Genetic Analysis of Systematic Mitochondrial Heteroplasmy in Rabbits

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ABSTRACT

One unusual property of rabbit mitochondrial DNA (mtDNA) is the existence of repeated 153-bp motifs in the vicinity of the replication origin of its H strand. Furthermore, every individual is heteroplasmic: it carries mtDNA molecules with a variable number of repeats. A systematic study of 8 females and their progeny has been devised to analyze mtDNA transmission through generations. The results suggest that three mechanisms are acting simultaneously. (1) Genetic drift in the germ line is revealed by the evolution of heteroplasmy when two major molecular forms are present in a female. (2) A high mutation rate (around 10^{-2} per animal generation) generating molecular diversity, by deletion and addition of repeated units, is required to explain the observation of heteroplasmy in every individual. Moreover, the rates of mutation from the most frequent type to the other types are unequal. The deletion of one unit is more frequent than a deletion of two units, which is in turn more frequent than a deletion of three. (3) Selection for shorter molecules in somatic cells is probable. The frequency distribution of mtDNA types depends on the organ analyzed (kidney-spleen and liver *vs.* gonads).

OWING to the maternal inheritance of mitochondrial DNA (mtDNA) in most metazoan species, the occurrence of intra-individual mtDNA heterogeneity (mitochondrial heteroplasmy) necessarily involves a mutational event in a germ line cell of a female. The mutations can lead to nucleotide substitutions (site heteroplasmy) or variations in the length of the whole molecule (length heteroplasmy). The evolution through generations of heteroplasmic states is the resultant of three phenomena: the rate of mutation, segregation through cell divisions and selection. Depending on their relative effects, heteroplasmy may or may not be resolved into homoplasmy.

The study of how heteroplasmy evolves requires pedigree and population analysis. The polymorphism (intra- and inter-individual) observed in wild populations is examined through the use of mitochondrial population genetic models (CLARK 1988; BIRKY *et al.* 1989), which require parameters such as the effective number of mitochondria, the mutation rates and the selective values of the different mtDNA forms. These parameters can be estimated from the analysis of (1) the frequency of heteroplasmic animals within a population, (2) the distribution of the mtDNA type frequencies in individuals and (3) the evolution of these distributions through generations.

Pedigree analysis performed on yeast (DUJON *et al.* 1974), paramecia (ADOUTTE and BEISSON 1972), Drosophila (SOLIGNAC *et al.* 1984; DE STORDEUR *et al.* 1989, NIKI *et al.* 1989), crickets (RAND and HARRISON 1986), cows (HAUSWIRTH and LAIPIS 1982) and bats (WILKINSON and CHAPMAN 1989) show that the parameters involved in the evolution of heteroplasmy may vary considerably among the species. Estimates of the effective number of mitochondria range from to a few hundred for insects to a few units for cows. The mutation rate is estimated at around 10^{-2} for bats and 10^{-4} for crickets per animal generation. In Drosophila, in some cases, it appears that selection acts toward the preferential fixation of a particular mtDNA type (DE STORDEUR *et al.* 1989).

Length heteroplasmy has already been reported for the European rabbit *Oryctolagus cuniculus* (ENNAFAA *et al.* 1987; BIJU-DUVAL *et al.* 1991). It is of a particular interest since every individual, whatever its geographical origin, is heteroplasmic, a situation referred to hereafter as "systematic heteroplasmy." Heterogeneity among rabbit mtDNA molecules originates from the presence of two units (LR, 153 bp and SR, 20 bp) repeated in tandem in two specific stretches near the H strand replication origin (MIGNOTTE *et al.* 1990). The number of each of these units is variable between molecules. The origin of this variability is still a matter of debate and mispairing slippage and/or intra- and inter-molecular recombination have been postulated. Mitochondrial DNA length polymorphism due to variation in the copy number of tandemly repeated sequences has been observed in a variety of species from several phyla (reviewed in RAND 1993). In some of these studies, mtDNA length heteroplasmy has also been reported, but with different frequencies, depending on the species.

The present study is devoted to the analysis of the evolution, through generations, of the systematic heteroplasmy oberved in *O. cuniculus* to understand its origin and maintenance. The results we present here confirm the systematic heteroplasmy for the long repeats through generations in all rabbits. The frequency

