Volume 17 Number 18 1989 Nucleic Acids Research

Rapid segregation of heteroplasmic bovine mitochondria

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Received May 30, 1989; Revised and Accepted August 16, 1989

ABSTRACT

By following the transmission of a heteroplasmic mitochondrial DNA mutation through four generations of Holstein cows, we have documented that substantial shifts in the levels of heteroplasmy can occur between single mammalian generations, that neutral mitochondrial genotypes can segregate in different directions in offspring of the same female, and that a return to homoplasmy may occur in only two or three generations. This apparently rapid rate of mitochondrial DNA segregation in mammals contrasts to the much slower rates observed previously in insects and suggest fundamental differences between taxa regarding the mechanisms of mitochondrial gene transmission.

INTRODUCTION

While the organization and evolution of the mitochondrial genome has been extensively studied in a variety of metazoan taxa, surprisingly little is known about the transmission genetics of mitochondrial genes beyond their maternal inheritance through the egg cytoplasm. The early observation of high levels of inter- and intraspecies variability in mtDNA sequences (1−3), coupled with an apparent absence of mitochondria heteroplasmy (multiple mitochondrial genotypes within an individual) led Uphold and Dawid (4) to propose that germ line segregation of mtDNA genotypes might be involved in the rapid generation of sequence diversity. The occurrence of mtDNA sequence differences between maternally related dairy cows (5,6) suggested that events occurring during oocyte maturation or early embryogenesis constituted a genetic founder effect or bottleneck in mtDNA inheritance which resulted in the rapid fixation of sequence differences in a maternal pedigree (6,7). This concept of mtDNA mutation followed by a rapid sorting of the genotypes from transiently heteroplasmic mammals, although generally accepted (8−10), is experimentally unproven. We have now tested this hypothesis by following the transmission of a heteroplasmic point mutation through four generations of Holstein cows. We show for the first time that substantial shifts in the levels of heteroplasmy can occur between single mammalian generations; that neutral mtDNA genotypes can segregate in different directions in offspring of the same female; and that a return to homoplasmy can occur in only two or three generations.

MATERIALS AND METHODS

Holstein cattle were obtained from the Dairy Research Unit herd at the University of Florida, Gainesville. Record keeping and factors affecting pedigree accuracy have been discussed (6). Crude mitochondrial pellets were isolated from brain or liver tissue through differential centrifugation, DNA was isolated and digested with HpaII or HpaII plus HaeII, and the fragments were separated by gel electrophoresis and blotted to Zeta-probe membrane as

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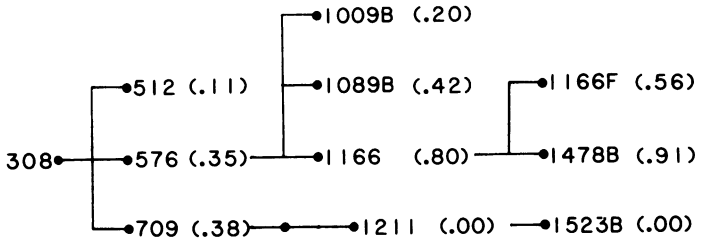

Figure 1. Maternal lineage of Holstein cows containing heteroplasmic mtDNA genotypes. Each genotype is expressed as a proportion of the mtDNA containing a cytidine residue at nucleotide 364 and hence lacking a Hpa II site here (see Fig. 2, Panel C). Animal identification numbers denote each individual; suffix 'B' denotes bull and suffix 'F' denotes fetus. Determination of heteroplasmic values is explained in the legend to Figure 2.

described previously (11). The pCLR clone was constructed as described (11); the pCLM clone was constructed by inserting the Bam H1-Eco RI fragment (nucleotides 16202−3357) into pSP64 (Promega Biotech, Madison, WI). Probes of these clones, made by nick-translation in the presence of α-^{32}P-dNTP's, were hybridized to blots overnight. Following high stringency washing, the membranes were autoradiographed. Quantitation of different mtDNA genotypes was done by scanning autoradiograms within the linear response range of the Kodak XAR-5 film with an Ultrascan laser densitometer followed by analysis with the software supplied (11). Scans of independent digests and blots of the same individuals varied typically by less than 10%. Except for animals 1211, 1523B and 1167, scans of autoradiograms of HpaII/HaeII digests (Figure 2, Panel B) were used only to confirm the results obtained with HpaII (Figure 2, Panel A) because the polymorphic bands were insufficiently resolved for confident measurement. In Figure 2, Panel A, all lanes except 1478B and 1009B are from the same blot; in Figure 2, Panel B, all lanes are from the same blot. In each panel, lanes are shown at different exposures to optimize visual inspection.

