Heteroplasmy and Polymorphism in the Major Noncoding Region of Mitochondrial DNA in Japanese Monkeys: Association with Tandemly Repeated Sequences[1]

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We have sequenced the major noncoding region of mitochondrial DNA (mtDNA) of four Japanese monkeys and have found length polymorphism in the sequenced region. The length polymorphism resulted from two tandem duplications of 160-bp sequences which contained the conserved sequence blocks 2 and 3 and the light-strand transcription-promoter region. We also found polymorphisms in this mtDNA region among 100 Japanese monkeys from 12 localities, 90 of which were analyzed using DNA amplified through the polymerase chain reaction. In two localities, we found individuals with heteroplasmic mtDNAs which had different numbers of the 160-bp repeats mentioned above. The 100 samples were classified into six types in terms of length and presence/absence of the recognition site of two restriction enzymes in the major noncoding region.

Introduction

Our previous restriction-enzyme analysis of mitochondrial DNA (mtDNA) derived from 10 Japanese monkeys (*Macaca fuscata*) revealed considerable differentiation among localities (Hayasaka et al. 1986). It also revealed length polymorphism with the length of ~200 bp in the major noncoding region (MNR) which was attributed to two independent mutational events (Hayasaka et al. 1986). To further clarify the nature of this length polymorphism, we have sequenced part of MNR in four types of Japanese monkey mtDNAs.

Since no functional genes have been located in MNR (Anderson et al. 1981, 1982; Bibb et al. 1981), this mtDNA region is relatively free of selective constraints. In fact, MNR of human mtDNA is known to show more polymorphism than any other region of mtDNA (Aquadro and Greenberg 1983; Greenberg et al. 1983; Horai and Hayasaka 1990). Besides the length polymorphism mentioned above, we also found polymorphic variants at restriction-enzyme recognition sites (Hayasaka et al. 1986). Thus, MNR of mtDNA is clearly suitable material with which to investigate intraspecific polymorphism in Japanese monkeys.

Utilizing the knowledge of nucleotide sequences and the polymerase chain reaction (PCR; Erlich et al. 1988; Saiki et al. 1988), we amplified part of MNR of 90 Japanese monkeys and analyzed the amplified DNA with two restriction enzymes. These analyses revealed that length polymorphism resulted from two tandem duplications of ap-

1. Key words: heteroplasmy, polymorphism, Japanese monkey, mitochondrial DNA, polymerase chain reaction.

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proximately 160-bp fragments and further established that there were heteroplasmic individuals in two localities. (Heteroplasmy implies the existence of two or more distinguishable mtDNA molecules within an individual.) Among 100 Japanese monkeys, 90 of which were analyzed in the present paper and 10 of which have been analyzed elsewhere (Hayasaka et al. 1986), we identified eight types of mtDNA and six types of heteroplasmic individuals.

Material and Methods

DNA Samples

Four types of mtDNAs (samples 4–7 in Hayasaka et al. 1986) used for cloning were prepared in our previous study. Total DNA was isolated from the buffy coat of blood samples (Blin and Stafford 1976), cultured B-cells (Davis et al. 1986), or liver (Marmur 1961). Twelve localities from which samples were obtained are shown in figure 1. The number of samples is shown in table 1. Three liver samples in Takahama, five in Gagyusan, and one each in Shodoshima and Yakushima are the same samples as had been analyzed elsewhere (Hayasaka et al. 1986). All liver samples and blood samples from the Takahama, Wakasa, and Arashiyama origins were taken from monkeys kept at the Primate Research Institute, Kyoto University, while other blood samples were taken from monkeys captured at each locality. The B-cell lines were established from blood samples also taken at Takasakiyama and Shimokita.

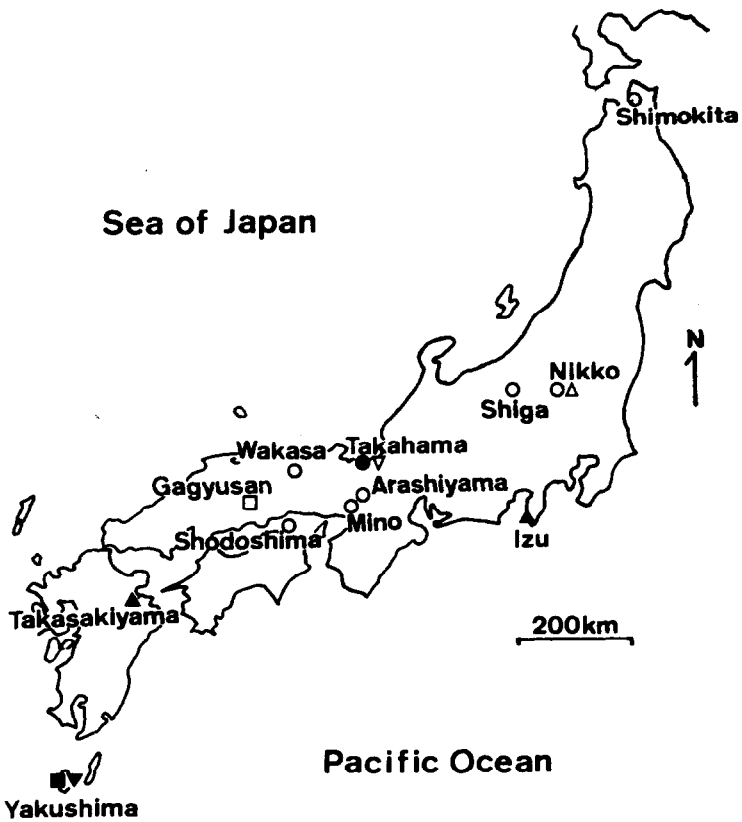


FIG. 1.—Distribution of eight types of mtDNA among 12 localities. Types I (●), II (○), III (□), IV (■), V (▲), VI (△), VII (▽), and VIII (▼) are represented.

Table 1
Sample Numbers and Types of mtDNA in 12 Localities

Locality	Sample[a]	mtDNA Type					
		I+VII	II	III	IV+VIII	V	VI
Shimokita	1 [1]		1				
Nikko	8 (1)		7				1
Izu	1					1	
Shiga	15 (1)		15				
Arashiyama	6		6				
Takahama	18 (3)	18					
Mino	1 (1)		1				
Wakasa	8		8				
Shodoshima	2 (2)		2				
Gagyusan	5 (5)			5			
Takasakiyama	3 [3]					3	
Takushima	32 (1)				32		
Total	100 (14) [4]	18	40	5	32	4	1

[a] Numbers in parentheses are number of liver cell samples; numbers in brackets are number of cultured cell samples.

