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Genetics

Mitochondrial DNA polymorphism in a maternal lineage of Holstein cows

(nucleotide sequence/cytoplasmic inheritance/silent mutation)

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ABSTRACT **Two mitochondrial genotypes are shown to exist within one Holstein cow maternal lineage. They were detected by the appearance of an extra *Hae* III recognition site in one genotype. The nucleotide sequence of this region has been determined and the genotypes are distinguished by an adenine/guanine base transition which creates the new *Hae* III site. This point mutation occurs within an open reading frame at the third position of a glycine codon and therefore does not alter the amino acid sequence. The present pattern of genotypes within the lineage demands that multiple shifts between genotypes must have occurred within the past 20 years with the most rapid shift taking place in no more than 4 years and indicates that mitochondrial DNA polymorphism can occur between maternally related mammals. The process that gave rise to different genotypes in one lineage is clearly of fundamental importance in understanding intraspecific mitochondrial polymorphism and evolution in mammals. Several potential mechanisms for rapid mitochondrial DNA variation are discussed in light of these results.**

All animal cells contain nuclear and mitochondrial genetic information which is transmitted to progeny. The division of nuclear genes during meiosis is a regular and equal process, reflected in the precise Mendelian segregation of the chromosomes. However, except for strict maternal inheritance in most organisms, the segregation of the extrachromosomal genetic elements (mtDNA in animal cells) is poorly understood.

Rapid intraspecific variation in mitochondrial genotypes has been noted for a number of mammalian species (1–20). In the majority of these studies, variation was detected by restriction site polymorphism and was explicable by simple gains or losses in sites. Nucleotide sequence data for several *Eco*RI polymorphisms in rat mtDNA confirmed this view (12, 21). In two cases, length polymorphisms suggesting insertional events were detected, and in both cases they mapped in or near the D-loop region (6, 9). The availability of such intraspecific mitochondrial genotypes has allowed the experimental demonstration of maternal inheritance in rats (8, 10, 22), *Peromyscus* species (15), and humans (23, 24) as well as in horse × donkey hybrids (25). These studies confirm the earlier conclusion of Dawid and Blackler (26) for *Xenopus*.

Intraspecific mtDNA variation is commonly assumed to arise between maternally isolated breeding populations. However, we have reported restriction site polymorphism within a single maternal Holstein lineage (18, 19). Such rapid changes cannot be explained in this manner and pose a problem for mitochondrial inheritance: Namely, it is not obvious how the mitochondrial genotype can vary so rapidly in the face of the high ploidy of mitochondrial genomes known to be present in both somatic (27) and germ-line cells (28). That is, if the parental molecules replicate more or less uniformly and are distributed evenly to daughter cells, the possibility of individual variants arising and becoming the majority population seems vanishingly small over short time spans.

To begin elucidation of the mechanism of mtDNA variation at a molecular level we have attempted to establish the shortest time span over which sequence changes can be detected by extending our study of mtDNA between maternally related Holstein cows and determining the exact nature of such nucleotide changes.

MATERIALS AND METHODS

Holstein Cows. All animals in this study were Holstein cows or bull calves registered with the Holstein-Friesian Association of America. With the exception of the founding maternal ancestor, Holstein H15, all animals in the H15 maternal lineage have been born, bred, and maintained at the University of Florida Dairy Research Unit under closed genetic conditions. The potential significance of multiple genotypes occurring in the H15 lineage demands that the accuracy of this lineage be documented. Three sets of records exist on the birth and genetics of every animal in the herd and these records for all animals in the H15 lineage are consistent with the data in Fig. 1. Calf switching between dams before animal tagging theoretically could lead to an artificial mixing of genotypes within a lineage. However, two types of data refute this possibility. First, we have similarly analyzed another Holstein lineage of 51 animals whose mtDNA also exhibits an *Hae* III digestion pattern distinguishable from patterns of all other Dairy Research Unit Holstein lineages and find no evidence for mitochondrial genotype mixing as would be expected if calf switching were as common as the H15 lineage data would require. Second, in dairy herds maintained for breeding studies, calf switching is extremely rare (approximately 1 switch in 500 births) both because of close management and because dams are extremely reluctant to switch. Thus, consideration of record-keeping procedures, birth records, mitochondrial genotypes between maternal lineages, and Holstein cow maternal behavior all attest to the accuracy of the H15 lineage.