RESULTS AND DISCUSSION

The rarity of individuals containing two different mtDNA genomes had initially prevented a direct examination of the rates of mtDNA segregation in heteroplasmic individuals. Examples of heteroplasmy have now been reported in diverse species including: drosophila (12−14), dairy cattle (15), frogs (16), crickets (17,18), lizards (19), humans (20,21), fish (22), and scallops (23). Many of these examples of heteroplasmic species are due to variations in the copy number of a repetitive sequence; this type of polymorphism is not a dependable genetic marker because copy number changes, possibly due to replication slippage, occur between animal generations (15). To avoid this problem, we identified a heteroplasmic Hpa II restriction site polymorphism within several maternally related holstein cows, and showed it to be a single guanosine/cytosine transversion within the D-loop region very near the phenylalanine tRNA (11). Three siblings, animals 512, 576, and 709 (Figure 1), differed substantially in the frequencies of the mtDNA genotype lacking the Hpa II site at bp 364 (genotype L) relative to the type containing the Hpa II site (genotype S)(11). This result provided indirect evidence for unequal partitioning of mtDNA genotypes during female germline development and suggested that mammals may segregate mtDNA genotypes more rapidly than insects. However, these data could not provide information on actual segregating rates.

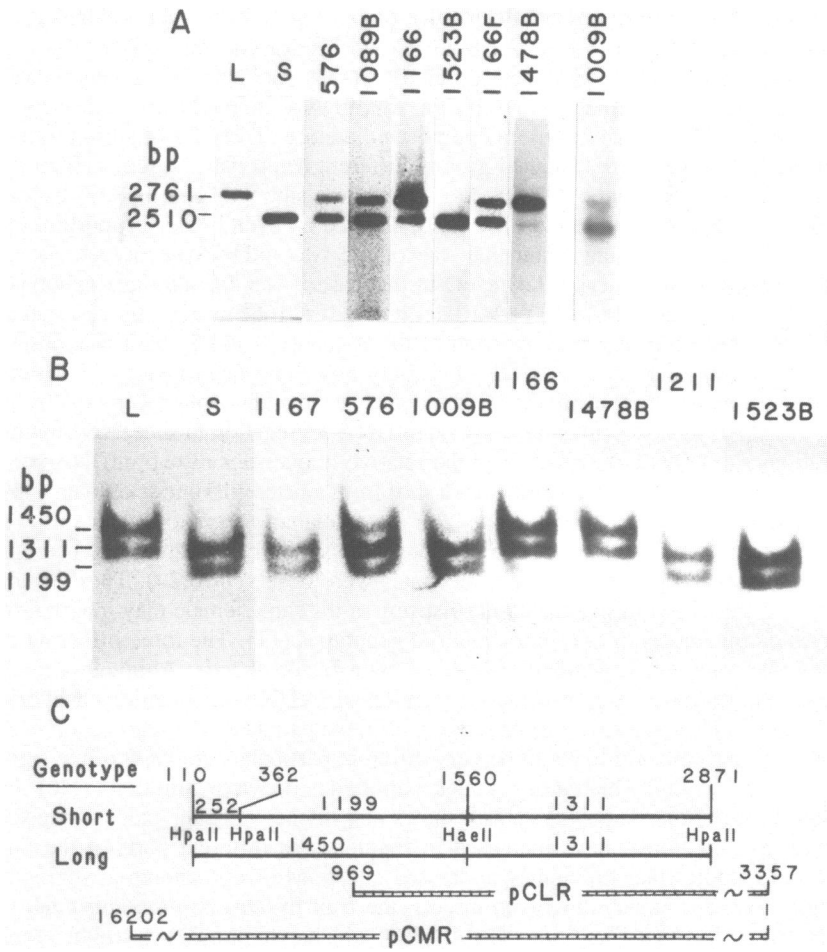

Figure 2. Southern blot of mtDNA samples isolated from the tissue of maternally related Holstein cattle. Animal numbers correspond to the pedigree shown in Figure 1. Panel A: HpaII digested samples probed with the pCLR probe (see below). Upper band is the 2.7 kb fragment lacking the extra HpaII site, 'L genotype'. Lower band is the 2.5 kb fragment containing the extra site, 'S genotype'. Panel B: HpaII/HaeII double digests of samples probed with the pCMR probe (see below). Upper and lower bands are diagnostic for S (1450bp) and L (1198bp) genotypes, respectively. The middle band (1131bp) present in all samples is a HaeII/HpaII fragment shared by both genotypes. Panel C: Restriction map and extent of each hybridization probe (hatched lines) in the polymorphic region of the bovine mitochondrial genome. The number above each indicated restriction site refers to its genome location and the numbers between sites indicate the distance in nucleotides between sites in each genotype. Animal 1167 is from an unrelated maternal lineage within the herd and serves to illustrate the existence of the homoplasmic S-genotype in most of the other animals examined.

We have extended our analysis of Hpa II restriction site heteroplasmy in this lineage through several generations in order to document experimentally how rapidly mtDNA segregates in mammals. MtDNA from two descendants of 709 and five descendants of

576 (Figure 1) were examined and the relative proportion of the S and L mtDNA genotypes of each were determined (Figure 2). Both F2 and F3 descendants of 709 (709 exhibited a L genotype frequency of 38%) had lost all detectable amounts of the L genotype (Figure 2), suggesting an extremely rapid return to mtDNA homoplasmy. Analysis of the descendants of 576 produced a more complicated picture. Three F1 offspring of 576 (576 contained 35% L genotype) showed grossly different heteroplasmic ratios (Figure 1). At one extreme, 1009B is only 20% L genotype while a sibling, 1166, is 80% L genotype. 