Evolution of Repeated Sequence Arrays in the D-Loop Region of Bat Mitochondrial DNA

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ABSTRACT

Analysis of mitochondrial DNA control region sequences from 41 species of bats representing 11 families revealed that repeated sequence arrays near the tRNA-Pro gene are present in all vespertilionine bats. Across 18 species tandem repeats varied in size from 78 to 85 bp and contained two to nine repeats. Heteroplasmy ranged from 15% to 63%. Fewer repeats among heteroplasmic than homoplasmic individuals in a species with up to nine repeats indicates selection may act against long arrays. A lower limit of two repeats and more repeats among heteroplasmic than homoplasmic individuals in two species with few repeats suggests length mutations are biased. Significant regressions of heteroplasmy, θ and π, on repeat number further suggest that repeat duplication rate increases with repeat number. Comparison of vespertilionine bat consensus repeats to mammal control region sequences revealed that tandem repeats of similar size, sequence and number also occur in shrews, cats and bighorn sheep. The presence of two conserved protein-binding sequences in all repeat units indicates that convergent evolution has occurred by duplication of functional units. We speculate that D-loop region tandem repeats may provide signal redundancy and a primitive repair mechanism in the event of somatic mutations to these binding sites.

ANIMAL mitochondria contain a circular 16-kb DNA molecule, encoding 13 protein, 22 transfer RNA (tRNA) and two ribosomal RNA genes (ANDERSON *et al.* 1981). The small size and compact organization of the mitochondrial DNA (mtDNA) genome has been suggested to be the result of selection for rapid organelle replication (HARRISON 1989; RAND 1993). However, recent discovery of length variation in the noncoding control region, which lies between the tRNA-Pro and tRNA-Phe genes, in a variety of vertebrate species (DENSMORE *et al.* 1985; MORITZ and BROWN 1987; BUROKER *et al.* 1990; HAYASAKA *et al.* 1991; WILKINSON and CHAPMAN 1991; ARNASON and RAND 1992; BROWN *et al.* 1992, 1996; HOELZEL *et al.* 1993, 1994; STEWART and BAKER 1994; XU and ARNASON 1994; YANG *et al.* 1994; CECCONI *et al.* 1995; PETRI *et al.* 1996; FUMAGALLI *et al.* 1996) is not consistent with this hypothesis and has not yet been adequately explained (WOLSTENHOLME 1992; RAND 1993). Because the proteins encoded by mtDNA genes play critical roles in oxidative metabolism and control region length might influence the rate of mtDNA transcription or replication (ANNEX and WILLIAMS 1990), the metabolic rate of the organism and possibly its survival could be affected by length variation.

Transcription of mitochondrial genes is initiated at two sites in the central, conserved portion of the control region (CHANG *et al.* 1985; KING and LOW 1987; CLAYTON 1992). Each strand of the mtDNA molecule, referred to as heavy (H) and light (L) based on differences in base composition, has different promoter regions that bind nuclear-coded proteins (GHIVIZZANI *et al.* 1993b; NASS 1995) and that differ in nucleotide sequence between species (KING and LOW 1987). Replication of the H-strand is primed by RNA transcribed between the L-strand promoter (LSP) and the H-strand origin of replication (O_H, CHANG and CLAYTON 1985). H-strand replication is usually terminated shortly thereafter at termination-associated sequences (TAS) resulting in short 7s DNA strands (DODA *et al.* 1981; CLAYTON 1991). 7s DNA strands remain associated with the L-strand and displace the original H-strand to create a three-stranded structure known as the displacement or D-loop. In mice, only 5% of replication events continue beyond the control region (BOGENHAGEN and CLAYTON 1978). Sequence-specific DNA binding proteins interact with TAS elements (MADSEN *et al.* 1993b) between two conserved regions, mt 5 (OHNO *et al.* 1991), which is also referred to as region J (KING and LOW 1987), and mt 6 (KUMAR *et al.* 1995). Initiation of replication of the L-strand occurs only when H-strand replication is two-thirds complete and the conserved O_L sequence, which in mammals lies between tRNA-Cy s and tRNA-Asn, is exposed (CLAYTON 1982).

While the function of the D-loop is not well under-

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stood (WOLSTENHOLME 1992), its structure and size are likely to influence mtDNA replication. A high proportion of triplex to duplex forms correlates with mtDNA copy number, mtRNA abundance and the rate of oxidative metabolism in different tissues (ANNEX and WILLIAMS 1990). The length of the 7s DNA strand, and therefore the size of the D-loop, varies depending on which TAS site is used for termination (DODA *et al.* 1981). Consequently, tandem repeats containing TAS elements should alter D-loop size. TAS elements occur within tandem repeats of evening bats (WILKINSON and CHAPMAN 1991), shrews (STEWART and BAKER 1994; FUMAGALLI *et al.* 1996), bighorn sheep (ZARDOYA *et al.* 1995), treefrogs (YANG *et al.* 1994), minnows (BROUGHTON and DOWLING 1994), sturgeon (BROWN *et al.* 1996), cod (ARNASON and RAND 1992; LEE *et al.* 1995), and seabass (CECCONI *et al.* 1995). Despite the distant taxonomic affiliations among these species, in most cases these R1 repeats (FUMAGALLI *et al.* 1996), Figure 1) are ~80 bp in length. In some fish and frogs the 80-bp repeat contains two or more smaller units. In several species, R1 repeats have been predicted to form thermodynamically stable secondary structures (BUROKER *et al.* 1990; WILKINSON and CHAPMAN 1991; STEWART and BAKER 1994; FUMAGALLI *et al.* 1996). R1 repeat duplications and deletions are thought to occur by competitive strand displacement among the three strands of the D-loop (BUROKER *et al.* 1990) resulting in a unidirectional mutational process (WILKINSON and CHAPMAN 1991).

Short, tandem repeats on the opposite side of the central conserved portion of the control region have also been reported in a variety of mammals including several carnivores (HOELZEL *et al.* 1994), pinnipeds (ARNASON *et al.* 1993; HOELZEL *et al.* 1993), pigs (GHIVIZZANI *et al.* 1993a), horses (ISHIDA *et al.* 1994; XU and ARNASON 1994), rabbits (MIGNOTTE *et al.* 1990; BIJU-DUVAL *et al.* 1991), shrews (FUMAGALLI *et al.* 1996), marsupials (JANKE *et al.* 1994) and bats (PETRI *et al.* 1996; E. PETIT, personal communication). These R2 repeats (FUMAGALLI *et al.* 1996) typically involve variable numbers of short 6- to 30-bp units, which often contain the 4-bp motif GTAC, and exhibit length variation similar to that described for nuclear microsatellite loci (CHARLESWORTH *et al.* 1994). Because these short repeats occur upstream from the origin of H-strand replication, they probably do not influence D-loop size. Consequently, their formation is more likely to be caused by replication slippage (LEVINSON and GUTMANN 1987; MADSEN *et al.* 1993a) than competitive strand displacement.