Cloning and Sequencing

*Cla*I fragments of mtDNA—2.4 kb for mtDNA types I and IV and 2.2 kb for mtDNA types II and III—which contained MNR were cloned into the *Acc*I site of pUC19 (Yanisch-Perron et al. 1985). The inserted fragments were further digested with *Tth*Hb81 and *Hae*III, and the resultant 650- or 810-bp fragments were cloned into pUC19. Further digestion with *Sau*3AI or exonuclease III digestion was used to prepare additional subclones for complete sequencing (Henikoff 1984; Yanisch-Perron et al. 1985). Nucleotide sequences were determined for both strands by the dideoxy chain-termination method (Sanger et al. 1977).

Amplification of MNR of mtDNA

Two oligonucleotides—Saru 4 (atcacgggtctatcacccta) and Saru 5 (ggccaggaccaagcctattt)—were synthesized using a DNA synthesizer (Applied Biosystems 380B) and were used for PCR. The PCR reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 1.25 mM each of four dNTPs, 20 pmol of two primers, 50 ng of template DNA, and 2.5 units of *Taq*I DNA polymerase in a total volume of 100 μl. A Thermal Cycler (Perkin Elmer—Cetus) was used for the amplification. The target fragment was amplified for 30 cycles. Each cycle consisted of 10 s at 94°C for denaturation, 10 s at 45°C for annealing, and 15 s at 72°C for elongation of DNA chains.

Electrophoresis

Five microliters of each amplified DNA was electrophoresed on 1.5% horizontal agarose gel [0.5 × TAE (tris-acetate-ethylenediaminetetraacetate); 1 × TAE = 0.04 M Tris, 0.02 M sodium acetate, 1 mM ethylenediaminetetraacetate (pH 8.0)]. Samples (5–10 μl) of amplified DNA were digested with *Kpn*I, *Hinc*II, *Apa*LI, or *Rsa*I. DNA fragments digested with *Kpn*I or *Hinc*II were separated on 1.5% agarose gels (0.5 × TAE), and those digested with *Apa*LI or *Rsa*I were separated on 5% polyacrylamide gel (0.089 M Tris, 0.089 M boric acid, 2 mM ethylenediaminetetraacetate). The frag-

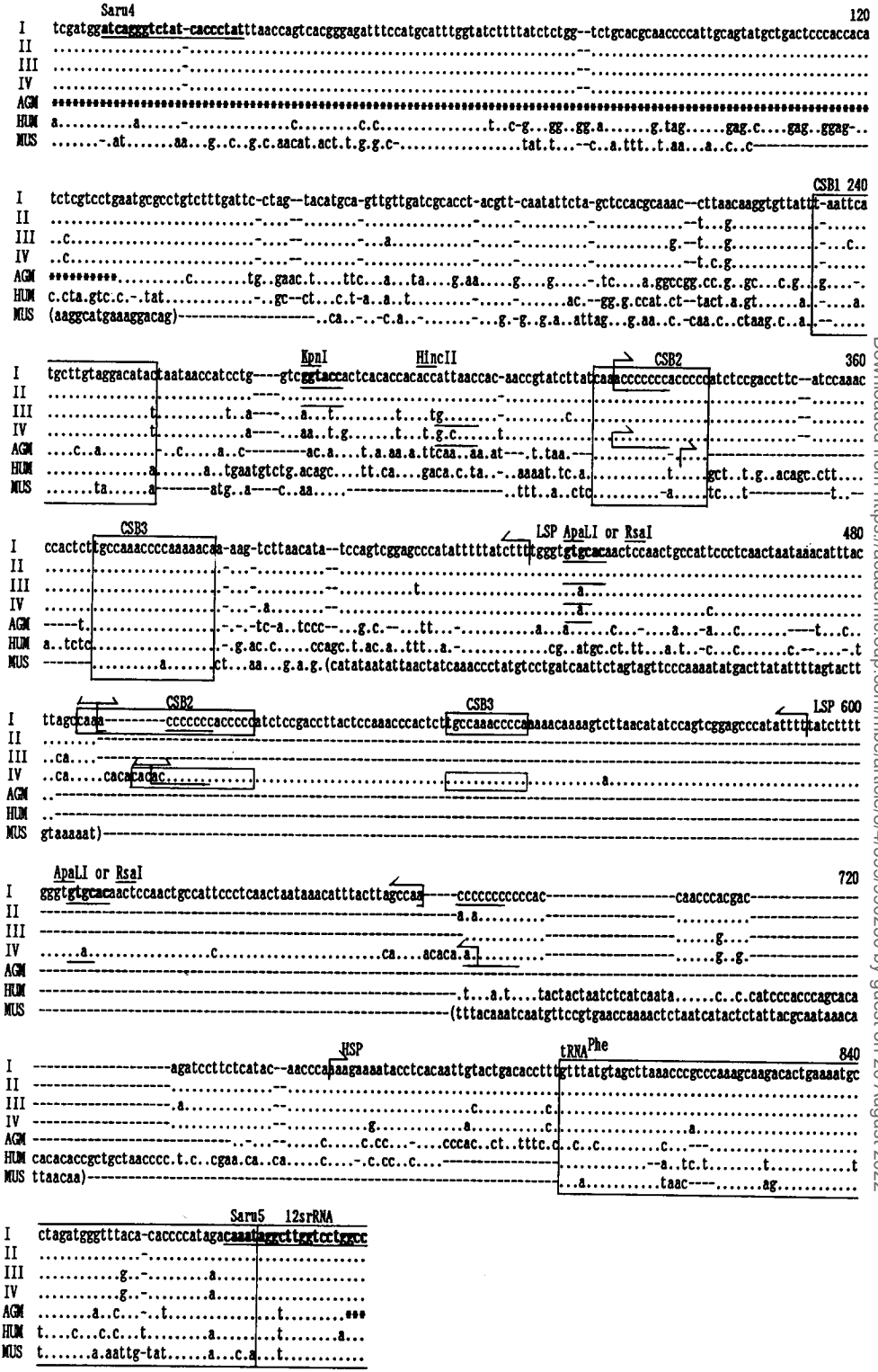


Table 2
Nucleotide-Sequence-Difference Values among Four Types of Japanese Monkey mtDNAs

	mtDNA TYPE			
mtDNA TYPE	I	II	III	IV
I		4	24	28
II	0.6		24	27
III	3.7	4.3		22
IV	3.7	4.2	3.4	

NOTE.—Numbers of nucleotide differences and percentages of nucleotide differences are shown above and below the diagonal, respectively. Additional 160-bp repeated segments in the types I and IV and deletions/insertions are excluded from sequence comparisons. Thus, the lengths of compared sequences are 646 bp among types I–IV and 645 bp between type III and the other types.

ments of DNA on gels were stained with ethidium bromide (2 μg/ml) and were visualized under ultraviolet light (302 nm).

