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Length Heteroplasmy of Sturgeon Mitochondrial DNA: An Illegitimate Elongation Model

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ABSTRACT

Extensive length polymorphism and heteroplasmy (multiple forms within an individual) of the D-loop region are observed in mitochondrial DNA of the white sturgeon (*Acipenser transmontanus*). The nucleotide sequence of this region, for both a short and a long form, shows that the differences are due to variable numbers of a perfect 82-bp direct repeat. We propose a model for the replicative origin of length differences, involving a competitive equilibrium between the heavy strand and the D-loop strand. This model suggests that frequent misalignment in the repeat region prior to elongation, facilitated by a stable secondary structure in the displaced strand, can explain both the polymorphism and heteroplasmy in this species.

MITOCHONDRIAL DNA (mtDNA) of animals is a highly conserved, compact genome carrying genes for 13 proteins, 22 tRNAs and two rRNA molecules (ANDERSON *et al.* 1981; CHOMYN *et al.* 1985, 1986). While the organization of these genes is generally maintained among vertebrate species, differences involving nucleotide substitutions and length variations are commonly observed both within and between species. Recently, a number of instances of sequence length heteroplasmy (multiple forms within a single individual) have been reported in invertebrates and lower vertebrates including several species of Drosophila (FAURON and WOLSTENHOLME 1976; SOLIGNAC, MONNEROT and MOUNOLOU 1983), Gryllus crickets (HARRISON, RAND and WHEELER 1985), scallops (SNYDER *et al.* 1987), frogs (MONNEROT, MOUNOLOU and SOLIGNAC 1984), Cnemidophorus lizards (DENSMORE, WRIGHT and BROWN 1985), crested newts (WALLIS 1987) and the fish, *Amia calva* (BERMINGHAM, LAMB and AVISE 1986) and *Alosa sapidissima* (BENTZEN, LEGGETT and BROWN 1988).

The origin and maintenance of these length differences are particularly intriguing. RAND and HARRISON (1989) suggest that frequent intermolecular recombination may cause heteroplasmy in crickets. The frequency with which heteroplasmy has been observed in animals suggests that it occurs rather easily, yet recombination, the principal mechanism by which *nuclear* reorganizations occur, is not evident in mitochondria of vertebrates (HAYSHI, TAGASHIDA and YOSHIDA 1985). An alternative mechanism, not requiring repair, is that these changes arise as a result of misalignment prior to replication.

We are studying mtDNA in white sturgeon (*Acipenser transmontanus*) from two perspectives: that of the genetics of populations along the west coast of North America and the evolution of mtDNA of a primitive vertebrate. Sturgeon are believed to have their origins in the early Jurassic and acipenserids are known from the Upper Cretaceous (GARDINER 1984). Similar to other lower vertebrates, sturgeon mtDNA has extensive length variation in the control or displacement region (D-loop). Individuals may be homo- or heteroplasmic for any number of discrete length variants of mtDNA.

In this study, we have cloned and sequenced the D-loop region of two extreme length variants. These data allow us to propose a novel model for the *intramolecular generation of heteroplasmy without recombination*. We suggest that length heteroplasmy may be the result of frequent competitive misalignment in the repeat region prior to replication.

MATERIALS AND METHODS

In 1987 and 1988, fresh liver or heart tissue samples were dissected from sturgeon caught by commercial and sports fishermen in the Fraser (British Columbia), Columbia (Washington) and Sacramento (California) Rivers. Mitochondrial DNA was isolated from the tissue by ethidium bromide/CsCl gradient ultracentrifugation for 60–72 hr (LANSMAN *et al.* 1981).

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Restriction endonuclease digestions of purified mtDNA were conducted using conditions recommended by the vendor (Boehringer Mannheim). Restriction fragments were end-labeled using the Klenow fragment of *Escherichia coli* DNA polymerase I and [α-^{32}P]dNTP (DROUIN 1980). The labeled restriction fragments were sized through a 0.9% agarose gel. After electrophoresis, the agarose gel was dried on Whatman 3MM filter paper in a gel drier. The dried gel was exposed to X-ray film (Kodak X-Omat AR) for 15 hr at room temperature and the restriction fragments were revealed by autoradiography (MANIATIS, FRITSCH and SAMBROOK 1982).