In this paper we present data on the presence and number of tandem R1 repeats among 41 species of bats representing most families in the order Chiroptera. By comparing sequence similarity between species with and without repeats we provide evidence for the evolutionary origin of R1 repeats in vespertilionine bats. We then compare the number of R1 repeats and heteroplasmy among seven species of vespertilionine bats in order to identify evolutionary processes that influence repeat array size. If the mutational process that gives rise to heteroplasmy is unbiased, we would expect homoplasmic and heteroplasmic individuals to have equal numbers of R1 repeats (BROWN *et al.* 1996). Deviations from this expectation indicate mutational bias or selection. Similarly, the proportion of heteroplasmic individuals is expected to be determined by a balance between mutation and organelle segregation (CLARK 1988; BIRKY *et al.* 1989) since paternal transmission is rare (HARRISON 1989; SKIBINSKI *et al.* 1994). Thus, variation in heteroplasmy should reflect variation in repeat mutation rate if the number of organelles per cell does not vary. Finally, we compare consensus sequences from vespertilionine bats with repeats to control region sequences of other mammals with and without repeats to determine if R1 repeated arrays have evolved multiple times in mammals and might influence organism function.

MATERIALS AND METHODS

Sampling locations: Bats were captured by netting at roosting and foraging sites in Europe, Malaysia, United States, Central America, South America, and Africa. *Nycticeius humeralis* were captured at six attic nursery colonies in Missouri and one in North Carolina (WILKINSON and CHAPMAN 1991). *Eptesicus fuscus* and *Myotis lucifugus* were captured in a single barn near the town of Princeton, Missouri. *Leptonycteris curasoae* and *L. nivalis* were captured in day roosts in Mexico; *Glossophaga soricina* was netted in Guanacaste, Costa Rica (WILKINSON and FLEMING 1996). Four species were captured in the Transvaal, South Africa: *Epomophorus crypturus* and *N. schleiffenii* were netted over streams near the town of Skukuzu in Kruger National Park while *Nycteris thebaica* and *Rhinolophus clivosus* were captured in a mine tunnel just south of Kruger National Park. Four species were also captured from a cave on Tamana Hill in Trinidad, West Indies: *Pteronotus parnelli, Mormoops megalophylla, Natalus tumidirostris* and *Phyllostomus hastatus. Saccopteryx bilineata* were captured at La Selva, Costa Rica. Six species were captured in peninsular Malaysia: *Hipposideros diadema, R. affinis, R. sedulus, Murina suilla, Nyctophilus gouldii* and *Kerivoula papillosa.* Seven species were collected in Germany: *Nyctalus noctula* in Brandenburg and Bavaria, and *E. nilssoni, M. myotis, M. bechsteini, Pipistrellus pipistrellus, P. nathusii* and *Vespertilio murinus* in Bavaria. Seven species were netted in Greece: *E. serotinus, N. leisleri, N. lasiopterus, Miniopterus schreibersi, P. kuhli, Tadarida teniotis* and *R. ferrumequinum.* Samples from *R. ferrumequinum* were obtained from Switzerland and Luxemburg.

DNA extraction, amplification and sequencing: A small piece of patagial membrane, ~10 mm^2, was excised from each individual with biopsy punches and stored either in a concentrated salt solution (SEUTIN *et al.* 1991) or 95% ethanol in the field. DNA was extracted from a tiny portion of each wing membrane sample using either Chelex (WALSH *et al.* 1991), a modified salting out procedure (MILLER *et al.* 1988) or a Qiagen DNA extraction kit following the manufacturer's protocol.

Control region mtDNA was amplified using two 22-bp primers, P and F (WILKINSON and CHAPMAN 1991). The P primer begins at position 15975 in the human proline tRNA gene (ANDERSON *et al.* 1981), while the F primer ends at position

16425 in a conserved sequence region found in the middle of the control region (Figure 1, (SOUTHERN *et al.* 1988). Double-stranded amplifications using PCR were performed as described in WILKINSON and CHAPMAN (1991) using AmpliTaq (Perkin Elmer) and 40 cycles of 95° for 1 min, 55° for 1.5 min, and 72° for 2 min in a Peltier thermal cycler. Amplification products were purified and concentrated using either ethanol precipitation or a silica gel-based method (GeneClean kit, QIAEX or Qiagen PCR-prep kit) following the manufacturers' instructions.

Double-stranded PCR products were sequenced by the dideoxy chain termination method using either γ^{35}S-ATP and Sequenase 2.0 (Amersham) or by cycle sequencing with ThermoSequenase (Amersham) using flourescent labeled primers and automated sequencers (LI-COR automated sequencer in Erlangen or an ABI automated sequencer at the Molecular Genetics Instrumentation Facility at the University of Georgia). Cycle sequencing was performed according to the manufacturer's protocol. A nested primer (*P** 5′-CCCCACCATCAACACCCAAAGCTGA-3′) was used to sequence PCR products generated with primers C and F (WILKINSON and CHAPMAN 1991) in a single direction for *S. bilineata, H. diadema, R. affinis, R. sedulus, R. ferrumequinum, T. teniotis, M. suilla, K. papillosa, M. schreibersi, E. nilssoni, E. serotinus, M. myotis, M. bechsteini, P. pipistrellus, P. kuhli, P. nathusii, V. murinus, N. leisleri, N. lasiopterus,* and *N. gouldii*. The three megadermatid sequences were provided by J. WORTHINGTON-WILMER. Control region sequence was obtained in both directions using both the P and F primers to initiate the sequencing reaction for the remaining 18 species.

R1 repeat estimation and comparison: The number of R1 repeats in the arrays of homoplasmic and heteroplasmic individuals was inferred by comparing PCR product sizes to a 100-bp ladder after agarose gel electrophoresis and ethidium bromide staining under UV. Expected repeat length was estimated from sequence information for each species. To test for differences in the frequencies of heteroplasmic and homoplasmic genotypes between species, we used contingency chi-square tests. We also used analysis of variance (ANOVA) to determine if the number of R1 repeats differed between heteroplasmic and homoplasmic individuals among the eight vespertilionid species for which the DNA of eight or more individuals was amplified. When determining repeat number we assumed that heteroplasmic individuals contained equal amounts of each repeat array detected on the gel.

Sequence comparison and analysis: All sequences were aligned with the help of the Higgins algorithm using the program MACDNASIS and were improved by subsequent manual alignment. When more than one individual of a species was sequenced, a consensus sequence was generated and then used for among species comparisons. In species having multiple R1 repeats, the flanking single copy region, as well as the first and last repeats (Figure 1), were aligned in a similar way. Throughout this paper we refer to the repeat nearest the central portion of the control region as the first repeat because it undergoes replication first. The last repeat refers to the repeat nearest the tRNA-Pro gene. Because prior studies of bats (WILKINSON 1992; WILKINSON and CHAPMAN 1991) with R1 tandem repeats have demonstrated that some process, such as competitive strand displacement (BUROKER *et al.* 1990), homogenizes repeat sequences in the middle of the array, we compared sequences between species with variable numbers of repeats by aligning all repeats between the first and last repeat to generate a single middle repeat consensus sequence. This method resulted in a consensus sequence for each of eight species of vespertilionine bats with repeats consisting of a first, middle and last repeat and flanking single copy sequences on each side of the repeats (Figure 2).

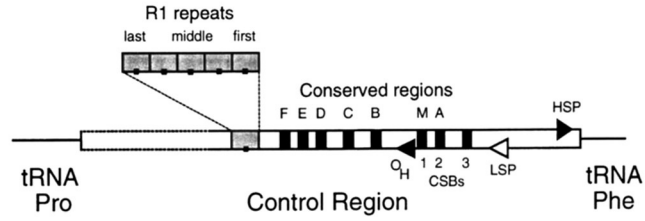

FIGURE 1.—Schematic organization of the bat mitochondrial control region. R1 repeated sequences (78–85 bp in length) in vespertilionine bats are located in the left domain of the control regon near the tRNA-Pro. Filled squares indicate termination-associated sequences (TAS) similar to those identified in humans, mice, and cows (DODA *et al.* 1981; KING and LOW 1987; MADSEN *et al.* 1993b). Conserved sequence areas in the central region (letters) and conserved sequence blocks (CSBs) are after SOUTHERN *et al.* (1988) and WALBERG and CLAYTON (1981), respectively. The origin of H-strand replication (O_H), the displacement loop (D-loop), and the L- and H-strand promotors (LSP, HSP) indicate approximate locations determined for human, cow, rat and mouse (CHANG *et al.* 1985; SACCONE *et al.* 1991).