Southern Blot Hybridization

One microgram of total DNA was digested with 25 units of *Kpn*I or *Cla*I, and restriction fragments were separated on 1% agarose gels (1 × TAE). DNA fragments on the gels were transferred onto Amersham Hybond N membranes (Southern 1975). DNA fragments on the membranes were probed with ^{32}P-labeled (Feinberg and Vogelstein 1983) purified mtDNA (1×10^6 cpm) in 6 × saline sodium citrate (SSC), 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 μg denatured salmon sperm DNA-/ml at 65°C overnight. The membranes were washed twice in 2 × SSC, 0.1% SDS at room temperature for 15 min, once in the same solution at 65°C for 15 min, and three times in 0.1 × SSC, 0.5% SDS at 65°C for 15 min and were exposed to Fuji RX X-ray films with Kodak X-Omatic intensifying screens. Enzymes used in the present study were purchased from Takara Shuzo (Kyoto), Nippon Gene (Toyama, Japan), U.S. Biochemicals (Cleveland), and Stratagene (La Jolla, CA) and were used according to the manufacturers' instructions.

FIG. 2.—Nucleotide sequence of major noncoding region of mammalian mtDNA. The L-strand sequences of the four types of Japanese monkey (I–IV) are aligned with those of human mtDNA (HUM [Anderson et al 1981], nucleotides 16564–16666), African green monkey mtDNA (AGM [Kawarya and Martin 1987], only one of the three copies of tandem repeats is shown), and mouse mtDNA (MUS [Bibb et al. 1981], nucleotides 15850–15887). All nucleotides are shown for the type I Japanese monkey mtDNA. For other sequences, dots indicate identities of nucleotide with the type I at each site, hyphens indicate deletions, and asterisks indicate regions which have not been sequenced. CSBs and genes (tRNA^{Phe} and 12s rRNA) are boxed and marked above the sequences. The 8-bp sequences (acccccccc) flanking the repeated sequences, the sequences for the two primers (Saru 4 and Saru 5), and the restriction-enzyme sites are underlined. Boundaries of the repeated sequences in type I and type IV mtDNAs of Japanese monkey and African green monkey and the positions of the L-strand and H-strand transcription-promoter regions (LSP and HSP) of human mtDNA are indicated by vertical lines with arrows. The parts of the sequence of mouse mtDNA that show no evidence of homology to primate mtDNAs are shown in parentheses.

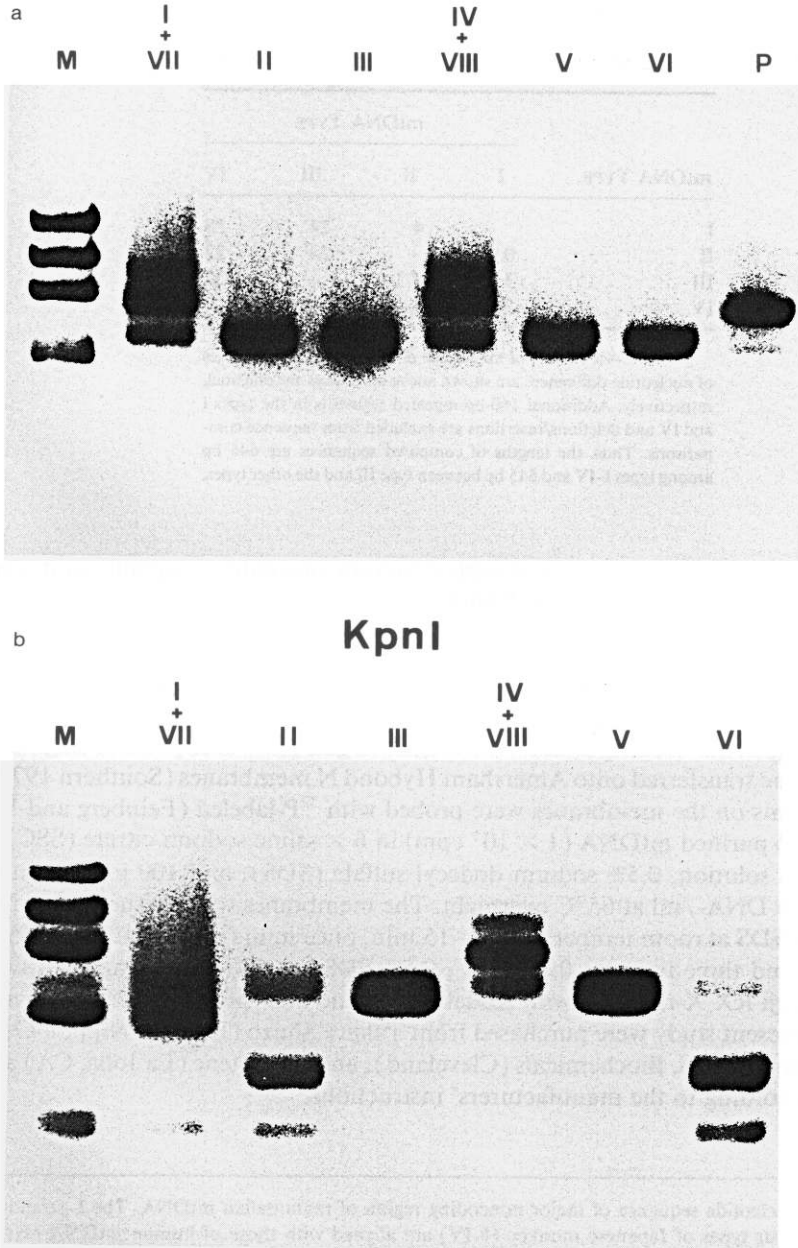
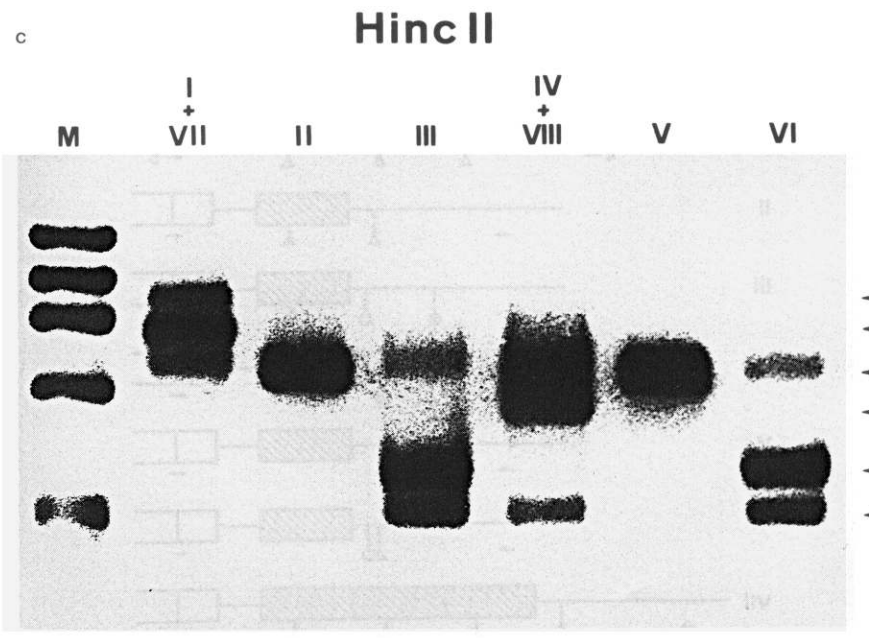


