \( S-364 \), analysis of leukocyte mtDNA.

\[ \text{HaeIII} \]

\( I-364(0) \) is derived from allele \( \text{Hpall} \), as in Figure 4. The 251-bp fragment marked with a circle in mitochondria of several other tissues. Figure 4 shows the \( \text{HpaII} \)-364 allele(s) present for allele \( S-364 \). Lane designations: \( L-364 \), \( PIUS 60\% \), \( S-364; 3, 90\% \) \( L-364 \) plus \( 10\% S-364 \); \( 4, 80\% L-364 \) plus \( 20\% S-364 \); \( 5, 60\% L-364 \) plus \( 40\% S-364 \); \( 6, 40\% L-364 \) plus \( 60\% S-364 \); \( 7, 20\% L-364 \) plus \( 80\% S-364 \); \( 8, 100\% S-364 \).

To address the possibility that mtDNA of leukocytes is not representative of other somatic cells, one \( L-364 \) mother that produced both \( S-364 \) and \( L-364 \) daughters was examined for the \( \text{HpaII} \)-364 allele(s) present in mitochondria of several other tissues. Figure 4 shows the \( \text{HpaII} \) digestion pattern of mtDNA from liver, kidney, brain, heart, and ovary of animal 2726 from lineage 75 (see Figure 8). Mitochondrial DNA from all tissues appeared to be homoplasmic for allele \( S-364 \), in agreement with the genotype assigned by analysis of leukocyte mtDNA.

**DISCUSSION**

The mechanism of mtDNA inheritance in mammals allows extremely rapid fixation of nucleotide sequence variants, with mutant genomes arising in two or three generations. This observation was first made of Holstein cattle maternal lineage H15 (Hauswirth and Laipis 1982), in which two different mtDNA sequences differing by the presence or absence of a \( \text{HaeIII} \) recognition site at nt 12,792 were found in closely related animals. In contrast to the simple example in which each sequence variant was found in a distinct branch of the lineage, pedigree analysis detected both nt 12,792 sequences in diverse branches. Thus, new mtDNA genomes must have been fixed in multiple, independent events. Further analysis of this lineage identified heteroplasmic animals containing a mixture of two distinct mtDNA sequence variants at nt 364 (Laipis, Van de Walle and Hauswirth 1988). One of these animals produced in the next generation progeny seemingly homoplasmic for a specific mtDNA sequence (Ashley, Laipis and Hauswirth 1989), thereby providing a direct observation of the rapid fixation of mtDNA sequence variants.

The current report presents another observation of this rapid fixation, with a variant tRNA\(^{\text{Glu}} \) gene (allele \( S-5602 \)) arising within a maximum of three generations. Furthermore, fixation of mutations at nt 364 was observed in bovine leukocyte mtDNA within a single generation, in the absence of any heteroplasmic intermediate animals. Animals evidently pure for a particular leukocyte mtDNA sequence produced offspring seemingly homoplasmic for a different leukocyte mtDNA sequence. Mitochondrial genome replacement in leukocytes was observed in 40% of the 32 mother-daughter pairs analyzed.

The failure to observe heteroplasmic animals as intermediates in the fixation process is in contrast to a previous study of sequence variation at nt 364 which found heteroplasy in one animal and its offspring (Laipis, Van de Walle and Hauswirth 1988; Ashley, Laipis and Hauswirth 1989). A possible explanation for this discrepancy is the source of mitochondrial DNA, leukocytes in the current study and brain or liver in the previous work. Both liver and brain tissue consist of diverse cell types, and thus may be derived from association of many embryonic cells. In contrast, the hematopoietic cell lineage most likely is derived from a smaller number of embryonic cells. Therefore, heteroplasmacy may be maintained in certain organ tissues but not in the developmentally homogeneous population of leukocytes.

This explanation is unlikely in light of the analysis of animal 2726 from lineage 75 (Figure 8), which was expected to be heteroplasmic because it produced both \( L-364 \) and \( S-364 \) daughters. However, mtDNAs from six different tissues of animal 2726, including liver, brain and leukocytes, all were homoplasmic for allele \( S-364 \). In this instance, therefore, a mtDNA sequence variant both arose and was fixed within one generation, at least in leukocytes. The data do not rule out, however, the possibility that mtDNA from certain tissues could be heteroplasmic even though leukocyte mtDNA is homoplasmic.

Persistent heteroplasmacy also has been observed in human pedigrees where mtDNA deletions or point mutations are associated with maternally inherited disease states such as myoclonic epilepsy and ragged-red fiber disease (MERRF), Leber's hereditary optic myopathy, and other mitochondrial myopathies (Holt, Harding and Morgan-Hughes 1988; Holt et al. 1990; Lott, Voljavec and Wallace 1990; Shoffner et al. 1990). These instances differ from that of nt 364 heteroplasy in bovine mtDNA in that
selection exists for one of the two sequence variants. In most of these cases the severity of the disease correlates with the degree of heteroplasmy. Thus, one sequence variant is likely to be nonfunctional or partially functional, providing selection for the fully functional alternative sequence. In contrast, nt 364 is located in a noncoding region of bovine mtDNA, and a functional difference between S-364 and L-364 is unlikely given their allele frequencies of 44 and 56%, respectively. Regarding the question of whether or not leukocyte mtDNA is representative of other tissues, it is worth noting that heteroplasmy was detected in human blood cells (HOLT et al. 1990; LOTT, VOLJAVEC and WALLACE 1990).