In digests with the enzyme *Bcl*I, one particular fragment showed discrete size variation among and, frequently, within individuals. A small fragment about 1.6 kilobase pairs (kb) and a larger 1.9-kb fragment originating from two different individuals were isolated by electrophoresis in low melting point agarose then cloned into Bluescript KS (Stratagene) and pUC19 cloning vectors. We have not observed any change in the length of either the 1.6- or 1.9-kb cloned fragments after repeated passage through *E. coli* (JM83). The fragments were sequenced in their entirety using dideoxynucleotide chain termination method primed from a series of overlapping synthetic oligonucleotides (SANGER, NICKLEN and COULSON 1977).

Nucleotide sequence analysis was done using Delaney and ESEE (CABOT and BECKENBACH 1989) DNA sequence programs. The program of ZUKER and STIEGLER (1981) was used to determine the most likely secondary structure and estimate thermodynamic stability.

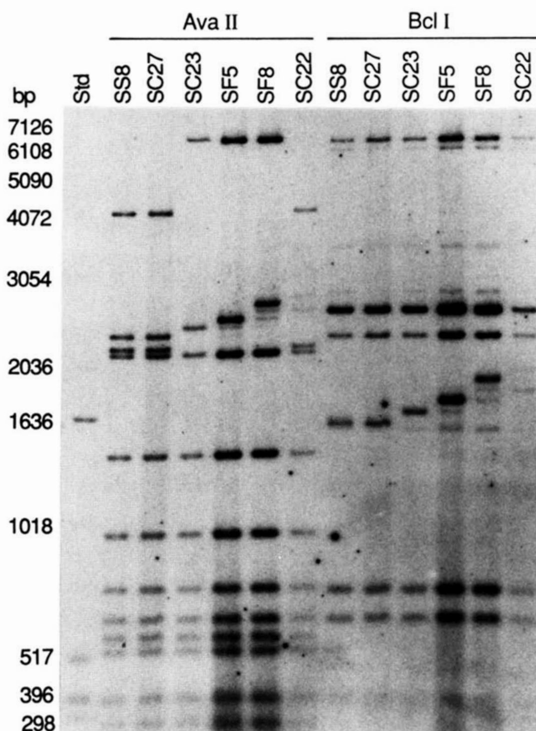

FIGURE 1.—Autoradiograph showing *Ava*II and *Bcl*I restriction endonuclease digests of six white sturgeon mtDNA genomes from individuals SS8, SC27, SC23, SF5, SF8 and SC22 (SS = Sacramento River; SC = Columbia River; SF = Fraser River).

RESULTS

Restriction endonuclease digest: Heteroplasmy of the D-loop was identified in two restriction endonuclease (*Ava*II and *Bcl*I) digests of white sturgeon mtDNA (Figure 1). The endonuclease, *Ava*II, revealed a series of length polymorphisms within and between individual fish. The fragment carrying the D-loop ranged in size from 2.4 to 2.6 kb. *Bcl*I digestion gave variable sized fragments ranging from 1.6 to 1.9 kb. In one individual (SF8), *Ava*II and *Bcl*I digests both revealed five different fragments of the D-loop region, ranging from 2.4 to 2.6 and 1.6 to 1.9 kb, respectively. In digests of mtDNA from individual SF5, three restriction fragments ranging from 2.4 to 2.6 and 1.6 to 1.75 kb, respectively, were observed. Evidently, five different length mtDNA genomes were present in SF8, and three differently sized genomes were present in SF5.

The *Ava*II digest also produced a restriction fragment length polymorphism in fragments not containing the D-loop. This polymorphism probably results from a nucleotide substitution. Individuals (SC23, SF5, SF8) lacking this *Ava*II site produce a 6.3-kb fragment while individuals (SS8, SC27, SC22) having the site have 2.2- and 4.1-kb fragments in their profiles (Figure 1).