DNA Isolation and Cloning. mtDNA from the livers or brains of culled animals or from liver biopsy specimens was prepared from purified mitochondria as described (29). *Pst* I digestion of bovine mtDNA yields two fragments, and *Bam*HI digestion yields three fragments (29). These were cloned into the *Pst* I and *Bam*HI sites, respectively, of pBR322 by standard techniques (30, 31). DNA was isolated from 2L cultures by a mod-

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Abbreviations: bp, base pair(s); H strand, heavy strand; URF, unidentified reading frame.

ified NaDodSO$_4$ lysis procedure (32) followed by acridine yellow-agarose column chromatography (33).

Restriction Endonuclease Digestions. All restriction enzymes were purchased from Bethesda Research Laboratories and digestion conditions were as suggested by the supplier. Restriction fragments were visualized by 3'-end-labeling with [α-^{32}P]dGTP and *Escherichia coli* DNA polymerase I large fragment (Boehringer Mannheim) (3), followed by electrophoresis and autoradiography.

DNA Sequence Determination. The chemical technique of Maxam and Gilbert (34) was used. Fragments for complementary sequence determination were made by labeling the *Bgl* II generated ends at their 5' ends with [α-^{32}P]ATP and polynucleotide kinase or at their 3'-ends as above, recutting with *Cfo* I in either case, and extracting the 262-base-pair (bp) *Bgl* II/*Cfo* I (37.9/36.7) fragment after electrophoresis on 6% polyacrylamide gels.

RESULTS AND DISCUSSION

Mitochondrial Genotypes within a Maternal Lineage. In order to delineate the shortest time span over which intraspecific mtDNA polymorphisms can be observed, we isolated mtDNA from 16 registered Holstein cows of a single maternal lineage (Fig. 1). Five animals in the H15 lineage have two *Hae* III fragments—*Hae* III L$_S$ and *Hae* III K' (see Fig. 2)—and will be referred to as L$_S$ genotype animals. In the 11 other animals, referred to as L$_L$ genotype animals, these two fragments are replaced by *Hae* III L$_L$. All of the 36 other *Hae* III fragments are unaltered between or within the two groups. Because both types of *Hae* III L fragments are not visualized in any single animal, we estimate that at least 95% of the mtDNA molecules in the analyzed animals contain the indicated *Hae* III fragment L.