Replacement of leukocyte mtDNA by a sequence variant in one generation implies the existence of heteroplasmic cells in the cell lineage between the germ line of the mother and leukocytes of the progeny, and that the heteroplasmic state is transient relative to the total number of cells in this cell lineage. If animal 2726 is representative of all animals, in that each tissue in the body contains the same homoplasmic mtDNA sequence, then the heteroplasmic state in the cell lineage is likely to be restricted to the germ line.

Leukocyte mtDNA replacement is a common event, requiring that the transient heteroplasmic state arises frequently. Sequence divergence occurred at nt 364 significantly more frequently than at any other site characterized in this study; the next most frequent polymorphism was at nt 15,738, where variation from the reference sequence was detected in 5% of the animals analyzed (G. L. LINDBERG and C. M. KOEHLER, unpublished results). In the reference sequence nt 364 is a C residue located immediately adjacent, on the 5′ side, to a consecutive sequence of G residues in the H-strand. This run of G residues is known to differ in length within tissues of individual animals (HAUSWIRTH et al. 1984). Thus, the polymorphic site is located in a plastic region of the mitochondrial genome, and may be subjected to abnormally high rates of mutation. From the D-loop sequences of approximately 100 animals, however, the nature of mutation at nt 364 seems to be constant. The only variation observed is insertion or deletion of an additional C residue 5′ to the run of G residues (data not shown). Either the mutation mechanism is such that insertion of a C residue is the only possible alteration, or insertion of another residue prevents further replication of the mutant molecule.

Relatively infrequent mutations at nt 364, and rapid formation of homoplasmic cells during development from germ line to mature leukocytes, are not sufficient to explain the observed frequency of mitochondrial genome replacement. Considering that animal cells contain several hundred copies of the mitochondrial genome, mutation at a rate as high as 1% would create a variant present as a small minority of the total mtDNA molecules. In this instance homoplasmic cells formed during embryogenesis would be expected to contain predominantly the original mtDNA sequence. The 40% frequency of genome replacement, however, suggests that the newly arisen variant quickly comes to comprise a significant proportion of the mtDNA molecules. Selection of a mutant allele by differential replication efficiency is ruled out by the observation that both S-364 and L-364 frequently appear de novo in daughters. The mechanism by which mutant mtDNA molecules could significantly increase in frequency in the few generations of heteroplasmic cells is not clear. Possible explanations for this phenomenon are (1) an extremely high mutation frequency at nt 364 approaching 40%, or (2) a gene conversion mechanism by which the new sequence is spread to other mtDNA molecules.

The prevalence of mutations at nt 364 during germ line transmission of mtDNA and leukocyte development suggests that heteroplasmy at this site also would arise during development of other somatic cells. The fact that heteroplasmy has been reported only in a few animals could be explained by the fact that whatever mechanism accounts for spreading of the mutant mtDNA sequence operates only in germ line cells. MICHAELS, HAUSWIRTH and LAIPIS (1982) suggest, for example, that amplification of mtDNA occurs during embryo development and the number of mitochondrial genomes increases approximately tenfold. Possibly the mechanism of mtDNA replication during amplification is different from that during somatic cell division, and the amplification mechanism causes a high mutation frequency at nt 364 whereas the somatic mechanism does not.

The following hypothesis is proposed to explain leukocyte mtDNA replacement in one generation, based on the concept of a hereditary bottleneck (HAUSWIRTH and LAIPIS 1982; ASHLEY, LAIPIS and HAUSWIRTH 1989). Amplification of mtDNA in germ line development proceeds such that a specific mutation at nt 364 occurs frequently. This mutation frequency may be extremely high, or for some other reason the mutant sequence spreads rapidly through the mtDNA population. As germ-line development or embryo development in the the leukocyte cell lineage proceeds, the number of mitochondrial genomes per cell decreases. At some point in the developmental pathway, prior to development of mature leukocytes, a progenitor cell becomes homoplasmic. If the homoplasmic observed for leukocytes extends to the entire animal, then the homoplasmic progenitor cell is likely to have arisen in the 16-cell stage embryo in which three cells are thought to be the clonal progenitors of all descendent somatic cells in the animal (MARKERT and PETTERS 1978; MINTZ 1970). These hypotheses can be tested using polymerase chain reaction methodology to characterize the mtDNA genomes of in-
individual oocytes and early embryos developing in vitro.

Genome replacement was observed directly only for sequence variants at nt 364. However, maternal lineages in which separate branches contain distinct mtDNA sequence variants (see Figure 3; HAUSWIRTH and LAIPIS 1982) could also have arisen in a single generation without heteroplasmic animals as intermediates. In these instances the frequency of mutation, or the rate at which the mutation spreads through the germ-line mtDNA population, might be less than for the nt 364 mutations. The high rate at which nt 364 mutations seemingly spread through the mtDNA population may be a consequence of their location in a noncoding, variable segment of the D-loop region, compared to other mutations such as S-5602 located in the tRNA\(^{\text{Gln}}\) gene. Decreasing the proportion of mutant mtDNA molecules in the germ-line cells would cause genomic replacement to occur less frequently, resulting in seemingly stable sequence divergence between branches of the lineage. The possibility that variant mitochondrial genomes can be fixed in one generation should be considered in analyses of evolutionary relationships based on divergence in mtDNA sequence.

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