FIG. 3.—Electrophoretic patterns of noncoding region of Japanese monkey mtDNAs amplified by PCR. Amplified DNA fragments undigested (a), digested with *Kpn*I (b), and digested with *Hinc*II (c) were separated on 1.5% agarose gels. Those digested with *Apa*LI or *Rsa*I (d) were separated on a 5% polyacrylamide gel. Numbers at the top of the panels denote mtDNA types. Numbers at the sides denote the approximate sizes (in kb) of fragments. M and P at the top of the panels denote, respectively, a molecular-weight marker (ø ×174 digested with *Hae*III) and the PCR products from the recombinant plasmid pUC19 containing the noncoding region of the type I mtDNA. In panels b and c, amplified fragments were digested partially.



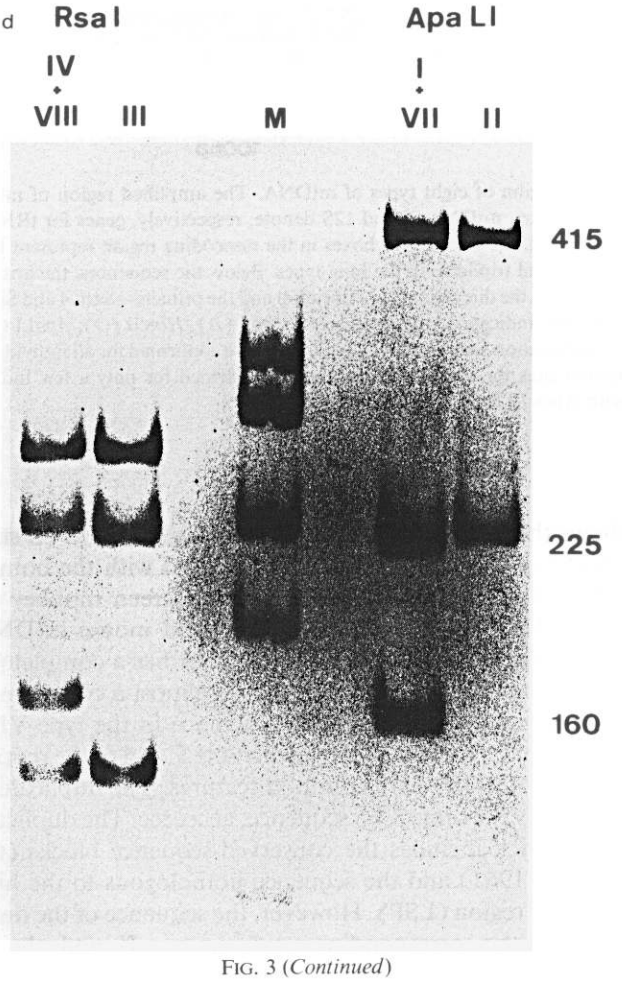


FIG. 3 (*Continued*)