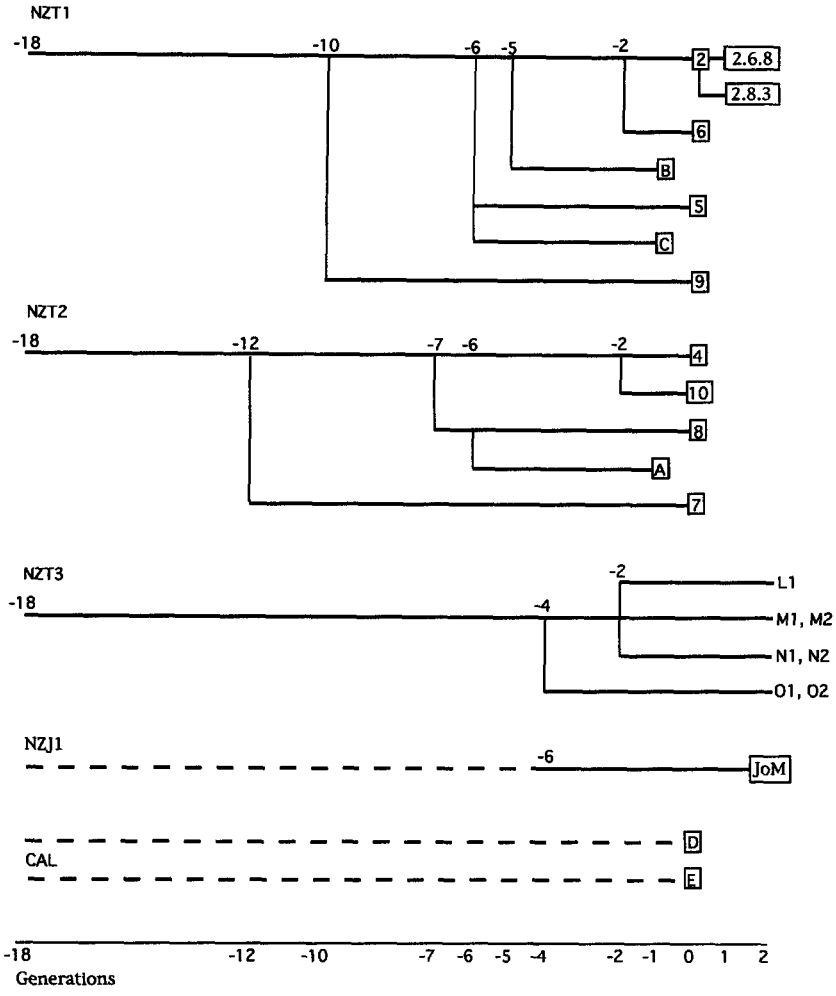

FIGURE 1.— Maternal pedigrees of the rabbits taken as parents. The identification numbers of the reproducing animals are framed. NZT1, NZT2, NZT3 and NZJ1 are the four maternal lineages to which all the New Zealand rabbits belong. Generation 0 is composed by the females (2, 4, 5, 6, 7, 8, 9 and 10) used as progenitors to start the experimental protocol. No data for female 5.

distributions within animals are compatible with a low mtDNA effective number (few hundreds) and a high mutation rate in germ cells. The comparison of the distribution in different organs of a same animal (kidneys-spleen *vs.* liver) suggests a selection pressure favoring shorter molecules in somatic cells. Moreover, it seems that, in some organs (kidneys-spleen and liver), there is a preferential accumulation of mtDNA which lose unit(s) of repetition, whereas accumulation of mtDNA which gain unit(s) of repetition appears to occur in the gonads.

MATERIALS AND METHODS

Origin of the animals: Most of the animals used in this study belong to a New Zealand rabbit strain (NZT), some to a second one (NZJ), and a few to a Californian strain (CAL) (see Figure 1). The New Zealand strains have been genetically separated from the Californian strains for over 100 years.

The animals derived from the NZT strain belong to a population selected for prolificity. Their pedigrees are known for 18 generations. The pedigrees of the animals derived from the NZJ strain are known for six generations. Pedigrees of the Californian animals are unknown.

Main experimental protocol: To analyze the state of mtDNA in parents and their descendants (F_1 and eventually F_2), eight families were initiated from eight females (2, 5, 6, 9, and 4, 7, 8, 10), chosen from two maternal lineages within the NZT strain: NZT1 and NZT2. Their relative relationships can be judged on Figure 1 through the number of generations which separates one from the other within each main maternal lineage.

Each of these eight females was crossed (1–8 times) with selected males, all issued from NZT strains, and a part of the whole set of the subsequent progenies was kept for mtDNA analysis. The females 2, 4 and 5 have produced, respectively, 65, 35 and 45 rabbits. From these animals, 51, 22 and 34 have been kept for mtDNA analysis. Five descendants from each of the other females (6, 7, 8, 9, 10) were also kept for mtDNA analysis.

Each descendant is numbered as follows: first the number of the female at the origin of the family, then the number of the litter to which it belongs, and finally its number within this litter. For example: animal 2.3.4. is a descendant of female 2; it was born within the third litter and was the fourth animal studied from that litter. The age (in days) of every animal sacrificed was recorded.

Within the family issued from female 2, a second generation was bred using two females 2.6.8 and 2.8.3 from the first generation and two males (D, E from the Californian strain, see Figure 1). From these crosses 9 and 15 descendants, respectively, were studied.

Complementary experiments: The main experimental protocol was intended to give some information about mtDNA type distribution within animals issued from the NZT1 and NZT2 lineages: males, females and their progeny. In order to

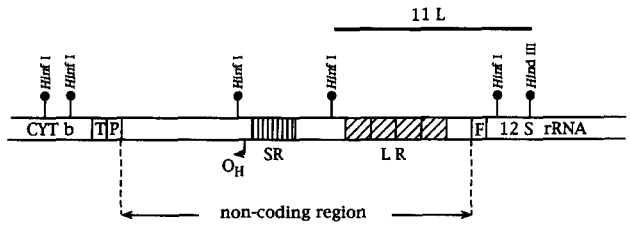

FIGURE 2.—*Hin*fI restriction map of the non-coding region. 11L, mtDNA fragment used as a probe for the long repeats; LR, long repeats; SR, small repeats; T, threonine tRNA gene; P, proline tRNA; F, phenylalanine tRNA; CYTb, cytochrome *b*, O_H, H strand replication origin.

generalize the results for the whole strain and even to other strains, an extension of the observations was needed. For this purpose, individuals from another New Zealand maternal lineage (NZT3) with different levels of relationships were involved in this study (L1, M1, M2, N1, N2, O1 and O2; Figure 1).

A female (JoM) of another New Zealand strain (NZJ), related to strain NZT, represents a fourth maternal lineage: NZJ1. This female has been studied along with seven of its descendants. Additional information was also obtained from mtDNA analysis of males belonging to the Californian strain (D and E).

Tissues examined: For each rabbit, mtDNA was individually isolated from the liver on the one hand and kidneys and spleen on the other hand. For some animals, mtDNA was extracted from ovaries (females 2.6.8, 2.8.3, 4.4.9, 5.3.9, 5.4.9, JoM, Jo.14.1, Jo.14.2, Jo.15.1, Jo.15.2) or testicles (males D, E). Data were not obtained for females 5 and 2.5.9 which died during the course of experiment.

Extraction of mtDNA: Extraction of mtDNA from the different organs was performed using the method previously described by BIJU-DUVAL *et al.* (1991) and involved the isolation of mitochondria, followed by purification of mtDNA through cesium chloride gradients.

Detection of long repeats: Individual mtDNA samples were digested with *Hin*fI to produce the shortest fragments: 182 + $n \times 153$ bp (n = number of repeats) which may contain the long repeats stretch (see the *Hin*fI restriction map of the non-coding region, Figure 2). After loading on vertical 2.5% agarose gels (Nusieve), electrophoresis was conducted for 16 hr at 70 volts. DNA was then transferred to a nylon membrane (Hybond N+, Amersham Corp.) according to the Southern method. The DNA marker was the plasmid Bluescript cut by *Hin*fI.