1478B, a son of 1166 and grandson of 576, showed an even higher proportion of the L genotype (91%) suggesting again that one of the two mtDNA genotypes was rapidly becoming fixed in the lineage; however, in this case it was the alternate genotype from that of the descendants of 709. Yet, a fetal bull calf (1166F), taken by cesarian section from 1166, showed a higher proportion of the S genotype (44%) than was observed in either its mother or brother (1478B). Precisely how long heteroplasmy will remain in measurable amounts must await the birth and analysis of additional progeny in this lineage.

The results of this experimental study on mtDNA segregation in successive mammalian generations show that large changes in the relative frequencies of two mtDNA genotypes can occur. They clearly demonstrate that a shift from a heteroplasmic state to an apparently homoplasmic state can occur within only 2 or 3 mammalian generations. Our earlier studies using different single base polymorphisms inferred a similarly rapid return to homoplasmy, but no actual heteroplasmic intermediate states were observed (6,24). These results also confirm the initial report that different offspring of the same female may inherit strikingly different complements of two mitochondrial genotypes (11). The three offspring of 576, for example, range in L genotype frequency by a factor of four. Although a number of previous studies have examined the segregation of mtDNA in somatic cell hybrids and found rapid fixation of a single genotype (e.g. 25), the parameters of segregation in somatic versus germline cells are likely to be very different particularly in view of the significant mtDNA amplification which accompanies mammalian oocyte maturation (7,26). Finally, the Hpa II restriction site polymorphism shows no evidence of being under strong selection pressure, as both forms have increased in frequency in different parts of the lineage.

The only studies that previously addressed questions of mitochondrial segregation in breeding experiments have involved insects, the fruit fly *Drosophila mauritiana* (12,13) and the field cricket *Gryllus firmus* (18), both of which exhibit mtDNA size heteroplasmy. A population genetics model applied to these data suggests that mtDNA genotype sorting is a stochastic process and a complete segregation to homoplasmy would require as many as 500 insect generations (13,18). Although the sample size of Holstein cattle involved in this study is necessarily smaller than that for insects, the data suggest that mtDNA transmission in mammals may be quantitatively different than in insects. Using the modified genetic drift model of Solignac et al. (13), it is possible to relate the variance in mtDNA genotype levels between generations to the number of generations required to fix or lose a genotype. The variance in frequency of the two mtDNA genotypes among the siblings 1009B, 1089B, and 1166 is calculated to be 0.09 and is typical of all first and second generation variances which ranged from 0.06 to 0.13. This is 2 to 20 times higher than the variance observed among offspring of 7 heteroplasmic female crickets (18). Based on the approaches of Solignac et al. (13) and Rand and Harrison (18) the number of generations (n) to fixation or loss of a genotype in a heteroplasmic animal can be estimated as the point at which the variance at the nth generation (Vn) is within 10^{-6} of $p(1-p)$,