To assess the possibility that mutation rate or population size may influence the number of R1 repeats, we used the last repeat in the array for seven vespertilionine species to estimate θ, the proportion of segregating nucleotide sites (WATTERSON 1975), and π, the heterozygosity per nucleotide site (NEI 1987). $\pi = \Sigma\, x_i x_j \pi_{ij}$, where x_i and x_j are the frequencies of the ith and jth type of sequences, respectively, and π_{ij} is the proportion of different nucleotides between the ith and jth type of sequence. Both of these statistics estimate the neutral parameter, L (NEI 1987), which for mitochondrial DNA is equal to $2N_{ef}\mu$ (RAND *et al.* 1994) at equilibrium where N_{ef} is the effective population size of females and μ is the rate of mutation. We compared sequences from the last repeat in the array because this repeat appears to be more conserved than any other repeat (see below) and can be more easily aligned between species, unlike the single copy sequence in the D-loop region, which differs markedly in length between species (Table 1). Because the sequence of the last repeat in the array could change as a consequence of a deletion event that removed the last repeat, θ and π are influenced both by the rate of nucleotide substitutions and by the rate of repeat duplication and deletion. In contrast, the proportion of heteroplasmic individuals is influenced only by the rate of repeat duplication and deletion.

To compare R1 repeat sequences between species with and without multiple R1 repeats we generated a consensus vespertilionine sequence for first, middle and last repeats using those eight species (*P. pipistrellus, M. bechsteini, M. lucifugus, M. adversus, N. humeralis, E. fuscus, N. gouldi, N. noctula*) for which the entire repeat region was sequenced (Figure 2). We then used the Lipman-Pearson algorithm and calculated the maximum percentage similarity between the three corresponding repeats in all other bats with multiple R1 repeats. To determine if repeat sequence similarity differs across repeats among bats with multiple R1 repeats we used the nonparametric Friedman test. For bat species without R1 repeats, we identified similar sequences to consensus first, middle and last vespertilionine repeat sequences by calculating maximum percentage similarities for all three repeats. To determine sequence similarity between bats and other mammals, we used the three vespertilionine consensus repeat sequences to search GenBank using the Blastn algorithm. We report maximum similarity values and, when available, the probability of obtaining such similarity by chance (ALTSCHUL *et al.* 1990).

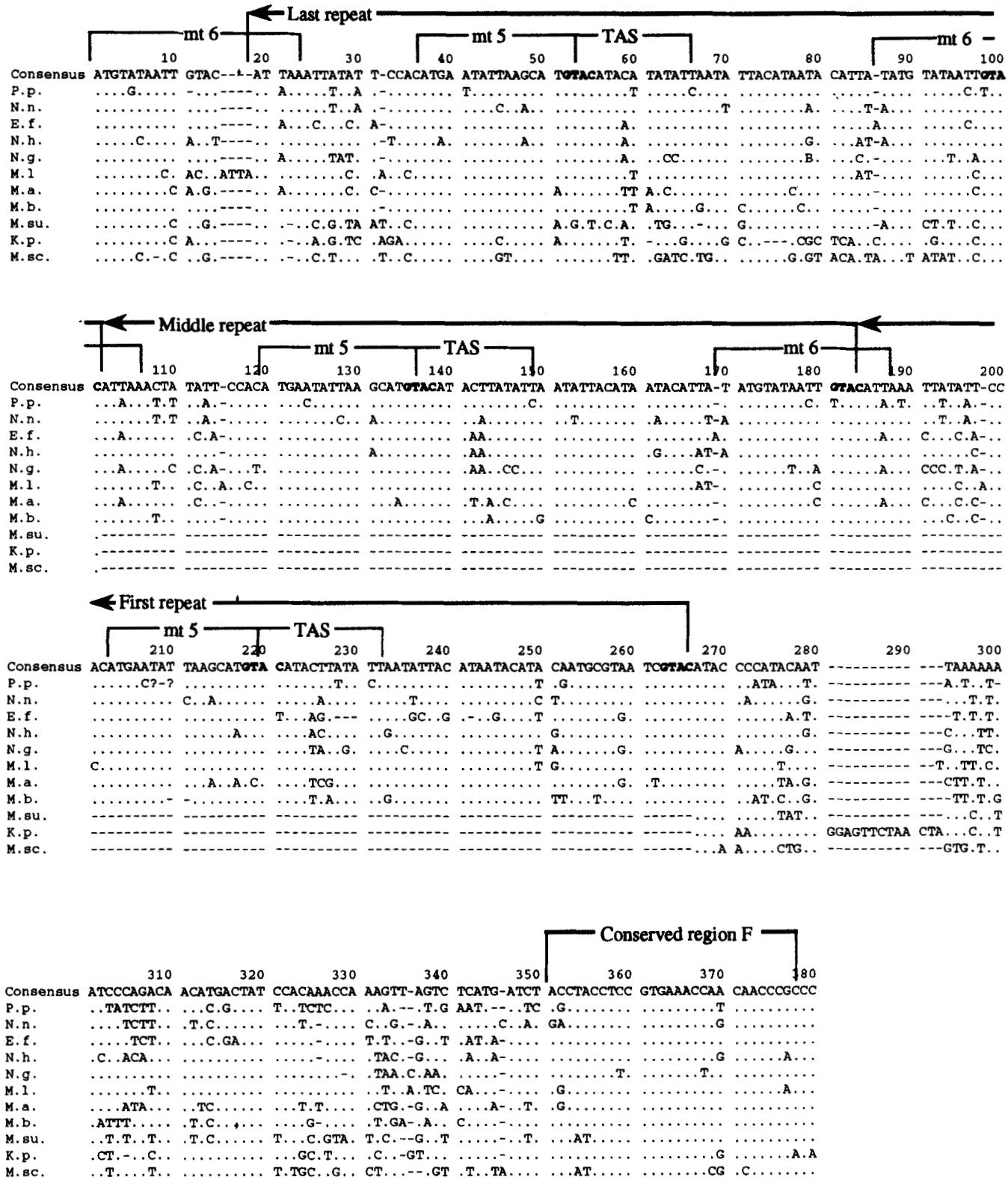

FIGURE 2.—Best alignment of consensus D-loop sequences for 11 vespertilionid species between the highly variable region nearest the tRNA-Pro gene and the end of conserved sequence block F. Only the consensus first, middle and last repeat are given for the eight species having multiple repeats (Nh, *N. humeralis;* Nn, *N. noctula;* Ef, *E. fuscus;* Ng, *N. gouldi;* Mb, *M. bechsteini;* Ma, *M. adversus;* Ml, *M. lucifugus;* Pp, *P. pipistrellus*). The most similar region in three species having no repeats (Msc, *M. schreibersi;* Msu, *M. suilla;* Kp, *K. papillosa*) is aligned to the last, most conserved repeat in the other eight species. mt 5 and mt 6 indicate conserved sequences identified in humans, cows, pig, rat and mouse. TAS, termination-associated sequences.