The identification of the long repeats (LR) was achieved after hybridization with a specific probe. A fragment including the long repeat domain, generated from a rabbit mtDNA clone, had been previously inserted in Bluescript (p11 L; MIGNOTTE *et al.* 1990) (Figure 2). Plasmid DNA was labeled by random priming with [α-^{32}P]dATP and hybridization was conducted overnight at 55°. The membranes were washed and autoradiographed for one night.

The presence, in the mtDNA extracted from one individual, of molecules differing in their lengths was easy to detect after autoradiography. The autoradiographs (see Figure 3, A and B) showed regularly spaced bands, with 153-bp steps, in the range of 488–1559 bp and a larger band (>2000 bp) near the top of the gel. This last band corresponds to the hybridization of p11L DNA with the *Hin*fI fragment outside of the non-coding region which includes part of the 12S rRNA gene (right side of Figure 2). The others originate from the digestion of molecules of various sizes containing the domain of the long repeats (LRs, left side of 11L in Figure 2). Depending on the number of repeats within this stretch, the length of the corresponding *Hin*fI fragment may vary from one molecule to another one. The length of the 947-bp *Hin*fI fragment, where 5 repeated units are present, was determined from the sequence of the cloned rabbit mtDNA molecule (MIGNOTTE *et al.* 1990). Taking into account the size of a repeated unit (153 bp) it is easy to determine how many of them are present in each of the observed bands and consequently to recognize the different kinds of molecules, as judged on their length, present in an extract. From this, it was possible to describe, for a given individual, the population of molecules it carried.

A partial digest of mtDNA from liver and kidneys-spleen (which has not been observed with DNA from ovaries and testicles) at the *Hin*fI site located between the two stretches of repeats (see Figure 2) generates a band the length of which varies from 1287 to 1667 bp. It is the sum of the 5LR *Hin*fI fragment (947 bp) and the fragment containing the small repeats (320 bp + $n \times 20$ bp, depending on the number of SR: n = 1–20). Thus, this fuzzy band masks any eventual fragment from mtDNA-8LR mtDNA-9LR (1406 and 1559 bp, respectively).

Frequency of molecules of different length in a mtDNA extract: The description of the population of molecules in a given individual can be improved by an estimate of the frequencies for each type of molecule. The relative quantity of molecules differing by their length were estimated through the intensity of the bands recognized, on the autoradiographs, as related to the domain of repeats. This was performed directly from the membrane, after washing, using a PhosphorImager (Molecular Dynamics). No correction for the effect of the length of the fragments on the relative frequencies has been done because the frequencies of the different fragments are very different in one sample. For two samples we compared only frequencies for the same type of fragment.

When the frequency of a type of molecules was found to be lower than 1%, the automatic quantification was considered not precise enough to give the number found as such. We have chosen to express these as: <1 for the values lower than 1% but much higher than 0.1%. Values around 0.1%, and even less, were noted: $\ll$1. In both cases, direct observations of the autoradiographs confirmed the existence of these bands and their relative intensities.

The accuracy of the measurements was appraised through two controls. First, we measured the frequencies of molecules for a given DNA sample loaded on two gels. This was done for 10 extracts. For frequencies between 1 and 10%, the error is less than ±1%. The frequencies <1% stayed always under this limit. Second, we examined an eventual saturation effect of the PhosphorImager screen. Two membranes (16 samples) were submitted to two exposures; the second exposure being 10-fold longer than the first one. The frequencies calculated for the two exposition time are very similar (less than ± 0.2% differences) which indicates no saturation effect.

RESULTS AND DISCUSSION

The analysis of the evolution of the systematic heteroplasmy observed in *O. cuniculus* was achieved through three main steps. (1) The description of the population of mtDNA molecules carried by each animal studied: for each mtDNA extract, different types of molecules were recognized, by the variation of the long repeat (LR) number, and their estimated relative frequency. (2) The examination of the evolution of mtDNA type frequencies through generations: animals

of chosen origins and their progeny (Figure 1) were studied through mtDNA extracted from liver on the one hand, kidneys and spleen on the other. Taking into account this general approach, the composition of the mtDNA molecule population observed for each individual is the consequence of events arisen in both the germ and somatic lines. (3) The examination of the evolution of mtDNA-type frequencies depending on tissue and age of the animals; mtDNA was extracted from different tissues of the same animals: liver, kidneys-spleen and testicles or ovaries, and from given tissues of animals of different ages.

Illustration of the experimental approach: mtDNA from the kidneys-spleen of the animal 2 [first line of Table 1 (see APPENDIX)] is given as an example. Five types of molecules are detected and characterized by the presence of 3, 4, 5, 6 and 7 repeat units. The mtDNA-5LR type is by far the most frequent (92%); mtDNA-4LR exhibited a higher percentage (5%) than mtDNA-6LR and mtDNA-4LR (2%); mtDNA-7LR being at the limit of detection (<1%).

Presentation of the whole set of results: The results are given in the APPENDIX, grouped by family. When females had more than one litter, only the results for one litter are given.

Genetic drift (in the family 7): Previous work on the evolution of heteroplasmy has shown that the time needed for the resolution of heteroplasmy may greatly differ depending on the species. The existence of genetic drift has been well documented in cows (OLIVO *et al.* 1983; HAUSWIRTH *et al.* 1984) and Drosophila (SOLIGNAC *et al.* 1984; DE STORDEUR *et al.* 1989). In the present study the observation of female 7 and its progeny has permitted us to examine this point.

mtDNA of female 7 was characterized by the presence of two types of molecules, mtDNA-4LR and mtDNA-5LR, with well defined frequencies (33 and 67%, repectively, in kidneys-spleen). Both types are still present in the offspring (7.4.1, 7.4.2, 7.4.3, 7.4.4, 7.4.5) with some individual variations in the relative proportions; the extremes being represented by individuals 7.4.3 with 25.5%/73.5% and 7.4.2 with 50%/48%, respectively for mtDNA-4LR/mtDNA-5LR (APPENDIX). The examination of mtDNA from liver confirmed these results. Such a fluctuation of the mtDNA type frequencies in family 7 suggests an effect of a relatively significant genetic drift in the germ cell line. By contrast, the fact that different tissues present similar situations leads us to conclude that the genetic drift through the first divisions of the egg (before the separation of the stem cells of the studied organs) must be low.