The distribution of length variants and heteroplasmy among populations and mtDNA genotypes will be discussed in a future publication. Briefly, 41% (52 of 128) of the sturgeon examined from the three river systems displayed length heteroplasmy for *Ava*II and *Bcl*I fragments as detected by hybridization of radioactive labeled sturgeon D-loop probes to Southern transfers of mtDNA. The remaining fish fall into discrete groups depending on the length of the D-loop region.

Sequence analysis: The complete sequence of the *Bcl*I 1.6- and 1.9-kb fragments showed that they span the region of the mitochondrial genome containing part of cytochrome B, threonine tRNA (tRNA^{Thr}), proline tRNA (tRNA^{Pro}), the D-loop and part of the phenylalanine tRNA (tRNA^{Phe}). The order and orientation of these genes is that typical of other vertebrate mitochondrial genomes. The sequences of the tRNA genes and cytochrome B gene are presented elsewhere (BROWN *et al.* 1989; GILBERT *et al.* 1988).

A portion of the sequence of the two fragments, extending from part of the tRNA^{Pro} through the D-loop, is given in Figure 2. The length difference is due to an 82-base pair (bp) sequence, present as four perfect direct repeat copies in the 1.9-kb *Bcl*I fragment, but only one in the 1.6-kb fragment. In addition, both fragments contain an imperfect and incomplete copy adjacent to the main portion of the D-loop region. Part of the repeat sequence has a 60% identity with the adjacent tRNA^{Pro} gene. Because of the imperfect repeat, the actual limits of the repeat sequence are somewhat arbitrary. The alignment in Figure 2 shows the similarity to tRNA^{Pro}. The perfect repeats

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                     * * ** *TAS I   * *  *****       ▼
1.6 kb        GCTAAGATTCTACATTAAACTATTCTCTGAC------CATGCTATGTTTAATCCACATTAATTTCTAGCCACCATACCATAAT (1)     77
1.9 kb        ...............................------.............................................. (1)     77
                                 <-tRNAPro-|
                 TAS II                                ▼
1.6 kb        GCTCACAAGC-ACATTAAATTGTTTAAGTACATAAGACATGCTATGTTTAATCCACATTAATTTCTAGCCACCATACCATAAT (2)    159

1.9 kb        GCTCACAAGC-ACATTAAATTGTTTAAGTACATAAGACATGCTATGTTTAATCCACATTAATTTCTAGCCACCATACCATAAT (2)    159
1.9 kb        GCTCACAAGC-ACATTAAATTGTTTAAGTACATAAGACATGCTATGTTTAATCCACATTAATTTCTAGCCACCATACCATAAT (3)    241
1.9 kb        GCTCACAAGC-ACATTAAATTGTTTAAGTACATAAGACATGCTATGTTTAATCCACATTAATTTCTAGCCACCATACCATAAT (4)    323
                                                                                  *    *  *
1.9 kb        GCTCACAAGC-ACATTAAATTGTTTAAGTACATAAGACATGCTATGTTTAATCCACATTAATTTCTAGCCATCATATCACAAT (5)    405

        TAS III
1.6 kb  GTTTCATCTACCATTAAATGTTATACACCATTATTTTTATGTGCACTAACATGATAAGCCTCCGATAACTTAAAATGTAGTAAGAGCCGAACATGGAGATAT   261
1.9 kb  ......................................................A..-....T.......................................   506

1.6 kb  GTCTAGAACATAAAGTTAATGAGATGAGGGACAATAACTGTAGGGATTCACAACTGAACTATTACTGGCATCTGGTTCCTATTTCAGGTCCATTAACAGTTA   363
1.9 kb  ......................................................................................................   608

1.6 kb  TTTCCCCATAACTGAACTATGTCTGGCATCTGATTAATGTTGGAGGTACTATGAAATCCATGACCCCACATGCCGAGAATCTTGTCAACATTTGGTATTTTT   465
1.9 kb  ......................................................G...........................................C...   710