We tested for possible sequence convergence by comparing sequence similarities between genera with and without repeats using the nonparametric Mann-Whitney U test.

RESULTS

Variation in array length among bats: PCR amplification and sequencing of D-loop mtDNA from 41 species of bats in 11 different families reveals that tandem repeats occur only in 18 vespertilionid bats (Table 1), all of which are typically placed in the subfamily Vespertilioninae (HILL and HARRISON 1987; KOOPMAN 1993). Species from each of the other three vespertilionid subfamilies exhibit significant sequence similarity with the consensus repeat sequence (Table 1), but do not contain multiple R1 repeats. Among the vespertilionine bats sampled, the repeated sequence varies between 78 and 85 bp with most species exhibiting 81-bp repeats. The size of the repeat changes independently of phylogeny as length differences occur within three different genera, Myotis, Eptesicus and Nyctalus, that are placed in different tribes (HILL and HARRISON 1987; VOLLETH and HELLER 1994).

Comparison of repeat array lengths among eight species of vespertilionine bats reveals that the modal number of repeats varies from five in *M. bechsteini* and *N. schleiffenii* to eight in *M. lucifugus* (Figure 3). A nested analysis of variance shows that the mean number of R1 repeats per individual differs significantly among species within tribes ($F_{4,790} = 210.9$, $P < 0.0001$), but not among tribes ($F_{3,4} = 0.12$, $P = 0.94$). *Post hoc* Tukey tests indicate that the mean number of repeats differs between the three species of Myotis and between two Pipistrellini species (Figure 4a). Thus, mean number of R1 repeats also varies independently of phylogeny.

The proportion of individuals that were heteroplasmic differs between the eight species of vespertilionine bats (Table 2, contingency $\chi^2 = 57.0$, d.f. = 7, $P < 0.0001$), but shows no consistent phylogenetic pattern. Partitioning the contingency table among species within tribes reveals that much of this effect is caused by heteroplasmy differences among species of Myotis ($\chi^2 = 41.5$, $P < 0.001$) and between the two Nycticeiini species ($\chi^2 = 4.34$, $P = 0.037$). Only the two Pipistrellini species failed to show any difference in heteroplasmy frequency ($\chi^2 = 1.3$, $P = 0.26$, average heteroplasmy = 43.3%).

Selection and mutation of R1 repeats: Either selection or biased mutation could cause the number of R1 repeats to differ between heteroplasmic and homoplasmic individuals. A two-way ANOVA on the number of R1 repeats in heteroplasmic and homoplasmic individuals among species reveals a significant interaction between species and heteroplasmy ($F_{7,782} = 6.2$, $P < 0.0001$) as well as the significant effect of species noted above ($F_{7,782} = 83.9$, $P < 0.0001$), but no main effect of heteroplasmy ($F_{1,782} = 1.1$, $P = 0.29$). Single species contrasts between heteroplasmic and homoplasmic individuals indicate significant differences in three of four species with extreme repeat numbers, but not in four species with intermediate repeat numbers (Figure 4b). The two species with fewest R1 repeats exhibit more repeats among heteroplasmic than homoplasmic individuals (*N. schleiffenii:* $F_{1,6} = 7.7$, $P = 0.0055$; *M. bechsteini:* $F_{1,243} = 10.4$, $P = 0.0013$), while one of the two species with the highest number of R1 repeats exhibits fewer repeats among heteroplasmic than homoplasmic individuals (*M. lucifugus:* $F_{1,16} = 21.6$, $P < 0.0001$).

The proportion of heteroplasmic individuals should correlate with the rate of length mutation in the absence of paternal transmission and with similar patterns of selection (CLARK 1988). Thus, a significant regression between heteroplasmy and average repeat number would suggest that length mutation changes with number of R1 repeats. Even if the rate of length mutation is unbiased, a minimum number of repeats will exert a directional bias to the mutation process that should result in higher repeat numbers as mutation rates increase. A weighted least squares regression reveals that heteroplasmy increases additively with repeat number (Figure 5a, $R^2 = 0.73$, $F_{1,5} = 13.4$, $P = 0.015$). Significance of this regression was estimated by weighting each species by the square root of the proportion of individuals represented by that species in the data set (WILKINSON 1992).

Additional evidence for an effect of mutation rate on repeat number comes from comparison of repeat number to θ and π, with both statistics calculated from last repeat sequences. A weighted least squares regression of θ on repeat number exhibited a significant positive relationship (Figure 5b, $R^2 = 0.70$, $F_{1,5} = 11.7$, $P = 0.0187$) as did the weighted regression of π on repeat number (Figure 5c, $R^2 = 0.90$, $F_{1,5} = 43.7$, $P = 0.0012$).

R1 repeat sequence similarities among bats: To determine if repeat position influences the rate of sequence divergence, as would be expected if mutation rates differ at opposite ends of the array, we compared consensus first, middle, and last vespertilionine repeats (Figure 2) to other species. Significant differences in sequence similarity were detected when each of the consensus repeat sequences was compared to the corresponding repeat from other vespertilionine species ($\chi^2 = 7.8$, d.f. = 2, $P = 0.020$, Friedman Test). The last repeat showed the highest median similarity (87%) followed by the middle (85%) and first (84%) repeats (Table 1). Thus, among those bats containing multiple R1 repeats, the repeat furthest from the origin of H-strand replication (Figure 1) appears to be more highly conserved.

In contrast, when each of the three consensus vespertilionine repeats are aligned to maximize similarity to non-vespertilionine bat sequences, a single repeat is detected, but percentage similarity does not differ among repeats ($\chi^2 = 4.3$, d.f. = 2, $P = 0.115$, Friedman Test). Maximum similarities for an 81-bp region ranged from

TABLE 1

Distribution of R1 tandem repeats in the Chiroptera

Family, subfamily, and species	n^a	Base pairs from tRNA-Pro to repeats	No. of repeats	Repeat size	Maximum repeat similarity[b]
Pteropodidae					
Epomophorus crypturus	2	55	1	—	64
Emballonuridae					
Saccopteryx bilineata	1	114	1	—	75
Nycteridae					
Nycteris thebaica	3	69	1	—	73
Megadermatidae					
Megaderma gigas	6	26	1	—	61
Megaderma spasma	2	24	1	—	57
Megaderma lyra	2	22	1	—	62
Rhinolophidae					
Rhinolophus clivosus	1	106	1	—	57
Rhinolophus ferrumequinum	11	106	1	—	70
Rhinolophus sedulus	4	106	1	—	74
Rhinolophus affinis	1	106	1	—	61
Hipposideridae					
Hipposiderus diadema	1	106	1	—	62
Mormoopidae					
Pteronotus parnelli	3	90	1	—	59
Mormoops megalophylla	17	102	1	—	63
Phyllostomidae					
Phyllostomus hastatus	5	29	1	—	65
Glossophaga soricina	1	24	1	—	68
Leptonycteris nivalis	4	24	1	—	68
Leptonycteris curasoae	49	24	1	—	68
Mollossidae					
Molossus molossus	2	29	1	—	77
Tadarida teniotis	1	41	1	—	72
Natalidae					
Natalus tumidirostris	20	31	1	—	78
Vespertilionidae					
Kerivoulinae					
Kerivoula papillosa	1	226	1	—	74
Murinae					
Murina suilla	2	100	1	—	77
Miniopterinae					
Miniopteros schreibersi	1	52	1	—	75
Vespertilioninae					
Barbastella barbastellus	1	98	2	85	82
Myotis myotis	191	155	3–7	82	89
*Myotis bechsteini**	245	203	3–7	81	93
*Myotis adversus**	4	162	4	81	89
*Myotis lucifugus**	19	31	5–9	82	96
*Nycticeius humeralis**	195	71	5–8	81	93
Nycticeinops schleiffenii	8	63	4–7	80	74
*Eptesicus fuscus**	20	67	5–6	82	94
Eptesicus nilssoni	2	80	3–4	81	86
Eptesicus serotinus	1	79	4	81	88
*Nyctophilus gouldi**	1	36	3	81	85
Vespertilio murinus	1	66	8	78	82
*Pipistrellus pipistrellus**	8	44	5–9	81	91
Pipistrellus nathusii	1	44	8	81	91
Pipistrellus kuhli	1	92	6	81	93
*Nyctalus noctula**	112	121	4–9	81	88
Nyctalus lasiopterus	1	139	6	81	86
Nyctalus leisleri	1	141	7	83	89

GenBank accession numbers for sequences reported in this table are U95318–U95355.