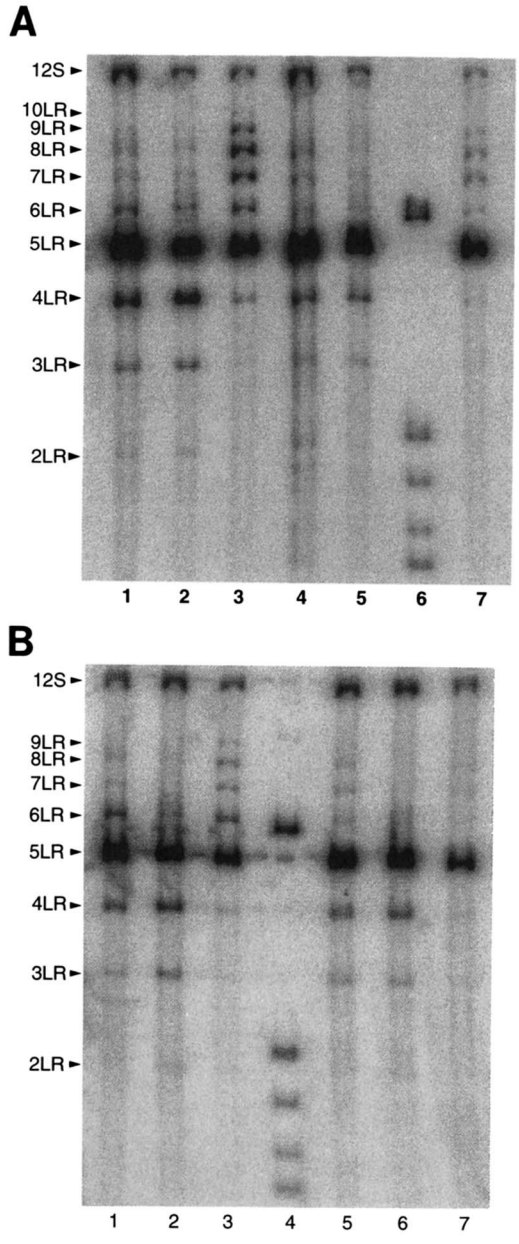

FIGURE 3.—Length heteroplasmy in *Hin*fI digests of mtDNA isolated from four rabbits (JoM and E, 2.6.8, D). (A) Lanes 1, 2, 3 = mtDNA extracted from the kidneys-spleen, liver and ovaries of the female JoM. Lanes 4, 5, 7 = mtDNA extracted from the kidneys-spleen, liver and testicles of the male E. Lane 6 = *Hin*fI digest of the Bluescript plasmid used as a marker. Molecules carrying 5 long repeats (5LR) are the most frequent mtDNA type extracted from each individual and each organ. In all cases, in the kidneys-spleen and liver, among the less represented types, the molecules with 4LR are more abundant than those with 3LR, themselves more abundant than those with 2LR. When observed the mtDNA type with 6LR is more abundant than those with 7LR. This mtDNA pattern is repeatedly found in most of the rabbits examined. In the ovaries and testicles, molecules with more than 5LR are present with higher frequencies than in kidneys-spleen and liver, but molecules with less than 5LR are present with lower frequencies. [It is difficult to estimate the occurrence of mtDNA with 8LR and 9LR since if they exist they would overlap with partial digest of mtDNA-5LR (see MATERIALS AND METHODS for additional explanation).] (B) Lanes 1, 2, 3 = mtDNA extracted from kidneys-spleen, liver and ovaries of the female 2.6.8. Lanes 5, 6, 7 = mtDNA extracted from the kidneys-spleen, liver and testicles of the male D. Lane 4 = *Hin*fI digests of the Bluescript plasmid used as a marker.

From the above data we can estimate the effective number of mitochondria (N_e) and its confidence interval following the equation:

$$V_n = p_0(1 - p_0)(1 - (1 - 1/N_e)^n \qquad (1)$$

with

V_n = the variance at the nth generation;

V_n = 0.01 when calculated with the mean frequency of mtDNA-4LR observed for the offspring of the female 7 (0.43; 0.55; 0.29; 0.46; 0.35)

n = the number of cell divisions in the germ cell line of a female;

$n \approx 20$ for rabbit (CHRETIEN 1966)

p_0 = the mean frequency of mtDNA-4LR observed for the female 7 (0.37) and its offspring (0.43; 0.55; 0.29; 0.46; 0.35)

that is: $p_0 = 0.42$. From (1), we calculate $N_e = 500$, and, with a confidence interval for V: $0.0036 < V < 0.0833$ (prob. 0.95), the confidence interval for N_e is:

$$50 < N_e < 1370.$$

Consequences of genetic drift: Due to the existence of genetic drift through generations, a female exhibiting more than 90% of a given type of mtDNA molecules must generate at the first generation a majority of individuals also with a high percentage of this form and a few homoplasmic ones.

Indeed, the other families, all derived from females with more than 90% of mtDNA-5LR (females; 2, 4, 5, 6, 8, 9, 10, 2.5.9, 2.6.8, 2.8.3) appear quite homogeneous with a high percentage of mtDNA-5LR, but have no homoplasmic descendants. This point will be discussed later.

Sporadic individual cases, such as 2.6.2, 2.6.3, 10.8.1, M2 (APPENDIX), seem to escape this rule. They exhibit mtDNA types longer than mtDNA-5LR at a frequency much higher than the rest of their families. If genetic drift in the germ cell line is expected to favor the far most frequent type of molecules, it may sometimes increase, by chance, the frequency of very rare types. The fact that, in all cases, longer molecules are observed whatever the origin of the mtDNA (liver and kidneys-spleen) supports this proposal.

High mutation rate: However, genetic drift is insufficient to explain all the observations. First, no homoplasmic descendant was found. Indeed, if genetic drift was the only force acting through generations it would lead inevitably to homogeneity for each type of mtDNA. This is obviously not true, since every rabbit examined so far, from either domestic or wild populations, is heteroplasmic. More precisely, the frequency distribution of the mtDNA-4LR type in the kidneys-spleen of the descendants of the females 2, 4, 5 is shown on Figure 4. These frequencies are low in most of the cases (as for the others animals) but never equal zero.