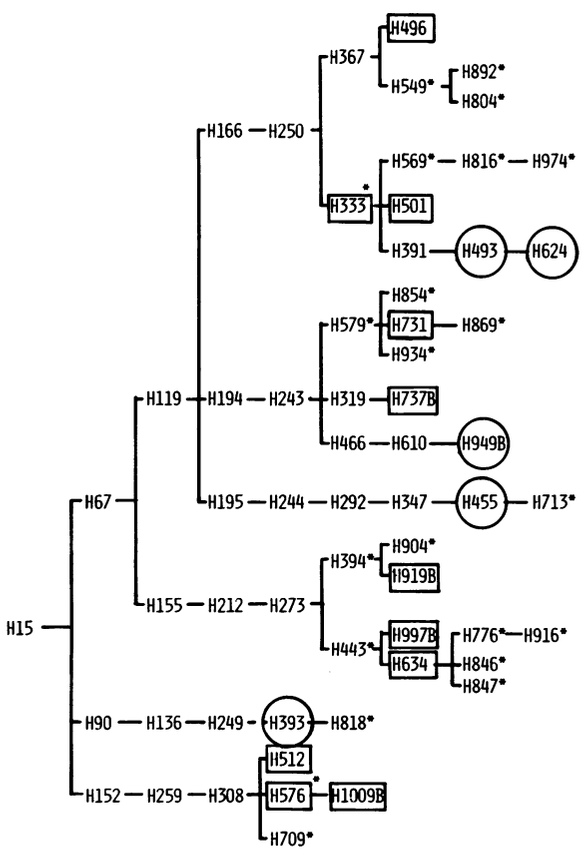

FIG. 1. Maternal lineage of Holstein cows defined by animal H15. Circled numbers, animals containing the *Hae* III L$_S$ fragment in their mtDNA; boxed numbers, animals containing the *Hae* III L$_L$ fragment; *, animals presently alive in the herd; numbers with a "B," bull calves.

The *Hae* III difference in the H15 lineage is apparently conservative in that the length of *Hae* III L$_L$ (543 bp) is equal to the sum of *Hae* III L$_S$ (510 bp) and *Hae* III K' (33 bp). This suggests that an extra *Hae* III site occurs in L$_S$ animals. If so, *Hae* III L$_S$ and *Hae* III K' should be physically adjacent and map to the same genome region as *Hae* III L$_L$. This is consistent with our previous studies (18, 19) which locate fragment *Hae* III L$_L$ between 36.9 and 40.5 map units and the two smaller fragments, *Hae* III L$_S$ and K' to between 37.1 and 40.5 map units, and to between 36.9 and 37.1 map units, respectively (Fig. 2). To test the extent of sequence variation between genotypes in this region we also mapped a number of other restriction sites both within (*Eco*RI, *Hin*fI, *Bgl* II) and immediately flanking (*Hin*dIII, *Hin*fI, *Sau*3A) this region (see Fig. 2) and noted that all sites and the distances between sites are unchanged between genotypes. Hence, no large-scale nucleotide sequence alterations have occurred between genotypes and we appear to have detected a limited nucleotide sequence variation leading to the creation of an *Hae* III site at 37.1 map units.

The pattern of appearance within the H15 lineage is of considerable interest. The simplest expectation is that one female progeny in the lineage was born with an altered genotype and thereafter all her female progeny bred true. If so, one branch of the H15 lineage should contain all five L$_S$ genotype animals. Instead, it is evident that the observed genotypic pattern is much more complex (Fig. 1). In fact, one branch defined by H333 has recent descendents of both genotypes. If it is assumed that the mitochondrial genotype is fixed at or near birth, the shift from L$_L$ to L$_S$ in the H333 branch occurred over a period no greater than the difference in birth dates between H333 and H493—that is, in less than 3 years, 8 months, and 19 days—which encompasses two generations.

A second unusual aspect of this polymorphism is that the present pattern of genotypes in the lineage requires multiple independent shifts between genotypes. Except for the H493/H624 mother/daughter pair, inspection of the lineage shows that no pair of L$_S$ animals belongs to a common maternal branch without L$_L$ animals. The most likely possibility is that H15 contained both genotypes whose mtDNA has segregated in its progeny, giving the observed mix of relatively pure L$_L$ and L$_S$ genotypes. The specificity and rapidity of the process leading to such shifts of mitochondrial genotype do not appear to be consistent with the simple, random segregation of mitochondrial alleles. A more complex mechanism must be invoked to explain this mitochondrial gene variation. To begin elucidating that mechanism it is necessary to determine the precise nucleotide change(s) that distinguish L$_L$ and L$_S$ genotypes.