[a] Number of individuals scored for R1 repeat number.

[b] Maximum percentage similarity calculated using the Lipman-Pearson algorithm between each species D-loop sequence and three consensus sequence arrays estimated from eight vespertilionid species (*) as illustrated in Figure 2. All similarity percentages are adjusted to match an 81-bp sequence.

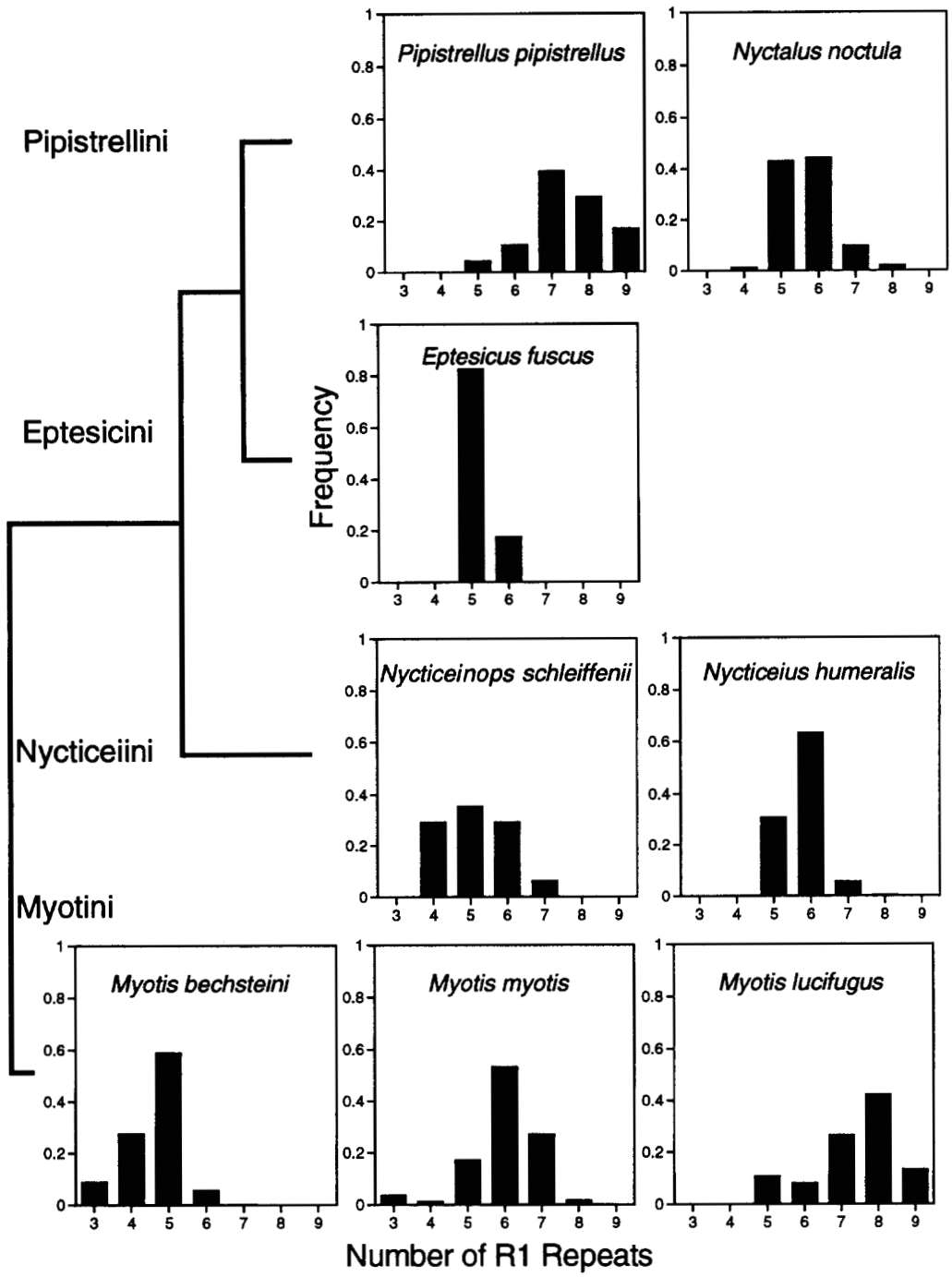

FIGURE 3.—Frequency of R1 repeats in eight vespertilionine species. Heteroplasmic individuals were counted as contributing equally to each repeat class. Phylogenetic relationships among four tribes (VOLLETH and HELLER 1994) are indicated by a cladogram.

57% to 77% (Table 1). When sequences of vespertilionids with and without multiple R1 repeats are aligned (Figure 2), three conserved regions, previously identified in the D-loop of other mammals as TAS (DODA *et al.* 1981), mt 5 (OHNO *et al.* 1991) and mt 6 (KUMAR *et al.* 1995), can be recognized within every repeat of each species. As has been reported for other mammals, some sequence differences occur among species in the TAS (Figure 2). Furthermore, in almost all species both the 5′ H-strand end and the middle of each repeated sequence contain the 4-bp palindrome GTAC (Figure 2).

With the exception of a 4-bp insertion in *M. lucifugus*, all bat species with R1 repeats also exhibit a highly conserved 14-bp partial repeat after the last repeat (Figure 2). Subsequent sequence between this partial repeat and the tRNA-Pro gene is difficult to align between species and exhibits considerable length variation, *e.g.*, from 36 bp in *N. gouldi* to 203 bp in *M. bechsteini* (Table 1). Similar length variation in this end of the control region also occurs in bats without multiple repeats (Table 1). Thus, the amount of single copy DNA in the left domain of the control region does not correlate with the number of R1 repeats. A partial repeat is not evident at the opposite end of the array. While the amount of

TABLE 2

Frequencies of R1 repeats for eight vespertilionine species

Species	f(3)	f(4)	f(5)	f(6)	f(7)	f(8)	f(9)	f(345)	f(45)	f(456)
Pipistrellus pipistrellus					2	1				
Nyctalus noctula			30	31	4				2	1
Nycticeius humeralis			40	97	3					
Nycticeinops schleiffenii		2	1							1
Eptesicus fuscus			15	2						
Myotis myotis			12	64	25	1		2	2	2
Myotis lucifugus					4	7	2			
Myotis bechsteini	21	53	122	5					27	1

Species	f(56)	f(57)	f(567)	f(67)	f(678)	f(78)	f(789)	f(89)	Prop. het.
Pipistrellus pipistrellus			1	1			1	2	0.63
Nyctalus noctula	27		10	2	1	4			0.42
Nycticeius humeralis	38		3	12		2			0.28
Nycticeinops schleiffenii	3			1					0.63
Eptesicus fuscus	3								0.15
Myotis myotis	24	2	16	37	2	2			0.47
Myotis lucifugus	3	1				1		1	0.32
Myotis bechsteini	15		1						0.18

f(45), number of individuals that are heteroplasmic for four and five repeats; f(5), number of individuals that are homoplasmic for five repeats. Prop. het, proportion of individuals heteroplasmic.

sequence between CSB-F and the first repeat is similar among vespertilionid species, with the exception of a 14-bp insertion in *K. papillosa,* little sequence conservation is apparent in this 75- to 85-bp region (Figure 2).