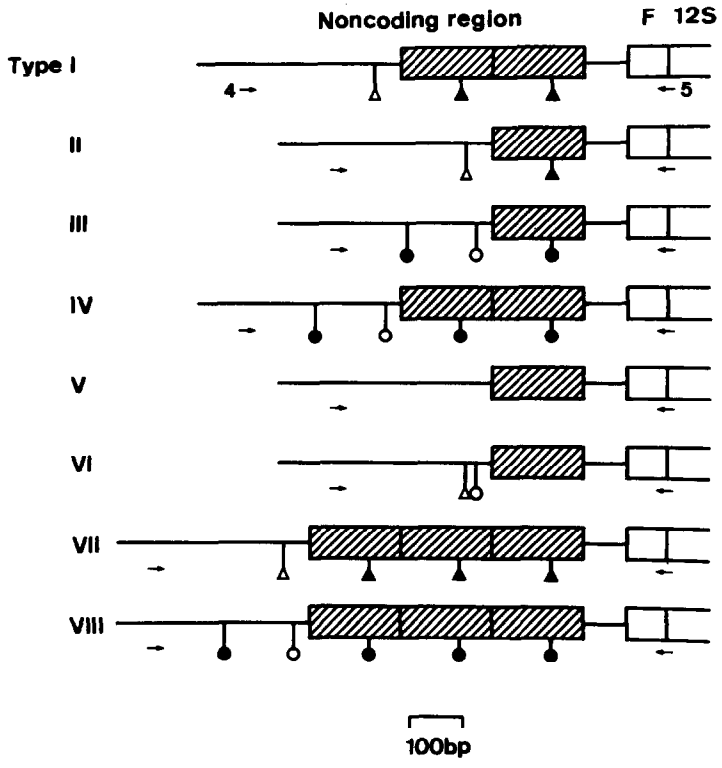


FIG. 4.—Amplified region of eight types of mtDNA. The amplified region of mtDNA is shown for each type of Japanese monkey mtDNA. F and 12S denote, respectively, genes for $tRNA^{Phe}$ and the small subunit of mitochondrial rRNA. The striped boxes in the noncoding region represent the segments which are supposed to duplicate and triplicate in the long types. Below the sequences, the arrows on the left- and right-hand sides indicate both the direction of amplification and the primers—Saru 4 and Saru 5, respectively—used in PCR. Vertical lines indicate restriction sites for *Kpn*I (△), *Hinc*II (○), *Apa*LI (▲), and *Rsa*I (●). Presence or absence of restriction sites for *Kpn*I and *Hinc*II were confirmed for all individuals, while presence or absence of restriction sites for *Apa*LI and *Rsa*I were confirmed for only a few individuals with types I+VII and II and with types III and IV+VIII, respectively.

Results

Figure 2 shows the nucleotide sequence of the light strand (L-strand) of MNR of the four types of Japanese monkey mtDNA aligned with the homologous region of human mtDNA (Anderson et al. 1981), African green monkey (*Cercopithecus aethiopus*) mtDNA (Karawya and Martin 1987), and mouse mtDNA (Bibb et al. 1981) mtDNA. The type I Japanese monkey mtDNA has a complete tandem duplication of a 158-bp sequence, while the type IV has almost a complete tandem duplication of a 167/168-bp sequence. The only difference in the type VI duplicated sequences is the number of c's in the poly c stretch at the 5′ end of the repeated sequences. Duplications in types I and IV share common features. The duplicated sequences in both types are flanked by the same 8-bp sequence, accccccc. The duplications occurred in the same region which includes the conserved-sequence blocks (CSBs) 2 and 3 (Walberg and Clayton 1981) and the sequence homologous to the human L-strand transcription-promoter region (LSP). However, the sequence of the duplicated region in type I is identical to the corresponding region in type II, and, throughout the se-

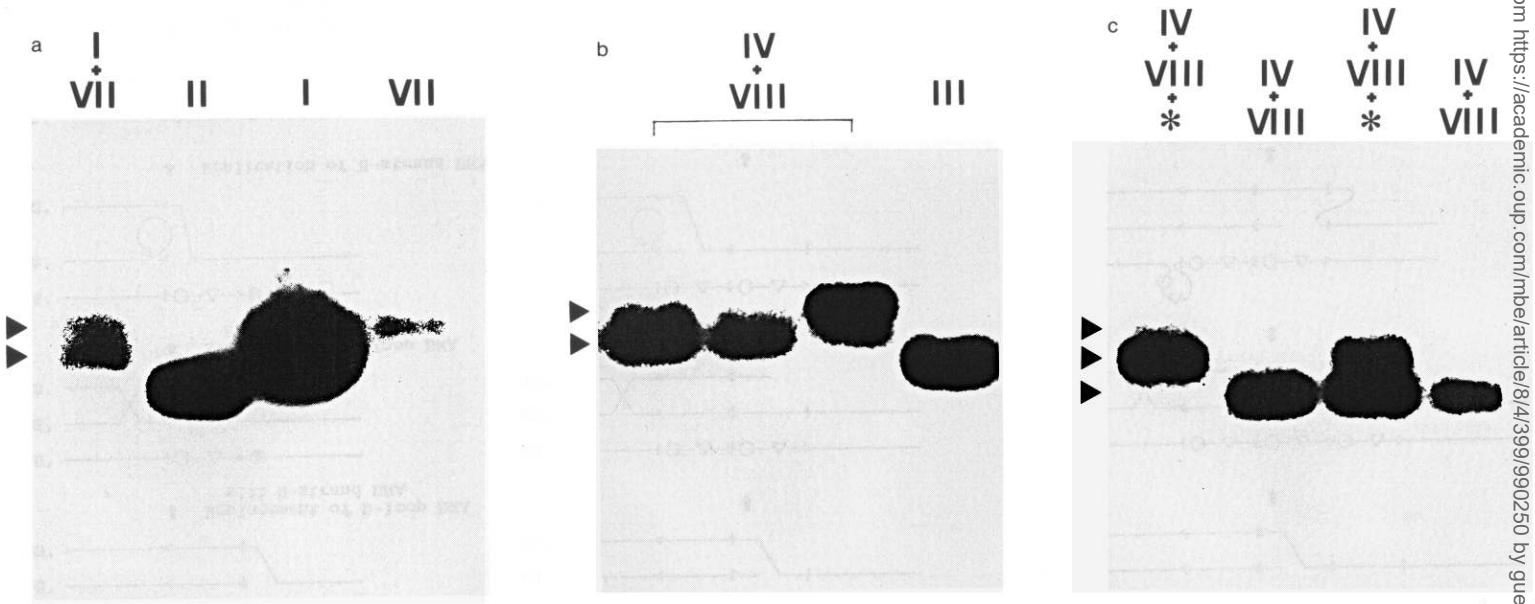
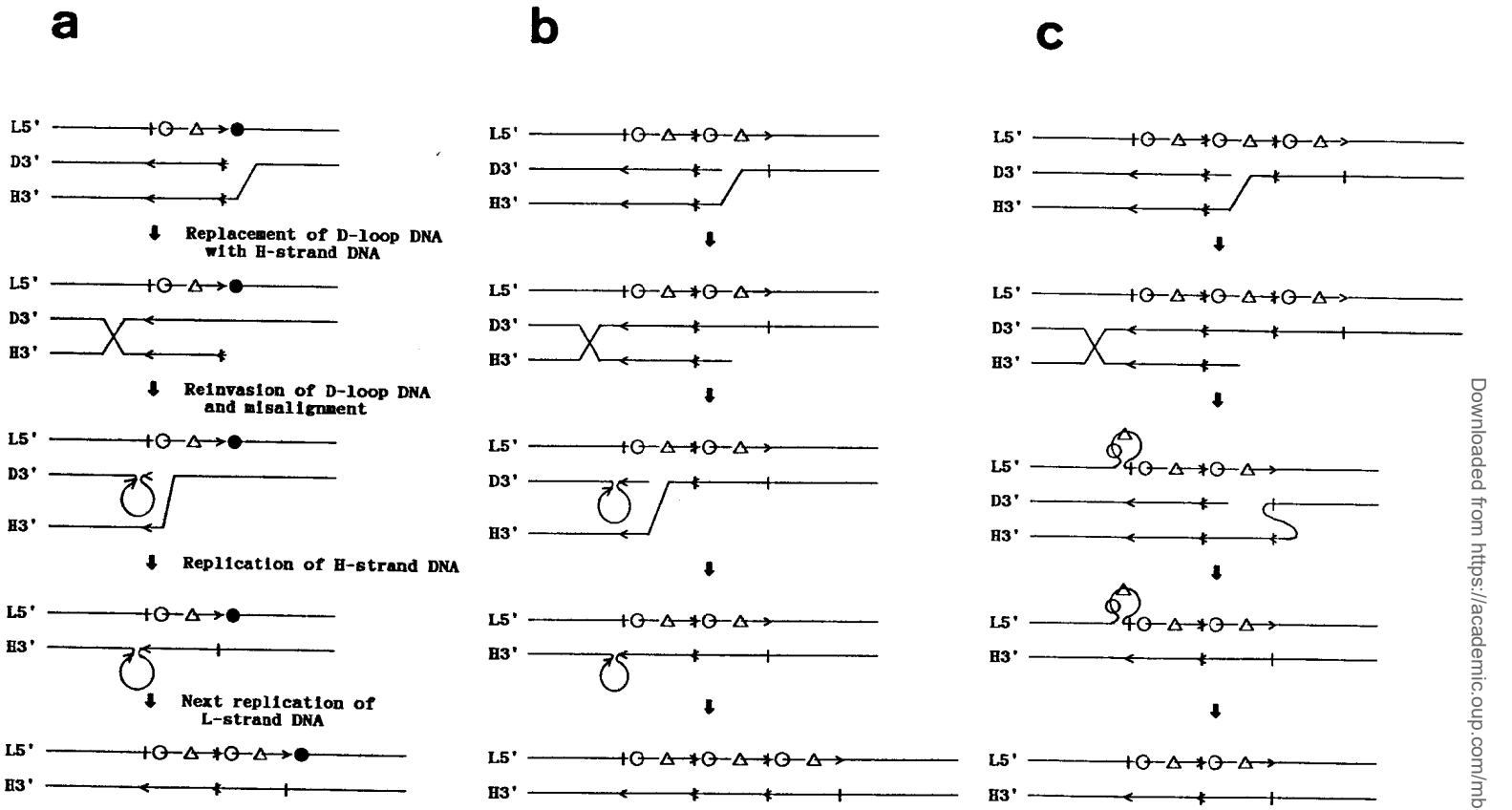