where p is the frequency of the genotype according to the equation,

$$Vn = p(1-p)(1-\alpha^n)$$

where

$$\frac{p(1-p)-Vn}{p(1-p)}$$

Therefore, fixation or return to homoplasmy can be estimated to occur when $\alpha^n \leq 10^{-6}$. The calculated time for loss of heteroplasmy is 28 generations using the offspring of 576 as typical of the lineage ($\alpha^1 = 0.61$). In both Drosophila and crickets the time to fixation was calculated to be 120 to 500 generations. The empirical data supports this difference between taxa; a heteroplasmic line of Drosophila maintained for 30 generations still exhibited heteroplasmy in more than half of the offspring. Yet, after only 3 generations, we observed one of the three F2 Holsteins to be homoplasmic for one genotype and another nearly so for the other genotype.

This difference in rates of mtDNA segregation between various animal taxa has been discussed previously (13,18) based on inferred mammalian segregation rates. This report provides experimental confirmation for rapid segregation of mammalian genotypes (6,24). Such differences in segregation rates reflect either differences in the number of segregating mtDNA molecules or in the number of cell divisions occurring during female germ cell development. Depending on how many germline cell divisions occur per animal generation (only estimates are available, e.g. (4)), the calculated number of segregating units for the Holstein cows in this study is between 20 (if 10 germ cell generations) and 100 (if 50 germ cell generations). This is a factor of 10 smaller than that calculated for insects. Although our sample size is small, there remains a clear and substantial difference in segregation rates and number of apparent segregating units in the mitochondria of mammals versus insects. We have presented evidence that a developmental bottleneck in the number of mtDNA molecules per mitochondrion during bovine oogenesis is apparently due to a lag in mtDNA accumulation relative to organelle accumulation (11,26). Random partitioning of organelles containing only one or a very few mtDNA molecules into early embryonic cells could account for the small number of apparent segregating units and lead to the observed rapid segregation of polymorphic mtDNA species in the progeny. It is not known whether a similar process occurs during insect oogenesis.

The origin and maintenance of mtDNA heteroplasmy in mammals has recently gained medical relevance due to the observation that patients with maternally transmitted muscle myopathies frequently exhibit heteroplasmy involving mtDNA deletions in muscle tissue (21,27). The variable penetrance of a maternally inherited encephalomyopathy has also been attributed to heteroplasmic mtDNA (28). These examples of mtDNA heteroplasmy in humans appear to be maintained during maternal transmission and, in contrast to the case reported here, may involve positive selection for the heteroplasmic state. Otherwise, it is difficult to reconcile persistence of heteroplasmy in view of our result showing a rapid return to homoplasmy when no apparent selective pressure is present. It will be interesting to follow mitochondrial genotypes in the offspring of these patients to see whether heteroplasmy is maintained. Whatever the outcome, it is clear that quantitative parameters of transmission and ultimate resolution of the heteroplasmic state in mammals will be important in understanding maternally inherited diseases.

Rates of evolution of DNA reflect both the mutation rate and the rate of fixation of newly arising mutations. Fundamental differences in the latter parameter for mtDNA, as suggested here by comparison of the mammalian and insect segregation data, are expected to contribute to variation in rates of evolution. Indeed, recent studies have demonstrated that the rate of mtDNA evolution relative to that of nuclear DNA does vary widely in different groups (29,30). In light of the growing use of mtDNA for estimating divergence times of species in evolutionary studies, it seems increasingly important that intrinsic rates of mtDNA evolution be estimated separately for comparisons among different animal taxa.

ACKNOWLEDGEMENTS

We thank Drs. C. J. Wilcox and M. Drost for providing animals and tissue samples. This work was in part supported by grants GM34825 and GM33537 from the National Institutes of Health and by Research to Prevent Blindness, Inc.

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