R1 repeat sequence similarities between bats and other mammals: A search of GenBank using the consensus vespertilionine first, middle and last repeats uncovered mtDNA sequences from nine additional orders of mammals with significantly similar sequences in the control region (Table 3). Examination of these sequences revealed the presence of R1 repeats between 74 and 80 bp in length in three additional orders: Insectivora, two genera and several species of shrews (STEWART and BAKER 1994; FUMAGALLI *et al.* 1996); Carnivora, domestic cat (LOPEZ *et al.* 1996) and mountain lion (M. CULVER, personal communication); and Artiodactyla, bighorn sheep (ZARDOYA *et al.* 1995). The maximum sequence similarity between any of the three consensus vespertilionine repeats and each mammal genus, excluding all vespertilionid genera, differs between orders ($H = 18.4$, d.f. $= 9$, $P = 0.031$, Kruskal-Wallis Test) and between genera with and without R1 repeats ($Z = 2.71$, $P = 0.0066$, Mann-Whitney U Test). The median maximum sequence similarity between vespertilionine repeats and other mammal genera with repeats is 79% ($n = 5$) and without repeats is 68.5% ($n = 38$). Although our sample of genera is not without phylogenetic bias, with the exception of bighorn sheep, which are in the order exhibiting the highest sequence similarity to the vespertilionine repeats, evolution of R1 repeats in mammals appears to involve sequence convergence. Sequence comparisons of species with and without repeats reveals that the three conserved sequence regions—mt 5, mt 6 and TAS—occur in the same order and relative position among repeats, even though their location within a repeat varies between orders (Figure 6).

DISCUSSION

Processes influencing the number of R1 repeats: The number of R1 repeats is not strongly influenced by historical factors because the modal number of R1 repeats varies extensively among closely related species, *e.g.,* Myotis. The evidence presented here is more consistent with repeat array length within a species being determined by a balance between selection and mutation. Selection is implicated both by the limited distribution of repeats within a species and by comparison of repeat number among heteroplasmic and homoplasmic individuals. A relatively ancient origin of R1 repeats in vespertilionine bats (see below) and a high rate of length mutation (WILKINSON and CHAPMAN 1991) should result in extensive variation in repeat number among species in the absence of stabilizing selection (unpublished simulation results). In contrast, R1 repeats in vespertilionine bats contain between two and nine repeats with every species exhibiting a unimodal distribution of repeats (Figure 3). Furthermore, fewer R1 repeats among heteroplasmic than homoplasmic *M. lucifigus,* one of the two species with high median repeat number, indicates that mitochondria with more than nine tandem repeats are at some selective disadvantage. Whether *P. pipistrellus,* the other species with high repeat numbers, actually differs from this pattern cannot be determined with confidence due to small sample size.

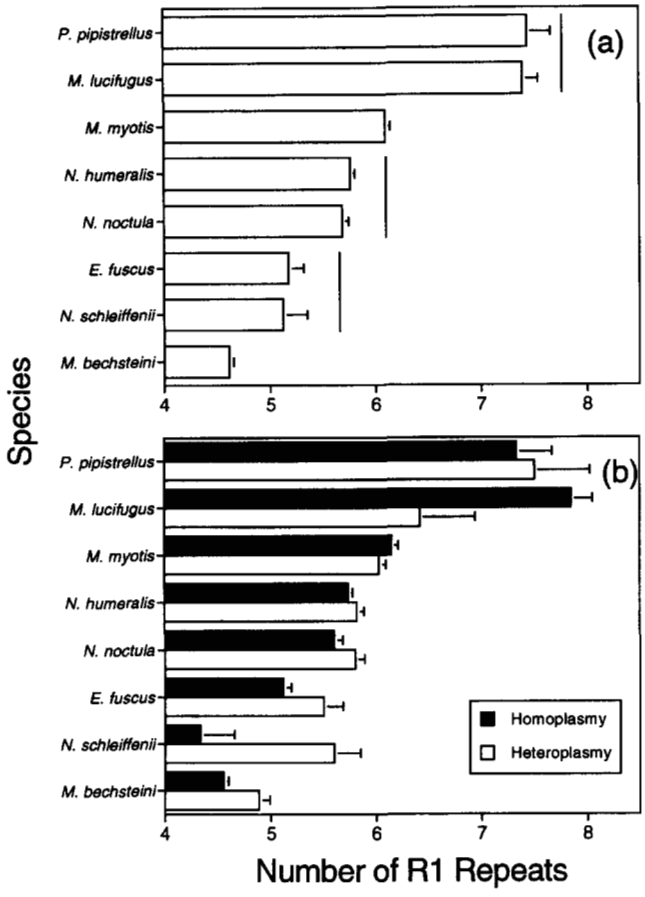

FIGURE 4.—(a) Mean (±SE) number of repeats for each of the eight vespertilionine species illustrated in Figure 3. Means that do not differ at the 5% level according to *post hoc* Tukey comparisons are connected by horizontal lines. (b) Average (±SE) number of repeats for heteroplasmic and homoplasmic individuals from each species.