FIG. 5.—Southern blot hybridization analysis of Japanese monkey mtDNA. DNAs were digested with *Kpn*I (a) and *Cla*I (b and c). Only the small fragments in each digestion are shown. Numbers at the top of the panels denote mtDNA types. Fragments representing heteroplasmies are indicated by arrowheads at the left-hand sides of the panels. Individuals heteroplasmic for three kinds of mtDNAs are indicated by asterisks.



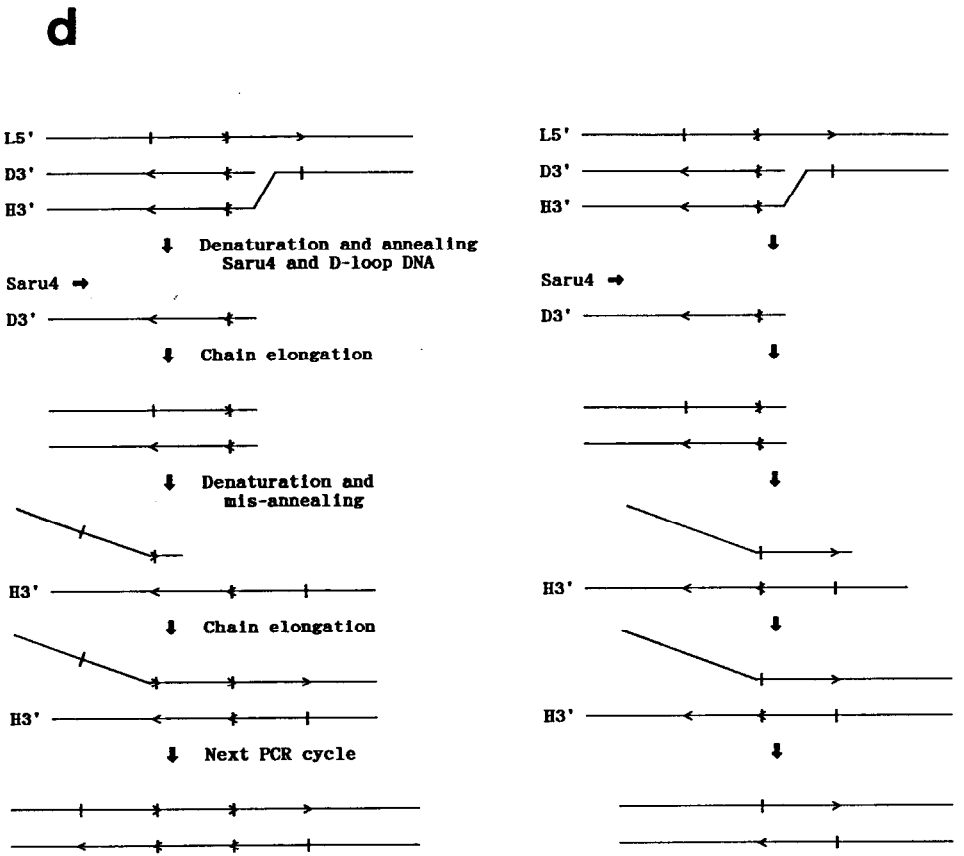


FIG. 6.—Possible mechanisms for duplication (a), maintenance of heteroplasmy (b and c), and artifact in PCR (d). L, H, and D denote, respectively, the L-strand, H-strand, and D-loop DNAs. Vertical lines and arrowheads indicate, respectively, 5′ and 3′ ends of the 160-bp repeated sequence. Circles, triangles, and black dots on the L-strand indicate, respectively, CSB2, CSB3, and the CSB2-like sequence.