1.6 kb  TATTCGGGTTTCCATTCACTGACATGTAGAACTCCTTCAGAGAAGAACAACAAGGTGGAACATACGCGACTGTCCGAGAGGATGAATAATGAATGGTACAAT   567
1.9 kb  ......................................................................................................   812

        CSB 1
1.6 kb  GACATATCCCTGATGTCACACATGGCCTGTGCTGTGTACAGAGAGATGTTTCACAGAGCCTGGTTTTA-TCTTTTCACATGACAATCATGGACGTTTACTAT   668
1.9 kb  ....................................................................A.................................   914

               CSB 2                                   CSB 3                           CSB 1
1.6 kb  CGACAAACCCCCTACCCCCTTATGTCGGACAGGCCTTATATTTCTTGTCAAACCCCAAAAGCAGGACTGACTTGTCATCGACATACCTTGATC           761
1.9 kb  .............................................................................................          1017
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FIGURE 2.—Aligned light strand sequences of part of the 1.6-kb (top) and 1.9-kb *BclI* fragments. Fragment designations are given on the left and nucleotide numbers on the right. Dots in the 1.9-kb sequence indicate identical nucleotides. Dashes indicate nucleotides that are absent from one of the sequences. The asterisks (*) indicate nucleotide differences from the 82-bp perfect repeat. The exact start of the first 82-bp repeat is somewhat arbitrary. The perfect repeat units could be regarded as starting as few as two or as many as 33 nucleotides from tRNA^{Pro}. The first and last nucleotides of the repeat illustrated in Figure 3A are indicated by vertical arrows. The alignment shown here starts within the tRNA^{Pro} and shows the sequence similarity between the tRNA^{Pro} and part of the repeat sequence. The terminal associated sequences (TAS I, II and III) and the consensus sequence blocks (CSB 1, 2 and 3) are underlined. There are six sequence differences between the 1.6- and 1.9-kb fragments in the putative control region: two insertion/deletions, three transitions and one transversion.

actually begin two or more nucleotides 3′ from tRNA^{Pro}.

The D-loop region contains well defined consensus sequence blocks (CSB) which have been seen in a number vertebrate mtDNAs (reviewed in CLAYTON 1984; DUNON-BLUTEAU, VOLOVITCH and BRUN 1985). In the sturgeon mtDNA both the order and sequence of these blocks is highly conserved. The sturgeon CSB 3 differs from that of Xenopus by a single nucleotide change (C to G) in the second to last nucleotide (DUNON-BLUTEAU, VOLOVITCH and BRUN 1985). CSB 2 from sturgeon contains an initial and final C pentamer rather than hexamer as found in Xenopus. The CSB 1 sequences are identical to those in Xenopus.

The repeat sequences, as well as the tRNA^{Pro} gene, contain sequences with high degree of identity to the terminal associated sequences (TAS) found at the ends of D-loop fragments. We find three variations of this sequence. The perfect repeats contain the sequence ACATTAAATTGTTT (designated TAS II, in Figure 2) which has a two nucleotide difference from the consensus TAS of mouse (DODA, WRIGHT and CLAYTON 1981). In the imperfect copy the sequence ACCATTAAATGTTA (TAS III) is the most highly diverged TAS sequence, while TAS I (ACATTAAACTATTC) in tRNA^{Pro} differs from TAS II by three transitions and from the mouse consensus by a single nucleotide. If TAS segments have functional importance in dictating the length of nascent DNA fragments in the D-loop, then individuals with five or six copies of the repeat would have a number of discrete length D-loop primer fragments.

Each perfect single strand repeat is capable of folding into a complex secondary structure of high thermodynamic stability (Figure 3A). In addition, adjacent repeats have internal folding capabilities, generating increasingly stable structures: one copy, −14.1 kcal/mole (Figure 3A); two, −34.4 kcal/mole (Figure 3B); three, −58.0 kcal/mole; and four, −78.3 kcal/mole. This property of the repeats may help stabilize them when single stranded, or when an additional repeat is present in one strand of a heteroduplex molecule.