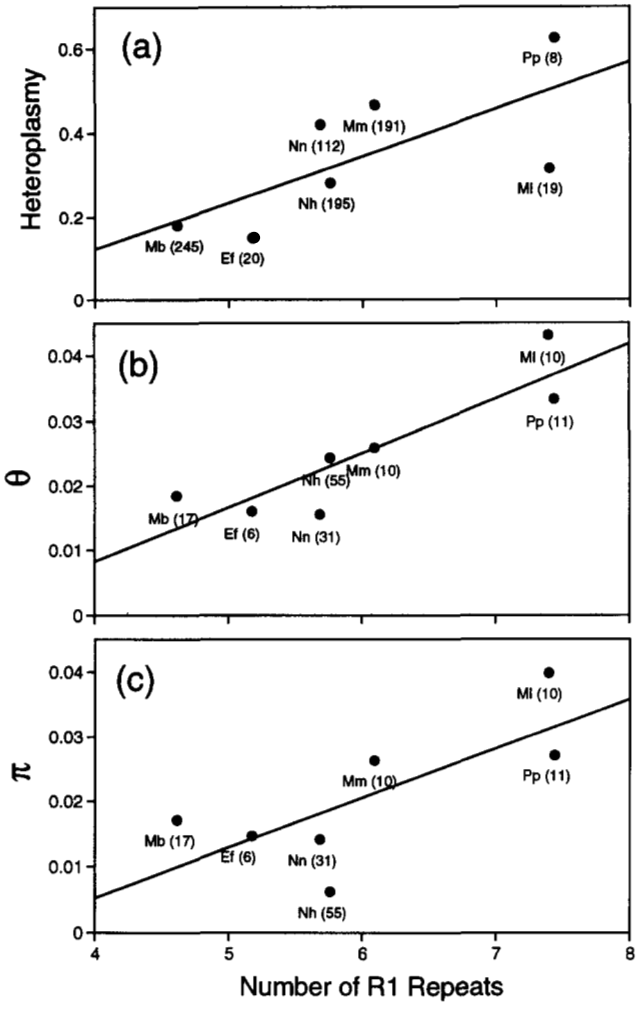

FIGURE 5.—(a) Least squares regression of proportion of heteroplasmic individuals on mean number of R1 repeats estimated from PCR product lengths for seven vespertilionine species (Nh, *N. humeralis;* Nn, *N. noctula;* Ef, *E. fuscus;* Mb, *M. bechsteini;* Mm, *M. myotis;* Ml, *M. lucifugus;* Pp, *P. pipistrellus*). Sample size is indicated in parentheses for each species. (b) Least squares regression of proportion of segregating sites, θ, estimated from the last repeat nearest the tRNA-Pro on mean number of R1 repeats. (c) Least squares regression of π on mean number of R1 repeats. The number of individuals sequenced is indicated in parentheses in b and c for each the seven species.

Two repeats appear to represent the lower limit to R1 repeat number in bats with multiple repeats (Table 1). Such a limit will cause biased length mutation toward increasing repeat number because duplication events will be more common than deletion events among individuals with two repeats. A lower limit to repeat number does not, by itself, predict the significant positive regressions observed between heteroplasmy, θ or π, and repeat number. These results are, however, consistent with a fixed probability that any repeat in an array will fold and either be duplicated or deleted during replication. Such a process would cause length mutation rates to increase additively with repeat number. Additional data are needed to determine if the regression of θ and π on number of repeats are also influenced by variation in population size.

BROWN *et al.* (1996) recently proposed a biochemical mechanism for how selection operates against mtDNA genomes containing multiple R1 repeats. If protein binding to conserved TAS sequences halts initiation of H-strand synthesis (MADSEN *et al.* 1993b), then multiple TAS sequences would be more likely to bind replication termination proteins. Assuming that 5% of D-loop strands lead to complete H-strand replication (BOGENHAGEN and CLAYTON 1978), the probability of H-strand replication should equal p^n where p is the proportion of D-loop strands initiating replication and n is the number of repeats with TAS elements (BROWN *et al.* 1996). Thus, D-loop strands from genomes with high numbers of repeats should rarely lack bound protein and consequently should be outreplicated by genomes containing few repeats in heteroplasmic individuals. This process should lead to a distribution of repeat numbers that is strongly skewed toward a single repeat (BROWN *et al.* 1996). Unfortunately, this mechanism, as described, does not account for variation in the number of R1

TABLE 3

Vespertilionine bat consensus repeat similarity to other mammals

Order, species	Common name	Maximum similarity[a]	Probability	Repeat no.	Repeat size
Artiodactyla					
Alces alces	Moose	69		1	
Bison bison	Bison	83	1.1 e-06	1	
Bos taurus	Cow	79	2.0 e-07	1	
Cervus elaphus	Red deer	77	3.1 e-05	1	
Cervus nippon	Sika deer	73		1	
Odocoileus hemionus	Mule deer	74		1	
Odocoileus virginiana	White-tailed deer	72		1	
Ovis canadensis	Bighorn sheep	75		4	74
Sus scrofa	Pig	80	1.8 e-07	1	
Carnivora					
Canis familiaris	Dog	67		1	
Felis catus	Cat	82	6.1 e-12	4	80
Puma concolor	Mountain lion	79		4–9	80
Cetacea					
Balaenoptera physalus	Fin whale	77	2.1 e-06	1	
Cephalorhynchus hectori	Southern dolphin	74	4.0 e-05	1	
Delphinus delphis	Common dolphin	73	4.1 e-05	1	
Globicephala melas	Pilot whale	73	9.5 e-04	1	
Megaptera novaeangliae	Humpback whale	74	2.2 e-06	1	
Phoecena phoecena	Common porpoise	72	0.02	1	
Tursiops truncatus	Bottle-nosed dolphin	73	9.5 e-04	1	
Insectivora					
Erinaceus europeus	Hedgehog	63		1	
Crocidura russula	White-toothed shrew	74	5.6 e-04	2–9	78
Sorex araneus	European shrew	80	4.9 e-10	5–6	78
Sorex cinereus	Shrew	81	1.4 e-13	5–7	79
Sorex haydeni	Shrew	80	2.0 e-05	5	79
Sorex hoyi	Pygmy shrew	80	3.4 e-11	5	79
Marsupialia					
Didelphis virginiana	Common oppossum	65		1	
Perissodactyla					
Diceros bicornis	Black rhinoceros	61	0.008	1	
Equus caballus	Horse	79	1.1 e-08	1	
Pinnipedia					
Arctocephalus forsteri	New Zealand fur seal	62		1	
Halichoerus grypus	Grey seal	62		1	
Mirounga angurostris	No. elephant seal	65		1	
Primate					
Homo sapiens	Human	54		1	
Rodentia					
Clethrionomys rufocanus	Bank vole	63		1	
Mus musculus	House mouse	65		1	

[a] Maximum sequence similarity percentages are relative to an 81-bp sequence.

repeats in vespertilionine bats (Figure 3). Even ignoring that the minimal repeat number in vespertilionine bats is two rather than one, neither individuals with eight repeats nor repeat distributions skewed toward larger repeat number, as occur in *M. lucifugus*, would be predicted (Figure 3). However, if p were to increase with repeat number, perhaps because a slightly larger D-loop somehow facilitates replication initiation, then p^n need not be maximal at $n = 1$. Furthermore, a positive relationship between p and repeat number would be consistent with the rate of repeat duplication and deletion increasing with repeat number noted above. Comparison of replication rates in mtDNA genomes differing in R1 repeat number, such as occur in vespertilionine bats, is clearly needed to test these ideas.

Origin and evolution of R1 repeats: We found multiple R1 repeats in all species of vespertilionine bats, but detected only a single R1 sequence in the three other vespertilionid subfamilies: Murinae, Miniopterinae and Kerivoulinae. Phylogenetic reconstruction of genera in these subfamilies based on chromosomal characters suggests that vespertilionine species with R1 repeats are