The parameters of the distribution (mean, μ, and standard deviation, σ) of mtDNA-4LR frequencies observed in the kidneys-spleen are as follows: $\mu = 3.5\%$, $\sigma = 2.1$ (51 observations), $\mu = 5.3\%$, $\sigma = 4.2$ (22 observations) and $\mu = 8.5\%$, $\sigma = 6.5$ (34 observations), respectively, for the descendants of the females 2, 4 and 5. In the liver these parameters are: $\mu = 5\%$, $\sigma = 3.8$ (35 observations), $\mu = 4.6\%$, $\sigma = 2.3$ (21 observations) and $\mu = 9.7\%$, $\sigma = 6.4$ (34 observations), respectively, for the descendants of the females 2, 4 and 5. No frequency less than 1% has been observed (this is also true for all the other rabbits studied).

Second, except for the female 7 and its descendants, mtDNAs of all the others animals were characterized by a predominant type: mtDNA-5LR with some less represented other forms. Frequencies of the types shorter than mtDNA-5L regularly decrease with mtDNA-4LR > mtDNA-3LR > mtDNA-2LR. The stability of this hierarchy between frequencies and the absence of homoplasmic individuals argue against the sole action of genetic drift.

Usually mtDNA-6LR frequency is higher than mtDNA-7LR frequency. In some cases, it is difficult to decide on the occurrence at low frequency (>1%) of mtDNA with 8LR or 9LR. A fuzzy band of low intensity, due to a partial digest, is observed at the presumed location of mtDNA fragments with 8LR and 9LR (see MATERIALS AND METHODS).

The stability of such a distribution, despite some evidence of genetic drift in the germ cells, can only be explained by a high mutation rate responsible for the regeneration (at each generation) of the rare mtDNA types from mtDNA-5LR (at least in the organs examined: liver and kidneys-spleen). The combined action of the two forces, genetic drift and mutation at a very high rate, may maintain through generations rare mtDNA types which would be rapidly lost if only genetic drift was acting. The systematic heteroplasmy observed in rabbits is therefore the consequence of such a phenomenon: purification may never be achieved since new molecules are perpetually generated through mutation and eliminated, with a high probability, by genetic drift. A similar hypothesis has been also proposed to explain the maintenance of length heteroplasmy and the rapid loss of restriction site heteroplasmy in cows (HAUSWIRTH *et al.* 1984; HAUSWIRTH and LAIPIS 1985).

The mutation rate per rabbit generation, from mtDNA-5LR to a given other type in this somatic cell lineages can be estimated. It corresponds to the frequency of this rare type if we assume that mtDNA-5 LR is present at almost 100% in the oocyte. It varies between 5.10^{-2} and 10^{-2} depending on the type generated (mtDNA-4LR or -3LR). These values are similar to those (around 10^{-2}) calculated, by different approaches, for frogs (MONNEROT *et al.* 1984) and bats (WILKINSON *et al.* 1991).

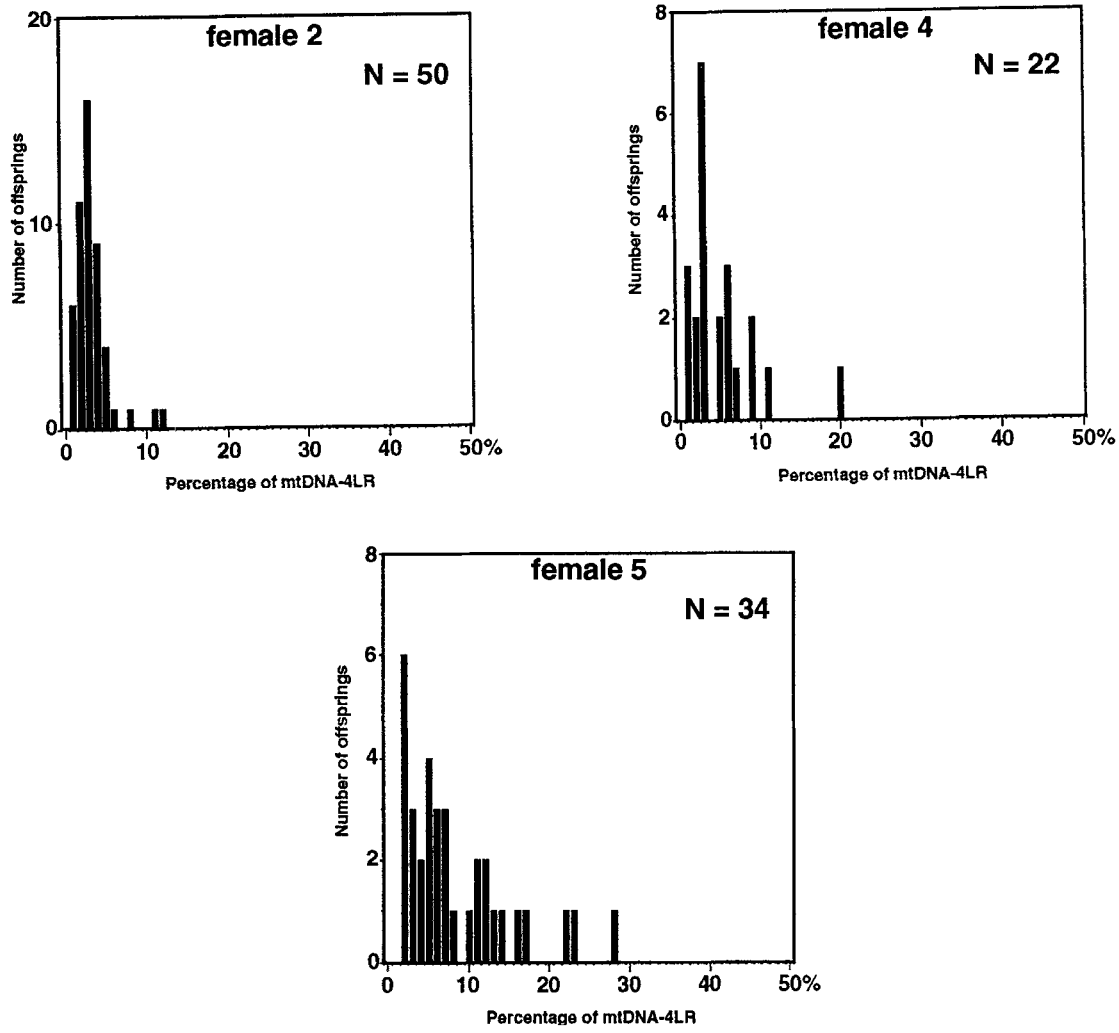

FIGURE 4.—Distribution of mtDNA-4LR frequencies observed in the kidneys-spleen of the descendants of the female 2, 4, 5. The mean frequency (μ) = 3.5%, standard deviation (σ) = 2.1 (51 observations), μ = 5.3%, σ = 4.2 (22 observations), and μ = 8.5%, σ = 6.5 (34 observations), respectively, for the descendants of the females 2, 4 and 5. No frequencies less than 1% have been observed (as for the others rabbits).