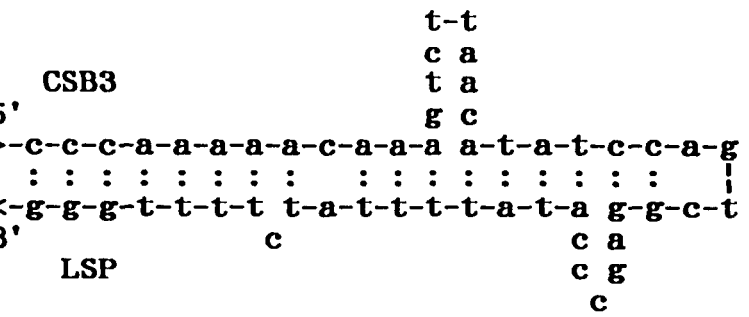


FIG. 7.—Possible secondary structure in 160-bp repeated sequence. The secondary structure is shown for the type I L-strand sequence which is located between nucleotides 385 and 445 in fig. 2.

quenced region, type I is much more similar to type II than to the other long type, type IV (table 2). Although type IV is similarly related to the other types, types III and IV share 16 unique nucleotide substitutions, which suggests that types III and IV are closely related. These findings confirm that there were two independent duplications, one of which produced type I and the other of which produced type IV, as previously suggested by our restriction-enzyme analysis (Hayasaka et al. 1986).

The CSBs which have been observed in several vertebrate mtDNAs were also identified in Japanese monkey mtDNA (fig. 2). Conservation of the CSBs confirms their importance in the switch from synthesis of the primer RNA to the D-loop/heavy-strand (H-strand) DNA (Chang and Clayton 1985; Chang et al. 1985). However, the regions 5′ to CSB1, between CSBs 1 and 2, and between LSP and $tRNA^{Phe}$ show extensive sequence divergencies, even between Japanese and African green monkeys, which diverged 10 Mya (Pilbeam 1984). This also supports other investigators' observations that MNR has accumulated many mutations (Aquadro and Greenberg 1983; Greenberg et al. 1983; Horai and Hayasaka 1990).

DNA fragments of 640 bp were amplified from DNA extracted from individuals of all localities but Takahama and Yakushima (fig 3a and table 1). These 640-bp fragments are in the range of size expected for mtDNA without duplication from the nucleotide sequences (fig. 2). These 640-bp fragments were classified into the following four types: type II with a *Kpn*I site and no *Hin*cII site, type III with a *Hin*cII site and no *Kpn*I site, type V with neither *Kpn*I nor *Hin*cII sites, and type VI with both *Kpn*I and *Hin*cII sites (figs. 3b and c and 4). Types II and III correspond to the respective types in our previous study (Hayasaka et al. 1986).

From DNA extracted from individuals from Takahama and Yakushima, three or more fragments were amplified for each individual (fig. 3a). One of them was 800-bp long, which was the size for mtDNA with duplication of the 160-bp sequence expected from the nucleotide sequences (fig. 2). The others were 640-, 960-, and 1,120-bp long. Southern blot hybridization analysis (fig. 5) revealed heteroplasmy of mtDNA in 12 of 18 individuals from Takahama and in 29 of 31 individuals from Yakushima. Two heteroplasmic monkeys in Yakushima had three mtDNA types which corresponded to the PCR fragments of 800, 960, and 1,120 bp. The others were heteroplasmic for two types which corresponded to the PCR fragments of 800 and 960 bp.

When we used the recombinant plasmid DNA with the 2.4-kb *Cla*I fragment of type I mtDNA as a template, 640- and 960-bp fragments occurred in the PCR product, in addition to the expected 800-bp fragment (fig. 3a). These artifactual fragments did

not disappear even after the annealing temperature was increased to 63°C. Thus, the fragments seen in PCR but not in Southern analysis are probably artifacts of PCR.

Because of the artifacts, amplified DNAs from Takahama and Yakushima individuals were not indistinguishable from each other, irrespective of whether they were homoplasmic or heteroplasmic for two or three types of mtDNAs. However, all DNAs amplified from Takahama monkeys had a *Kpn*I site but no *Hinc*II site, while all amplified DNAs from Yakushima monkeys had a *Hinc*II site and no *Kpn*I site (fig. 3b and c). An 800-bp fragment with a *Kpn*I site corresponds to type I, and an 800-bp fragment with an *Hinc*II site corresponds to type IV of our previous study (Hayasaka et al. 1986). We designated fragments >800 bp with a *Kpn*I site as type VII and those with a *Hinc*II site as type VIII (fig. 5). Since types I and VII always coexisted in the PCR-amplified DNA, those individuals who had both types we regarded as having a single type, I+VII. Similarly, since types IV and VIII always coexisted in the PCR-amplified DNA, those individuals who had both types we regarded as having a single type, IV+VIII (table 1).

Nucleotide sequences (fig. 2) showed that there was an *Apa*LI site and an *Rsa*I site in the repeated sequences of types I and IV, respectively. *Apa*LI digestion of amplified DNA of types I+VII and II (fig. 3d) revealed that type I+VII had a unique 160-bp fragment in addition to the two fragments shared by both types. *Rsa*I digestion of amplified DNA of types IV+VIII and III (fig. 3d) showed that type IV+VIII had a unique 160-bp fragment in addition to the three fragments shared by both types. These observations suggest that types I and VII have tandem duplication and triplication, respectively, of a 160-bp sequence and that types IV and VIII have tandem duplication and triplication, respectively, of another 160-bp sequence (fig. 4).

As described above, the 100 analyzed samples were classified into six types—types I+VII, II, III, IV+VIII, V, and VI. (table 1). Type II was widely observed in seven localities from central to northeastern Japan (fig. 1 and table 1). The other types were observed in one or two localities (fig. 1 and table 1). Except for the cases of heteroplasmy, only one type of mtDNA was observed in the eight (of nine) localities from which more than one sample was taken (table 1).

Discussion

The majority of Japanese monkeys have the short mtDNA, as is also the case for three other species of macaques—rhesus monkey (*M. mulatta*), crab-eating monkey (*M. fascicularis*), and Formosan monkey (*M. cyclopis*) (Hayasaka et al. 1988; authors' unpublished data). Thus, it is more plauseble to derive the long types of mtDNA from the short types via duplications than to derive the short types from the long types via deletions.