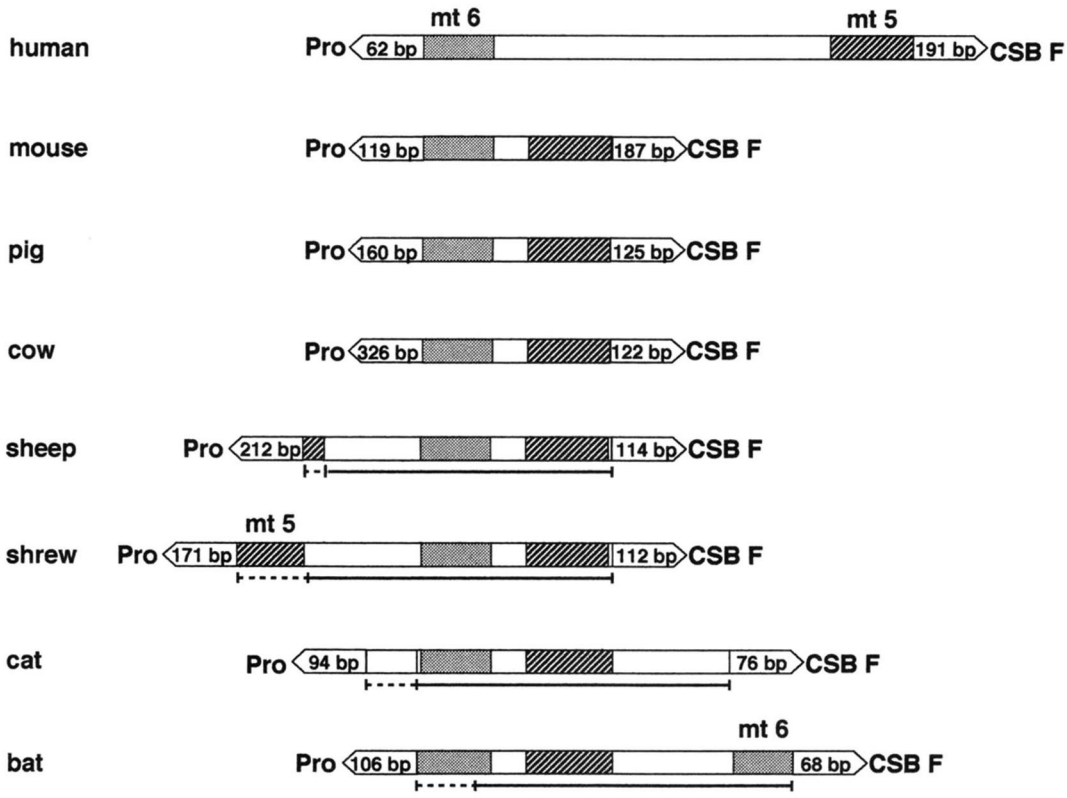

FIGURE 6.—Location of conserved elements, mt 5 and mt 6, within the left domain of the mammalian mitochondrial control region. For species having multiple R1 repeats a single repeat is shown and underlined (——, for whole repeat; ---, partial repeat). The figure is based on sequences from human (ANDERSON *et al.* 1981), mouse (PRAGER *et al.* 1993), pig (MACKAY *et al.* 1986), cow (ANDERSON *et al.* 1982), bighorn sheep (ZARDOYA *et al.* 1995), shrew, *Crocidura russula* (FUMAGALLI *et al.* 1996), cat (LOPEZ *et al.* 1996) and bat, *N. noctula* (this study).

monophyletic (VOLLETH and HELLER 1994). Unfortunately, the characters used by VOLLETH and HELLER (1994) do not contain sufficient information to resolve the placement of the genus Myotis either within or outside a vespertilionine clade. Nevertheless, recent phylogenetic analysis using 803 bp of ND1 mitochondrial DNA sequence for 15 vespertilionid species (F. MAYER, unpublished data) confirms that Myotis is the sister genus to a monophyletic clade containing all other vespertilionine genera used by VOLLETH and HELLER (1994). The three remaining vespertilionid subfamilies join basal to Myotis in this analysis. Thus, current evidence strongly supports monophyly of R1 repeat arrays in bats.

In contrast, the presence of multiple R1 repeats with similar sequences in vespertilionine bats, shrews, cats and bighorn sheep suggests recurrent evolution of repeat arrays in mammals. The alternative hypothesis of R1 repeat array loss in most daughter taxa of a common ancestor to vespertilionine bats, shrews, cats and sheep is unlikely for two reasons. R1 repeat sequences among vespertilionine bats, shrews and cats have converged, not diverged, with phylogenetic distance. Furthermore, we found no evidence that multiple R1 repeats have ever been been lost in any species of vespertilionine bat, cat (M. CULVER, personal communication) or shrew, where sequences for several related species have been examined.

The presence of three conserved sequence elements—TAS (DODA *et al.* 1981), mt 5 (OHNO *et al.* 1991) and mt 6 (KUMAR *et al.* 1995)—in all cases of bat R1 repeats and in all mammalian control regions we examined further suggests that R1 repeats arose from sequence duplication of functional units within the mitochondrial genome rather than from recent genetic exchange between the mitochondrial and nuclear genomes as has recently been noted for other taxa (LOPEZ *et al.* 1996; SORENSON and FLEISCHER 1996). Although the function of these sequence elements in regulating mtDNA replication is unclear, at least two different nuclear-coded proteins have been identified that bind to these elements (MADSEN *et al.* 1993b; KUMAR *et al.* 1995). Furthermore, while the sequence of the repeated unit differs in vespertilionine bats, shrews, cats, and bighorn sheep, the order and spacing of the mt 5, mt 6 and TAS sequence elements in two repeats is identical in these and other mammalian species except humans (Figure 6). The order of these conserved elements may be critical for forming stable secondary structures. Although R1 repeats from fish, shrews and vespertilionine bats differ in sequence, each have been predicted to form stable secondary structures with remarkably similar size and shape (BUROKER *et al.* 1990; WILKINSON and CHAPMAN 1991; STEWART and BAKER 1994; PETRI *et al.* 1996). These observations suggest that successful protein binding in this part of the control region probably involves similar secondary structures in all vertebrates. Greater sequence similarities between the last repeats in the array within a species (WILKINSON and CHAPMAN 1991), as well as among different vespertilionine species, further suggest that the last repeat may be the most important functional unit in the array.

Initial duplication of an R1 repeating unit may have

occurred through a modification of the competitive strand displacement model (BUROKER *et al.* 1990) in which a partial repeat near the tRNA-Pro gene participated in duplication. If the proto-repeat folded into a stem-loop structure during replication, then the partial repeat sequence could anchor the new H-strand to the beginning of the repeat on the L-strand, thereby yielding a duplication. Although the beginning and ending point of the repeat unit was defined differently in vespertilionine bats and shrews (WILKINSON and CHAPMAN 1991; STEWART and BAKER 1994; FUMAGALLI *et al.* 1996), if sequences are aligned in the direction that replication occurs, a partial repeat of similar length can be identified in bats, shrews, cats and bighorn sheep after the last repeat (Figure 6). A partial repeat is also found in the closest relatives (Miniopterinae, Kerivoulinae and Murininae) of those bats having multiple R1 repeats (Figure 3).

Possible selection on R1 repeats: R1 repeat sequence, size and number convergence between vespertilionine bats, shrews, cats and bighorn sheep, as well as the absence of array loss, suggest that multiple R1 repeats may provide some selective advantage, rather than just represent an example of selfish replicating elements. Exactly how selection operates, however, is unclear because R1 repeat sequences may undergo selection at multiple levels due to competition among mitochondria within individuals, as well as competition among individuals with potentially different metabolic abilities. To the extent that successful organelle transmission depends on replication rate, larger organelle genomes containing many R1 repeats should be at a selective disadvantage compared to smaller genomes within an individual.

In contrast, selection among individuals may favor an increase in R1 repeat numbers for at least two reasons. One possibility is that multiple R1 repeats could compensate for deleterious mutations during the lifetime of an individual. Mitochondrial DNA is well known for its high mutation rate (BROWN 1985) and lack of repair mechanisms (WOLSTENHOLME 1992). Multiple R1 repeats may provide a redundant signal if a mutation in one repeat alters the binding ability of a regulatory protein. Alternatively, concerted evolution caused by repeat duplication and deletion could eliminate damaged repeat sequences. If either process occurred during the lifetime of the animal, then multiple repeats might increase longevity. Some effect on longevity seems likely because the rate of deletions and point substitutions in the mtDNA genome increases with age in humans (BAUMER *et al.* 1994; LEE *et al.* 1994; KADENBACH *et al.* 1995) and mice (TANHAUSER and LAIPIS 1995).

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