DISCUSSION

The observation of perfect repeat structures in the D-loop of sturgeon requires a mechanism that can maintain perfect sequence identity despite the fact that this region is subject to rapid evolutionary change (BROWN, GEORGE and WILSON 1979; UPHOLT and DAWID 1977). Except for the conserved control regions, the D-loop is the most rapidly evolving portion of the molecule. In addition, the mechanism must give rather similar probabilities of gain or loss of repeat units to account for the existence of the repeats. If the mechanism for loss has a higher probability of occurring, the repeats would be rapidly eliminated from the genome.

In vertebrates, a D-loop strand is synthesized, dis-

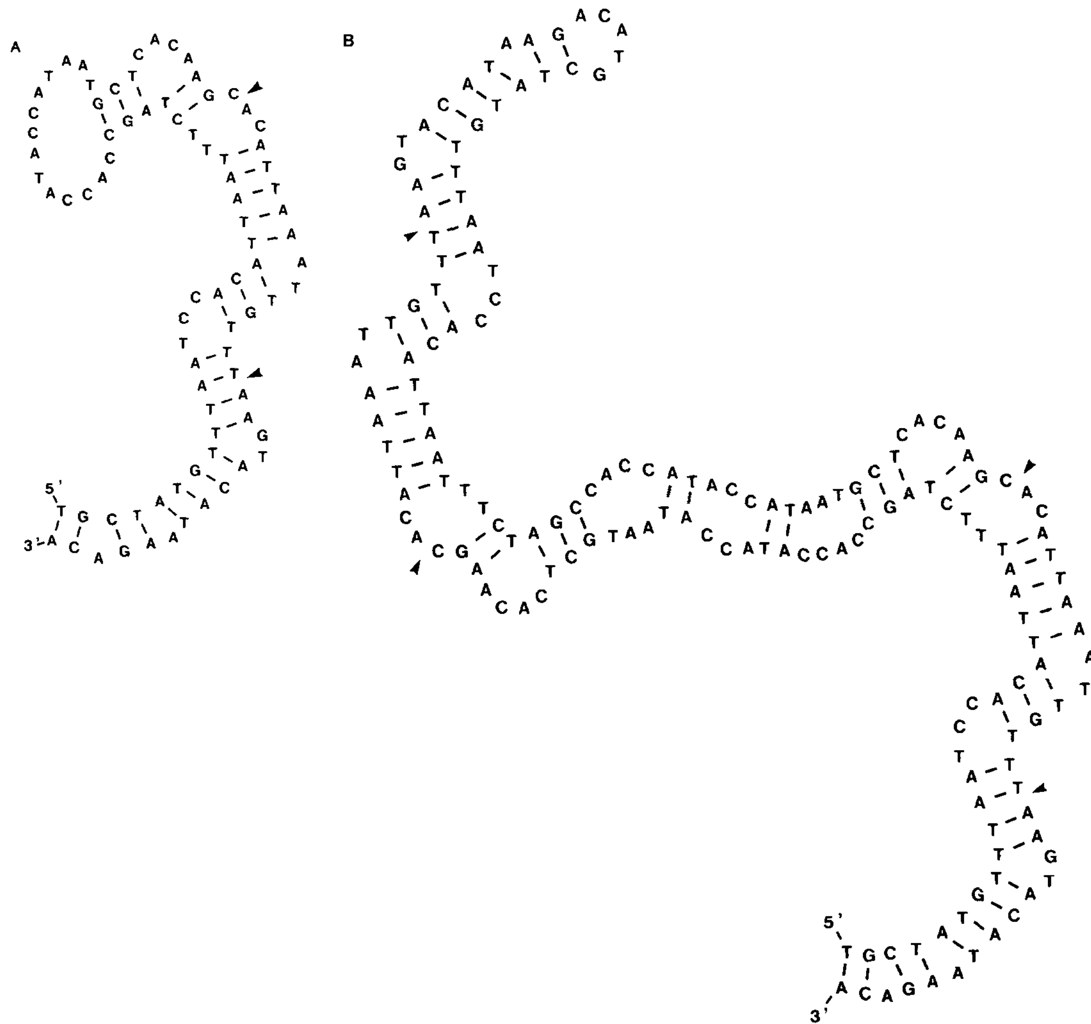

FIGURE 3.—(A) Internal pairing capability of a light strand repeat sequence. The limits of the repeat (starting 4 bp 3′ from tRNA[Pro]) shown here maximizes the stability of the hairpin structure and ensures base-pairing to the termini of the repeat element. Alternative choices of repeat start points result in structures, when folded, that have lower stability and protruding termini. The ends of the TAS sequence are indicated by arrowheads. (B) Alternative folding for two adjacent light strand repeats forming a single long hairpin.

placing the H-strand (heavy strand) from the L-strand (light strand) (CLAYTON 1982). The D-loop strand extends from the consensus sequence blocks (CSB) to a point downstream from the TAS sequence. When multiple TAS sequences are present, the D-loop strand on different mtDNA molecules