Selection: We wanted to know if the mtDNA types were selectively neutral. The analysis was done only for the mtDNA-5LR and mtDNA-4LR type which are represented at high enough frequencies to be estimated precisely.

When the frequency of mtDNA-4LR in the liver is plotted against that found in the kidneys-spleen, a good correlation between the values is observed (R^2 = 0.90), confirming a parallel evolution of this frequency in these two organs. More interestingly, the value of the regression coefficient: 0.742 indicates that the frequency of mtDNA-4LR in kidneys-spleen is, in a vast majority of the cases, lower than in the liver (Figure 5). These differences are significant, a Wilcoxon signed rank test gives z = 6.694, prob. = 0). Two possible explanations may account for this result. First possibility: the mutation rate of mtDNA-5LR to mtDNA-4LR is higher in the liver than in the kidneys-spleen, or for a same mutation rate the mtDNA turnover is higher in the liver than in the kidneys-spleen. Second possibility: the strength of selection for mtDNA-4LR *vs.* mtDNA-5LR is higher in liver than in kidneys-spleen. Both mechanisms can of course act simultaneously.

In the case of no selection and no mutation, the frequency variations of the mtDNA-4LR type observed in the two organs should be due solely to genetic drift, but the value of the regression coefficient should equal to 1.0 (R^2 depending on the importance of the genetic drift as for measurement errors).

We tried to detect the possible effect of selection for mtDNA-4LR against mtDNA-5LR by comparing the consequences of (1) variation in the mutation rate and (2) variations in the strength of selection, in liver and in kidneys-spleen based on the difference between the mtDNA-4LR frequencies observed in these two sets of tissues.

If mutation is the unique force that increases the frequency of mtDNA-4LR, with a higher mutation rate

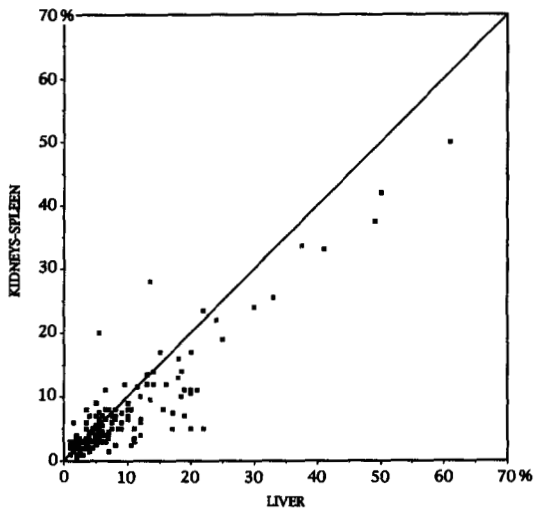

FIGURE 5.—Comparison of mtDNA-4LR frequencies in the liver and in the kidneys-spleen of all the rabbits analyzed (161 observations). A regression analysis gives: $Y = 0.74X + 0.4$, $R^2 = 0.90$, SD = 0.03 on the estimate of the regression coefficient. These values are clearly correlated but the frequency observed in the liver is higher than in the kidneys-spleen. See discussion for possible explanations.

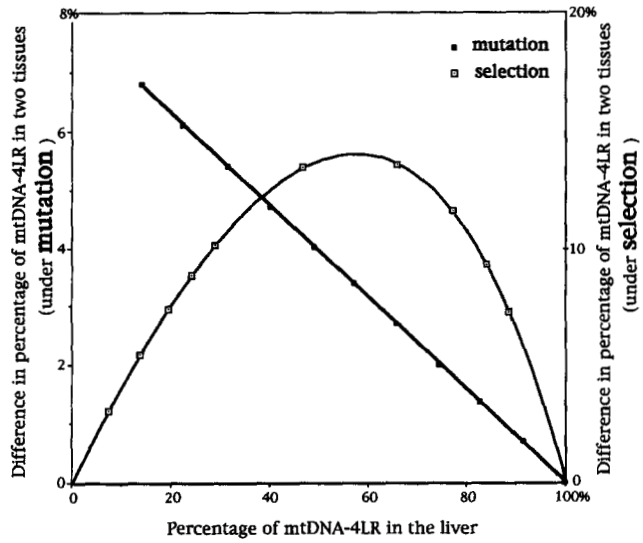

FIGURE 6.—Numerical illustration of the effect of unequal selection strengths or mutation rates in two organs on the difference between the observed frequencies of mtDNA-4LR. These differences are plotted against the frequencies of mtDNA-4LR observed in one organ (the liver). In this example, the number of generations (cycles of replication) is 30. In the case of selection, selection coefficients have been taken equal to 1.05 for mtDNA-4LR in the kidneys (1.0 for mtDNA-5LR) and equal to 1.07 for mtDNA-4LR in the liver (1.0 for mtDNA-5LR). For mutation, mutation rates of mtDNA-5LR to mtDNA-4LR were equal to 0.0025 in the kidneys and 0.005 in the liver.

from 5LR to 4LR in the liver than in kidneys-spleen, and a higher initial frequency of the 4LR variant, one expects less difference in the frequency of 4LR between liver and kidneys-spleen after a fixed period of mutation. This is due to the fact that a higher initial frequency of 4LR dictates a lower frequency of 5LR, which is presumed to be the "source" of new 4LR variants by mutation. But if we consider the variation of the selection strength for mtDNA-4LR against mtDNA-5LR as the reason for the frequency difference of mtDNA-4LR in liver and in kidneys-spleen, then the higher are these frequencies in the organs at a given age, the higher is the difference between the frequencies of mtDNA-4LR in the two organs. The effect of selection increase with the frequency of the type selected (to around 60% of mtDNA-4LR, after which this difference decreases). A numerical example is given in Figure 6 to illustrate this proposal. We applied this reasoning to our data. The differences observed between the mtDNA-4LR frequencies in the liver and in the kidneys-spleen were plotted against the frequencies of mtDNA-4LR observed in the liver (Figure 7) or in the kidneys-spleen (similar result not shown). We selected the values obtained for adult animals (more than 100 days old) and standardized the difference by accounting for the age of the animals by dividing the difference by the age. A significant correlation ($R^2 = 0.73$) betwen these data is observed. The slope of the line of best fit, positive and significantly different from zero, shows that the difference increases with the frequency in the liver (or in the kidneys-spleen). Consequently mutation is not the sole driving force and this implies some selection of mtDNA-4LR against mtDNA-5LR.