Nucleotide Sequence of the Two Mitochondrial Genotypes. MtDNA from four animals within the H15 lineage (H493, H512, H634, H1009B), two of each *Hae* III genotype, was amplified by cloning and compared by *Hae* III digestion (Fig. 2). In no case thus far examined has the *Hae* III restriction pattern of cloned mtDNA varied from the animal mtDNA (in total, 55 independently derived clones from 9 of the 16 animals). The nucleotide sequence of the region around the *Hae* III-37.1 site on the heavy strand in alkaline CsCl (H strand) is compared between cloned DNA from H949B (L$_S$ genotype) and H634 (L$_L$ genotype) in Fig. 3. The entire coding strand sequence we have determined for the L$_L$ genotype in this region is: (*Bgl* II site) 5' GA, TCC, TTC, ATA, CTA, AAC, CCA, AGC, GAC, TCA, AAC, ATA, CCC, TTG, ATT, GGA, CTA, GCA, TTA, GCT, GCA, ACC, GGA, AAA, TCC, GCC, CAA, TTT, GGT, CTC, CAC, CCG, TGA, CTT, CCC, TCT, GCA, ATA, GAA, GGC,

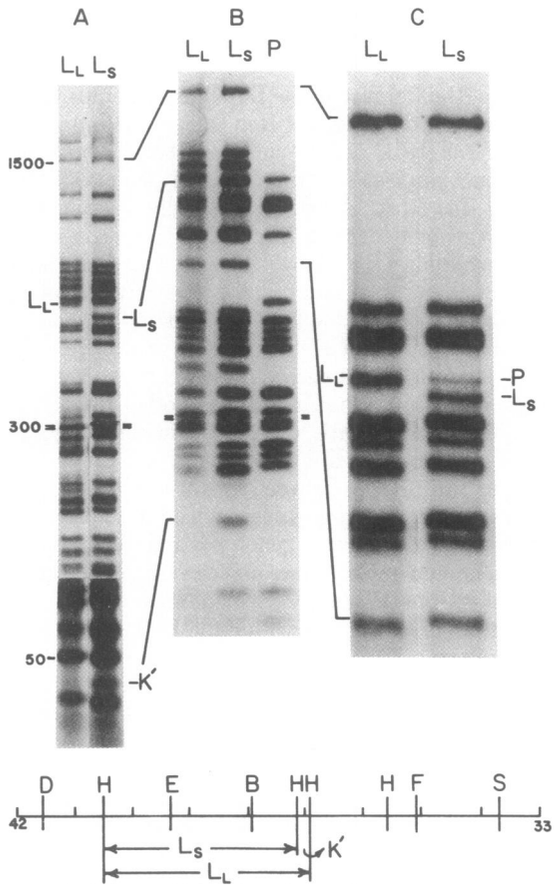

FIG. 2. Characterization of the *Hae* III polymorphism in animals from the H15 maternal lineage by polyacrylamide gel electrophoresis and autoradiography of the 3'-end labeled fragments. (*A*) Representative digests of animal mtDNA run on 6–12% discontinuous gel (double horizontal line marks the change from 6% to 12% polyacrylamide); lane L_L is the pattern for H455, an animal whose mtDNA does not contain a *Hae* III site at 37.1 map units; lane L_S is the pattern for H634, which does contain this site. Indicated fragment lengths in base pairs (bp) are determined relative to digested pBR322 DNA run on the same gel (not shown). (*B*) Representative digests of the *Pst* I 19.2/62.2 mtDNA fragment cloned into pBR322 and run on a 6% gel. Lanes: L_L, from H512, demonstrating retention of the L_L fragment; L_S, from H493, demonstrating retention of the L_S and K' fragments; P, *Hae* III digest of the cloning vehicle pBR322 DNA. (*C*) The same two digests as in *B* but run on a 4% gel so that fragments L_L and L_S can be resolved. A pBR322 DNA fragment (band P in lane L_S) comigrates with fragment L_L. The corresponding bands in each panel are indicated. At the bottom is a physical map of the region surrounding *Hae* III fragment L. Cleavage sites are shown for *Hin*dIII (D), *Hae* III (H), *Eco*RI (E), *Bgl* II (B), *Hin*fI (F), and *Sau*3A (S). The map extends from 42 to 33 map units and is divided into divisions of 1 map unit (about 160 bp). For reference to the complete sequence of bovine mtDNA (unspecified breed) the *Hin*dIII site is at 12,180 bp, the *Bgl* II site is at 12,707 bp, and the *Sau*3A site is at 13,450 bp from the H-strand origin of replication in the direction away from D-loop expansion (35).