can terminate downstream from any of the TAS sequences (DODA, WRIGHT and CLAYTON 1981). An individual with multiple copies of the repeat will therefore have several different lengths of D-loop strands. The resting state of the D-loop region is triple stranded, with the D-loop strand and the H-strand competing for base pairing with the single L-strand. It is known that the D-loop strand may be completely displaced from the L-strand and is subsequently replaced by a newly synthesized D-loop strand (reviewed in CLAYTON 1982). It is also known that nascent H-strands, synthesized during replication, initiate at the same point as the D-loop strands (ROBBERSON, CLAYTON and MORROW 1974; TAPPER and CLAYTON 1981), although it is not known whether they actually use D-loop strands as primers.

To account for the gain or loss of repeat units, we assume that there is a dynamic competitive equilibrium between the D-loop strand and the H-strand for base pairing with the L-strand. If the D-loop strand is partially displaced by the H-strand, and then successfully reinvades, misalignment in the repeat region may easily occur. If the D-loop strand is partially displaced, the repeat units would form relatively stable hairpin loops, shortening the displaced strand (Figure 4A) and increasing the likelihood of misalignment. Alternatively, the paired H- and L-strands can form a cruciform structure (Figure 4B). If a misaligned D-loop strand is extended into a nascent H-strand during replication, gain (Figure 4A) or loss (Figure 4B) of a repeat unit will occur.