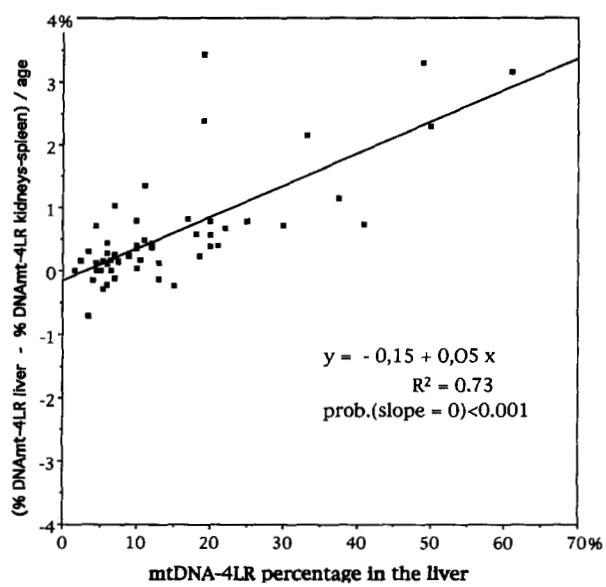

FIGURE 7.—Plot of the difference between the frequencies of mtDNA-4LR (liver–kidneys-spleen) corrected for the age of the animals (in months) against the frequencies of mtDNA-4LR in the liver. Data are obtained from adult rabbits (more than 100 days old). A regression analysis gives: $n = 52$, $Y = 0.05X - 0.151$, $R^2 = 0.73$, SD = 0.007 on the estimate of the regression coefficient.

Variation depending on tissues (gonads *vs.* others organs): For some individuals (4.4.9, 5.3.9, 5.4.9, 2.6.8, 2.8.3, JoM, Jo.14.1, Jo.14.2, Jo.15.1, Jo.15.2, D, E; see APPENDIX) the distributions of mtDNA type frequencies

for mtDNA extracted from ovaries or testicles have been compared with those for mtDNA extracted from liver and kidneys-spleen. All mtDNA studied exhibit a large majority of mtDNA-5LR, whatever their origin, but the relative proportions of the other forms differ. When mtDNA from kidneys-spleen or liver presents the hierarchy already described, a different distribution is observed in the testicles and ovaries of the adult animals: the molecules longer than mtDNA-5LR are more frequent than those shorter than 5LR (Figure 3, A and B). The absence of longer molecules than 6LR in the ovaries of four young females (Jo.14.1, Jo.14.2, Jo.15.1, Jo.15.2) descendants of the old female JoM for which we observe high frequencies of these types, argues for their regeneration during the development (APPENDIX).

The existence of differences in the type of mutations occurring (ovaries, testicles/liver, kidneys-spleen), and the selection in somatic cells (kidneys-spleen and liver) as a consequence of the differentiation state of cells remains however to be proven.

General conclusion: Considering the whole set of data, we observe that the type mtDNA-5LR is maintained as the predominant one in various, even unrelated mtDNA lineages. This suggests that neither high mutation rates nor intensive selection for shorter molecules at the long repeat stretch level are active in the germ cell line, whereas these two forces are active in the somatic cells. The biological significance of this difference has to be understood.

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APPENDIX

The frequencies of the different types of repeated stretches in mtDNA are shown in Table 1.