CCA, ACT, CCC, GTC, TCA, GCA, CTA, CTC, CAT, TCA, AGC, ACA, ATA, GTG, GTA, GCA, GGT, ATC, TTC, CTA, ATC, CGT, TTC, TAT, CCC, CTG, AGA, GAA, AAC, AAT, AAA, TAC, ATC, CAA, T 3'. The open reading frame is indicated by commas. This sequence agrees with that of Anderson *et al.* (35) except for the point mutation creating the *Hae* III site difference (sequence in italics).

The deduced sequence is identical for both genotypes except that at the *Hae* III-37.1 site a guanine residue in H949B is an adenine residue in H634. This base transition accounts directly for the loss of this site in the L_L genotype (3'-C-C-A-G-) and

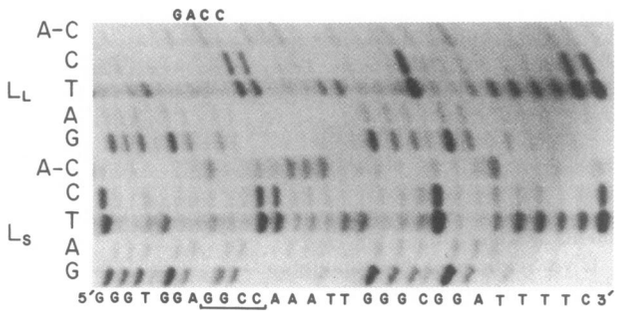

FIG. 3. Autoradiograph of a nucleotide sequencing gel comparing the region containing the mtDNA *Hae* III polymorphism. The fragment sequenced is Bgl II/*Cfo*I 37.9/36.7 3'-end labeled at the *Bgl* II site (84 bp from the *Hae* III site of interest). Lanes are marked according to the base-specific reactions (34): G, guanine specific; A, adenine + guanine; T, thymine; C, cytosine + thymine; A > C, adenine > cytosine. The sequence labeled L_L is from H634 mtDNA *Bam*HI 27.9/48.8 fragment cloned into pBR322. The sequence labeled L_S is from the equivalent fragment in a *Pst* I 19.2/62.2 cloned fragment of H512. The *Hae* III site accounting for the difference between L_S and L_L genotypes is indicated in the top sequence and underlined in the bottom sequence. Several fragments were analyzed from both 3'- and 5'-end labeled *Bgl* II sites to get complementary sequences. A summary of all clones sequenced is shown in Table 1.

this difference is maintained in H493 (L_S genotype) and H512 (L_L genotype).

The functional significance of this base substitution was established by locating its map position relative to three previously mapped loci on the bovine genome—large and small ribosomal RNA genes and the D loop (36)—and then oriented relative to the gene organization of human (37, 38) and mouse (39) mtDNA. The gene organization in these mammalian species is known to be completely conserved in *Bos taurus* (35). The L_L/L_S base substitution, so aligned, falls within unidentified reading frame 5 (URF-5), which is transcribed from the H strand. This is a region of about 1,800 bp flanked upstream by a leucine tRNA gene and downstream by another putative coding region (URF-6), which is transcribed from the complementary L-strand (37, 39). URF-5 is so designated because it contains a reading frame without mitochondrial stop codons (37, 39) and because a polyadenylylated mRNA maps to this region in the human and mouse systems (40, 41). The 224-bp Holstein sequence extending from the *Bgl* II site to 160 bp beyond the base substitution site is within URF-5. This sequence contains stop codons in two of the three reading frames which allows an equivocable assignment of an open reading frame. The 5'–3' coding strand sequence for the L_L genotype is TTT, GGT, CTC, and for the L_S genotype it is TTT, GGC, CTC. According to mitochondrial codon usage (42), both GGU and GGC code for glycine. Thus, the base substitution that distinguishes *Hae* III genotypes will not alter mitochondrial coding properties and