Extensive length polymorphism and heteroplasmy associated with the repeated sequences have been reported for a number of different animal species (nematode [Wallis 1986], insects [Solignac et al. 1983; Harrison et al. 1985; Rand and Harrison 1986], mollusk [Snyder et al. 1987], fish [Birmingham et al. 1986; Buroker et al. 1990], amphibian [Birmingham et al. 1986], and reptiles [Densmore et al. 1985; Moritz and Brown 1987]). However, extensive population studies on human (Cann and Wilson 1983; Horai and Matsunaga 1986) and rodent mtDNA (Ferris et al. 1983; Lansman et al. 1983) failed to detect intraspecific length polymorphism with sizes >100 bp. Cases of heteroplasmy have been observed in mammalian mtDNA (Hauswirth and Laipis 1982; Brown and DesRosiers 1983; Greenberg et al. 1983; Olivo et

al. 1983; Hauswirth et al. 1984; Boursot et al. 1987; Holt et al. 1988), but none of these cases resulted from differences in the number of repeated sequences.

Since the majority of monkeys in Takahama and Yakushima were heteroplasmic for their mtDNA, we suggest that the heteroplasmy has been maintained in the monkeys in these localities for generations. Heteroplasmy can be maintained for generations when recurrent mutations result in transitions from one type of heteroplasmic mtDNA to another type. Otherwise, segregation and/or selective expansion of a few mtDNA molecules either in germ cells or during early development (Olivo et al. 1983) results in the fixation of one type of mtDNA in an individual.

In human, the synthesis of a species of the D-loop DNA starts from the region 3′ to LSP (Chang and Clayton 1985). In Japanese monkey mtDNA (fig. 2), the 8-bp sequence accccccc, just 3′ to the 160-bp sequence, shows partial homology with CSB2 which serves as a initiation site of D-loop DNA synthesis. Thus, D-loop DNA synthesis might start from this CBS2-like sequence block and follow a course similar to that depicted by Buroker et al. (1990). If the 5′ end of this species of the D-loop DNA was displaced by the H-strand, were reinvaded, and were misaligned with CSB2 on the L-strand, then, as replication of the H-strand DNA continued, mtDNA with the duplicated 160-bp sequences would be generated after the next replication (fig. 6a). Although the 160-bp repeated sequence in Japanese monkey mtDNA cannot form the complete internal pairing throughout its length that was seen in the repeated sequence in sturgeon mtDNA (Buroker et al. 1990), a 54-bp stretch within it can form a strong secondary structure (fig. 7). This secondary structure could work for the stabilization of the single-stranded DNA that resulted from the misalignment. If this were the case, the 8-bp sequence accccccc, which was part of CSB2 and flanked both ends of the repeated sequences, could play a very important role in the duplication. Karawya and Martin (1987) reported the tandem triplication of a 108-bp sequence which in African green monkey mtDNA also contained CSB3 and part of CSB2. However, we could not find similar characteristics in its repeated region.

mtDNA with three or more copies of the 160-bp sequences could also be generated, by a similar process, from mtDNA with two copies. As CSBs 2 and 3 locate within the repeated sequence, 5′ ends of some of the D-loop DNAs are also probably mapped within the repeated sequence (Chang and Clayton 1985). Thus, the displacement of the D-loop strand by the H-strand and the subsequent reinvasion and misalignment of the D-loop strand could make a triplicated mtDNA from the duplicate (fig. 6b). A similar process could also reduce the number of repeated sequences (fig. 6c).

We observed that all heteroplasmic monkeys found in our study had mtDNAs with two or more copies of the 160-bp sequence and that none of them had mtDNA without the repeated sequence. A possible explanation for this observation is that an mtDNA mutation from one copy of the 160-bp sequence to two copies (fig. 6a) rarely occurred, while mtDNA mutations among two or more copies of the repeated sequence (fig. 6b and c) have occurred frequently enough to overcome the rapid genotypic shifts of mtDNA within individuals (Olivo et al. 1983).

Although the primer RNA molecules covalently bound to the D-loop DNA have not been identified in human (Chang and Clayton 1985), they have been identified in mouse (Chang et al. 1985). Thus, we suspect that part of primer RNA may also covalently bind to D-loop or H-strand DNA during its synthesis in Japanese monkeys. These primer RNA molecules could stabilize the pairing between the misaligned D-loop and L-strand DNAs in the processes shown in Figure 6b and c, since, in these cases, the primer RNA was (1) transcribed from LSP which was located in the repeated

sequence (Chang and Clayton 1985) and (2) thus still complementary to the L-strand DNA after the misalignment. On the other hand, the primer RNA could not be involved in stabilizing the misaligned strands shown in figure 6a, since the primer RNA was no longer complementary to the L-strand DNA. This difference in stability might explain the difference in the occurrence of these mutational events that has been mentioned above.

The existence of the D-loop DNA may result in artifact fragments in the PCR-amplified DNA. As is seen in figure 2, the 5′ primer, Saru 4, can anneal with the D-loop DNA, and amplification of the complementary strand stops at the 5′ end of the D-loop DNA. After denaturation, most of the DNA molecules complementary to the D-loop DNA anneal perfectly to the H-strand mtDNA, although some molecules might fail to do so. If so, their misalignment could result in the extra bands seen in the PCR product (figs. 3a and 6d). However, this process cannot explain the artifact derived from the recombinant plasmid DNA. In this case the secondary structure in the repeated sequence mentioned above (fig. 7) might have something to do with the artifact.

Although the existence of an artifact made our classification of the 100 samples complicated, our analysis has proved that PCR is a useful tool for population genetical studies of wild animals. In fact, it took only 1 wk to complete the amplification and restriction analysis of the 90 samples. In contrast to the results from our previous study (Hayasaka et al. 1986), which showed that each of four types of mtDNA had been observed only in one locality, two types—II and V—were observed in seven and two localities, respectively. However, only a limited region of mtDNA and only a small number of restriction enzymes were used in the present study. By further analyses using more restriction enzymes on a larger region of mtDNA, it should be possible, with relatively small investment of time and labor, to determine the phylogenetic relationships among the four short types and to classify the subtypes within each type.

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