This mechanism forms a heteroduplex molecule

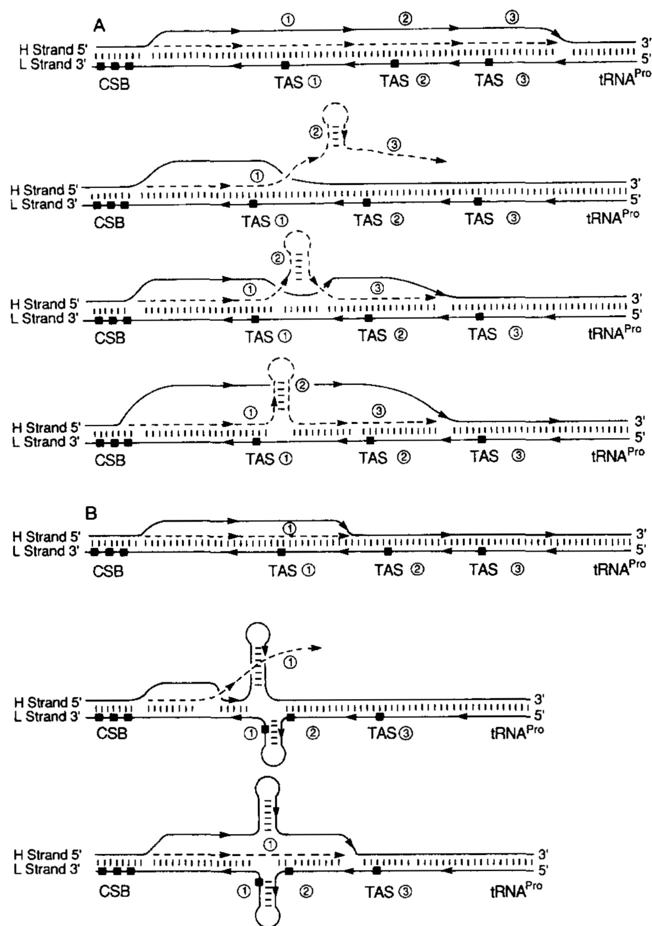

FIGURE 4.—(A) Addition of a repeat when a long D-loop strand is partially displaced by the H-strand. The arrowheads on each strand indicate the endpoints of the repeats, based on the termination points for the D-loop strands. When the repeat region is partially displaced, it would tend to form relatively stable hairpins. If the 3′ end of this shortened strand reinvades, it will most likely do so at an upstream copy of the repeat. Base pairing both upstream and downstream from the hairpin ensures precise alignment with any copy of the repeat. (B) Loss of a repeat when a short D-loop strand is partially displaced by the H-strand. If the 3′ end of the D-loop strand reinvades at a downstream copy, the L-strand will be forced into a hairpin loop. If the D-loop strand in this configuration is extended into a nascent H-strand, a copy is lost. Both processes, (A) and (B), result in a heteroduplex molecule. The heteroduplex would be resolved into molecules of two different sizes at the next replication, resulting in heteroplasmy.

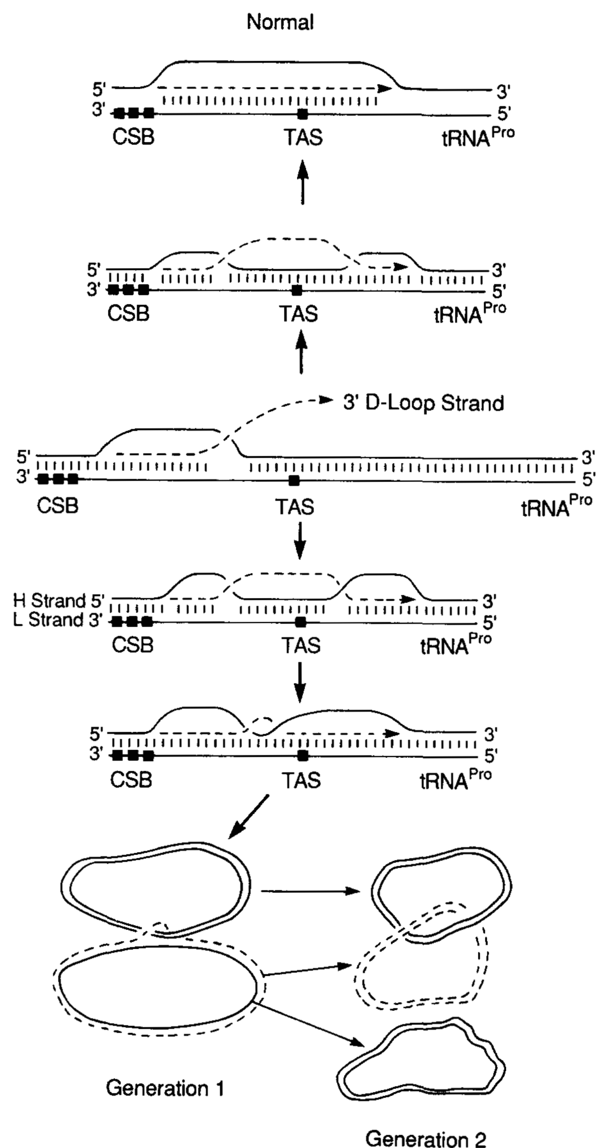

FIGURE 5.—The model outlined in Figure 4 requires that the D-loop strand and the H-strand be in a dynamic, reversible equilibrium for pairing with the L-strand. If this mechanism is correct, the free end of the D-loop strand could reinvade from either side of the H-strand. If it reinvades from the same side the result is normal replication (upper alternative). If the D-loop strand is looped around the H-strand (lower alternative), the result is catenated dimers.

with one or more repeat units unpaired. The heteroduplex would be stabilized by the internal base pairing capability of the repeats. Resolution of the heteroduplex would occur at the next replication.