Table 1. Summary of clones subjected to sequence analysis

Animal	Genotype	Clone	Sequence polarity
H493	L_S	115	3'
H493	L_S	116	3'
H493	L_S	116	5'
H493	L_S	117	3'
H455	L_S	241	3'
H949B	L_S	351	3'
H512	L_L	321	3'
H512	L_L	321	5'
H512	L_L	322	3'
H634	L_L	955	3'

should render either genotype functionally equivalent in the animal. It therefore is possible to conclude that we have observed the appearance of two mitochondrial genotypes within a single maternal lineage of Holstein cows related by a neutral or silent mutation.

Mechanisms for Rapid mtDNA Polymorphism. There have been numerous studies of intraspecific mtDNA polymorphism; however, this report concerns maternally related individuals in which genotypic shifts occurred over recorded time spans. The rapidity and the occurrence of multiple shifts raise a number of questions regarding the stability and inheritance of the mitochondrial genome. It has been suggested that mtDNA evolves more rapidly than nuclear DNA because the mutational rate is greater for nuclear DNA (43). However, mutation rate alone is unlikely to account totally for our observations. First, the apparent minimum rate of change, 2×10^{-5} base substitution per base pair per year, is a factor of 10^3 times higher than the evolutionary rate (43). Second, the fact of multiple identical but independent adenine-to-guanine transitions is too specific according to any known mutagenic mechanism. Hence, other fundamental mitochondrial properties must also contribute to produce rapid polymorphism.

Two potential sources of mtDNA variation are paternal mitochondria and effect of the nuclear genes on mitochondrial properties. Although paternal contribution to the mitochondrial genotype is very small, if not negligible (15, 21–24), Holstein breeding data allows its consideration. Fifteen bulls fathered the 60 H15 animals. No bull was in the H15 lineage. Therefore, we conclude that all bulls fathering H15 animals have the L_S genotype because it appears universally outside the H15 lineage. If it is assumed that H15 was a pure L_L and that the L_S genotype was a direct result of an L_S mating, a shift occurred in a minimum of 4 of the 60 matings. This frequency is not consistent with total lack of such shifts upon many more similar matings of other mammals (15, 21–24). Additionally, in another maternal Holstein lineage (see *Materials and Methods*) in which such shifts would also be observable by *Hae* III digestion, none have happened in a total of 22 matings. However, we cannot rule out the possibility that a maternal nuclear gene predisposes the shift between mitochondrial genotypes because it has occurred thus far only in the H15 lineage. Specific breeding studies could be established to test this hypothesis.