TABLE 1

Frequencies of the different types of repeated stretches in mtDNA

	Organ														
	Kidneys-spleen, no. of repeats							Liver, no. of repeats							
Animal	2	3	4	5	6	7	8	2	3	4	5	6	7	8	Age (days)
2		2	5	92	2	<1		1	2.5	9	87.5	≪1	≪1		511
2.6.1	<1	2	98	<1					<1	4	96	<1			79
2.6.2		<1	3	91.5	5.5				<1	4	87	9			79
2.6.3		<1	4	89	7					6.5	86.5	7			79
2.6.4		<1	5	95					3	8.5	88.5				79
2.6.5		<1	3	97	<1				<1	3	97	≪1			79
2.6.6		<1	3.5	94	2.5	≪1			<1	3.5	96.5				79
2.6.7			2	98						1	99	<1			79
2.8.1			3	97	≪1	≪1			1	4.5	94.5				352
2.8.2		<1	4	96	≪1	≪1			1.5	7	91.5				352
4		1.5	6.5	90	2	<1		<1	2	12	86	≪1	≪1		448
4.4.1		≪1	1.5	98.5	≪1				<1	4	96	≪1			49
4.4.2	≪1	<1	6	93	1	≪1			<1	5.5	94.5	≪1	≪1		49
4.4.3		1	5	86	3	5			1	3.5	93.5	1	2		49
4.4.4		<1	6	87.5	4.5	2			<1	9	90	1	<1		49
4.4.5		≪1	1	99					<1	2.5	97.5	<1			49
4.4.6		<1	5.5	92	2.5	<1			2	6	90.5	1.5	≪1		49
4.4.7		1	9	86	4	1					No data				49
4.4.8		<1	20	78	2	≪1				5.5	94.5				49
4.4.9		≪1	4	96	<1	≪1					No data				420
5				No data							No data				
5.5.1		<1	2.5	97.5	<1	<1			1	8	91	≪1	≪1		91
5.5.2		≪1	2.5	97.5	<1	<1				1	99				91
5.5.3		≪1	3	96	1	<1			1	5.5	93.5	≪1	≪1		91
5.5.4		≪1	10	90	<1	≪1			1	18.5	80.5	≪1	≪1		91
5.5.5		<1	5	95	<1	≪1			≪1	7.5	92.5	≪1	≪1		91
5.5.6		≪1	2	96	1	1			<1	1	99	≪1	≪1		91
5.3.9	<1	2	11	87	<1	<1		<1	3	20	77				476
5.4.9	<1	1	4	95	<1	<1		<1	1.5	6.5	92				434
6		<1	3	97	<1	≪1		<1	2	11	87	≪1	≪1		497
6.8.1		<1	6	94					3.5	12	84.5				91
6.8.2		1	14	85					3.5	18.5	78				91
6.8.3		<1	3	97					1	4	95				91
6.8.4		<1	2	98					<1	1	99				91
6.8.5		1.5	3	95.5					1	4.5	94.5				91
7			33	67				<1	3	41	56	≪1	≪1		329
7.4.1		<1	37.5	62.5	≪1	<1			1	49	50				105
7.4.2		2	50	48					1	61	38	<1			105
7.4.3		1	25.5	73.5					<1	33	67				105
7.4.4		<1	42	58	<1	<1			2	50	48				105
7.4.5		<1	33.5	66.5	<1	≪1			<1	37.5	62.5				105
8		<1	5	95				3	7	17	73	≪1	≪1		440
8.7.1		<1	7.5	90.5	2				<1	17	81.5	1.5			77
8.7.2		<1	4	96					<1	6	94				77
8.7.3		<1	4	96						2	98				77
8.7.4			1.5	98.5					<1	4	96				77
8.7.5			8	92						15.5	84.5				77
9		1	3	96	<1	≪1		<1	1	6	93	≪1	≪1		322
9.3.1		≪1	<1	100					≪1	<1	100				49
9.3.2		<1	3	97					<1	6	94				49
9.3.3		≪1	3	97					≪1	5.5	94.5				49
9.3.4		≪1	1	99					<1	3	97				49
9.3.5		≪1	7	93					<1	8	92				49
10		1	5	94	<1	<1		2	8	20	70	≪1	≪1		583
10.8.1		1	1	89	≪1	9			2	2	88	≪1	8		49
10.8.2		<1	3	97	≪1	≪1			<1	2.5	97.5				49
10.8.3		<1	3	97					<1	5	95				49

TABLE 1

Continued

Animal	Organ: Kidneys-spleen, no. of repeats 2	3	4	5	6	7	8	Liver, no. of repeats 2	3	4	5	6	7	8	Age (days)
10.8.4			1.5	98.5					<1	2.5	97.5				49
10.8.5			0.5	99.5					<1	2	98				49
JoM	≪1	1	10	86	3	<1		≪1	2	18.5	75.5	4	<1		1085
Jo.11.1			4	96	≪1					4	96	≪1			61
Jo.11.2		<1	2.5	95.5	2	≪1			<1	4	94	<1			61
Jo.11.3			1	99					<1	2	98				61
Jo.14.1		≪1	1	99					1.5	5.5	93				130
Jo.14.2		1	6.5	89.5	3	≪1			1.5	9	83.5	6			130
Jo.15.1		<1	7	91.5	1.5	≪1			<1	8.5	88.5	3			73
Jo.15.2		≪1	1.5	98.5	≪1	≪1			2	6	92	≪1			73
2.6.8	≪1	<1	4	91	5	<1		≪1	2	12	86	<1			549
2.6.8.2.1		<1	9.5	86.5	4				<1	13.5	83.5	3			87
2.6.8.2.2		<1	6.5	87.5	6				<1	8	86	6			87
2.6.8.2.3		<1	2.5	91.5	6				<1	5	89.5	5.5			87
2.6.8.2.4		<1	3	93.5	3.5				<1	2	94	4			87
2.6.8.2.5		<1	4	93	3				<1	4	95	1	≪1		87
2.8.3	<1	1	8	91	<1	≪1		<1	2	10.5	87.5				451
2.8.3.1.1		<1	8	92	<1	<1			<1	6.5	93.5	≪1	≪1		77
2.8.3.1.2		<1	3	97					<1	3	97				77
2.8.3.1.3		<1	4	96	≪1	≪1			<1	5	95	≪1			77
2.8.3.1.4		<1	2.5	97.5					<1	2	98	<1			77
2.8.3.1.5		<1	4.5	95.5						5.5	94.5	≪1			77
L1	<1	3	12	85	≪1	≪1		<1	4	13	83	≪1	≪1		251
M1	<1	1	8	91	≪1	≪1		<1	1.5	7	91.5	≪1	≪1		252
M2	<1	1.5	7	82.5	9	≪1			1.5	10	82.5	6	≪1		252
N1	<1	4	19	77	≪1	≪1		<1	8	25	67	≪1	≪1		231
N2	<1	1.5	7	91.5	≪1	≪1		<1	4	10	86	≪1	≪1		231
O1	<1	2	17	78	3	≪1		<1	2	15	83	≪1	≪1		254
O2	<1	1	24	71	4	≪1		<1	3.5	30	66.5	≪1	≪1		254
A		1	5	87	6	1		3.5	11.5	22	62	1	≪1		770
B		1.5	11	86.5	1	<1			3	21	76	≪1	≪1		749
C		2	10.5	88.5	1	1		1.5	10.5	20	68	≪1	≪1		763
D	<1	3	9	88	<1	<1		<1	2	10	88	≪1			910
E	<1	1	6.5	88.5	2	2		≪1	<1	6.5	93.5	≪1	≪1		903

Animal	Ovaries or testicles, no. of repeats 2	3	4	5	6	7	8	9	10	Age (days)
Male										
D		<1	<1	100	<1	<1	<1	<1		910
E		≪1	<1	96	<1	2	2	<1		903
Female										
449			<1	90.5	<1	3	6.5	<1		420
539		<1	4	91	1.5	2	1.5			476
549			1	89	2.5	2.5	4	1		434
2.6.8			1	88	6.5	1.5	2	1		549
2.8.3			1	98	1	<1				451
JoM		<1	1	74	5	7.5	9.5	3	<1	1085
Jo.14.1		<1	3.5	96.5						130
Jo.14.2			2.5	96	1.5					130
Jo.15.1			2	98						73
Jo.15.2			1	99						73