This model takes advantage of the unique properties of the D-loop region of the mtDNA molecule, and of the presence of the TAS sequences in each repeat unit in the sturgeon mtDNA molecule. The model accounts for the precision of the repeat units, since perfect register of the misaligned strands is assured by base pairing both upstream and downstream from the putative single stranded hairpin loop. The mechanism also accounts for the abundance of repeat copies in sturgeon. When two or more copies of the region are present in a mtDNA lineage, increase in copy number is at least as easily accounted for as loss of a repeat unit. The model is fundamentally distinct from other models based on polymerase stalling in homopolymer regions (HAUSWIRTH *et al.* 1984) or replicative misalignments that can result only in the deletion of repeat sequences (EFSTRATIADIS *et al.* 1980).

The model allows us to make several predictions. First, if heteroplasmy arises by this mechanism, we would expect the minimum number of repeat copies in a heteroplasmic individual to be three, with the central, active copies perfectly conserved. Consequently, the smallest *Bcl*I fragment within a hetero-

or homoplasmic individual would be 1.6 kb. This prediction has been confirmed by hybridization of radioactive sturgeon D-loop probes to Southern transfers of *Bcl*I digested mtDNA of 128 individuals. The smallest fragment observed in 76 homoplasmic individuals and 52 heteroplasmic individuals was 1.6 kb. Second, we would predict that the first and last copies could diverge somewhat in sequence, as long as perfect register pairing at both ends of the D-loop strand is maintained. Third, we would predict that the central repeat(s) should evolve in a concerted manner. That is, distant clonal lines with multiple repeats may show nucleotide differences, as long as the mechanism is not disrupted, but all central repeats within a clonal line should be identical.

If our dynamic, competitive equilibrium model is correct, it might be useful to consider what would happen if reinvasion by the 3′ end of the D-loop strand occurs from the other side of the H-strand (Figure 5). The result is a D-loop strand wrapped around the H-strand. If such an alignment is extended into a new H-strand, the new strand will be catenated through the old H-strand. There is evidence for such linked mtDNA molecules (CLAYTON 1982). Repeat units are not necessary for this result, just a competitive equilibrium between the H-strand and D-loop strand for base pairing on the L-strand.

Heteroplasmy has always been regarded as a necessary, but transient, stage for any evolutionary change in the mitochondrial genome. It has been noted that the majority of changes distinguishing the sequences of species are point mutations (BERMINGHAM, LAMB and AVISE 1986). Yet the majority of cases of heteroplasmy are length differences (HALE and SINGH 1986; reviewed in MORTIZ, DOWLING and BROWN 1987). The only well-documented case following a point mutational variant through several generations (HAUSWIRTH and LAIPIS 1982) showed that, in cows, segregation sorts out the different sequences in five or fewer generations. Heteroplasmy for length variation, in contrast to nucleotide substitutions, is apparently persistent across generations (HARRISON, RAND and WHEELER 1985; SOLIGNAC *et al.* 1987). We believe that it is necessary to consider three different mutation rates: that for point mutations; that for size changes not mediated by repeat sequences and secondary structure; and that for which direct repeat sequences facilitate recurrent mutations.

The precision with which recurrent duplications can occur for direct repeats containing TAS sequences, and having relatively stable secondary structure, is evident from the precision of the four repeats in the large fragment of the sturgeon D-loop reported here. If these results can be generalized, then the frequency and apparent persistence of size heteroplasmy across generations may be a result of high recurrent mutation, maintaining the heteroplasmic condition in the face of a tendency for rapid segregation to eliminate it.

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