One alternative to the involvement of paternal mitochondria or maternal nuclear genes is the mode of segregation of mtDNA molecules. Several models for mtDNA transmission have been proposed (44). These analyses are based primarily on data from lower eukaryotes and they treat mitochondrial transmission as an intracellular population problem in which mtDNA molecules segregate randomly at each cell division. If parental mtDNA molecules are transmitted in a random distribution, there is a finite probability that any viable mutant may become established as the major mitochondrial genotype in a progeny, given a sufficient number of generations. Clearly, if an altered mitochondrial gene confers an advantage to the cell, the number of generations required to establish that genotype is expected to be markedly reduced. This appears to be the case for the selection of chloramphenicol-resistant mitochondrial mutants (45, 46). If the mutant is selectively neutral, as may be the case for the Holstein genotypes, a statistical analysis would be possible if the number of transmitted mtDNA molecules and the number of female germ-line cell generations, which together define the number of segregation events, were known for mammals. It has been estimated that a mammalian germ cell contains a minimum of 100 mtDNA molecules and that there are no more than 50 germ cell generations (6). These assumptions appear to be reasonable in view of the estimates for the number of mtDNA molecules in somatic cells (27) and for the total number of primary bovine germ-line cells (47). By assuming random segregation and using these values, Upholt and Dawid (6) estimated that it would take at least 20 generations to obtain a pure mtDNA population from a mixed population. We observe a shift from an L_L genotype to an L_S genotype in as few as two and in never more than five generations. The maximal rate for observing a shift in mitochondrial genotypes according to the random segregation model is therefore 1/10th the experimental rate. It is unlikely that either the germ cell generations are greater than 50 or the number of mtDNA molecules in germ cells are fewer than 100. Hence, the totally random segregation model does not appear to apply. However, if the segregation mechanism favored one genotype, this model would work well. For example, the L_S genotype may have a replicative or selective advantage relative to the L_L genotype. This could account for the H333/H493 shift and is at least formally possible because other sequence differences may be linked to the *Hae* III difference.

Another segregation mechanism which may complement random segregation can be postulated based on the fact that mitochondria and mtDNA are amplified during mammalian oocyte development. Piko and Matsumoto (28) calculated that the mature mouse oocyte contains 100–1,000 times more mtDNA than is found in somatic cells. In preliminary studies we also find about a 100-fold increase of mtDNA in mature bovine oocytes. If this amplification uses a limited number of template molecules, a genetic "found effect" may be operative—that is, amplification of one or a few mtDNA molecules will yield one predominate genotype in the mature oocyte. Thus, assuming mitochondria in germ-line cells are to some degree heterozygous in this lineage, differential amplification could lead to a shift from one mitochondrial genotype to another in a single generation. The amplification model has another attractive aspect. It is well established that a large fraction of mammalian oocytes undergo spontaneous degeneration during development (48). Within this model, a potential explanation is provided because random template choice could lead to oocytes containing functionally impaired mitochondria. Such an oocyte would not survive. The model therefore tests for mitochondrial fitness and simultaneously serves to "dilute" much unrepaired genetic damage at each generation. Therefore, it has the combined features of generating rapid genotypic shifts while maintaining a relatively pure and functional genotype. It is conceivable that amplification and random segregation processes may occur in tandem to generate rapid polymorphism.

The amplification model makes several predictions which appear to be testable. (*i*) mtDNA sequences, particularly in animals that have recently undergone a polymorphic shift, should be polymorphic within an individual. Clearly, good candidates exist within the H15 lineage for this purpose but we have yet to observe a mixed genotype at greater than the 5% level. (*ii*) mtDNA from an individual oocyte may be relatively pure but may exhibit variable sequences between oocytes from an individual. Again, animals with recently shifted mitochondrial genotypes are predicted to be the best candidates. (*iii*) If mtDNA is "recloned" at each generation, multiple neutral mutations may appear simultaneously. To our knowledge, none of these predictions has been tested in any animal system, but members of the H15 lineage offer an attractive resource for this purpose.

It remains unclear exactly how the type of rapid polymorphism we observe interfaces with mtDNA evolution. One might postulate that such rapid variation in individual bases are steps in the evolutionary process, but we cannot yet say precisely how common intralineage mtDNA polymorphism really is. For ex-

ample, restriction analysis in two other large maternal lineages has not yet revealed similar mtDNA variations. However, we are presently limited to restriction site analysis which surveys only about 2% of the nucleotides in the genome. On the other hand, the existence of intralineage variation suggests that a mechanism exists that could be used to fix rare but functionally significant changes in the mitochondrial genome.

Note Added in Proof. After submission of our manuscript, we became aware of a recent publication also concluding that the segregation of mtDNA molecules is not totally random (49). Those authors proposed that random replication of organelle DNA molecules may be responsible for the apparent rapid segregation of organelle genes and that this may aid in fixing new mutations.

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