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In vivo and *in vitro* evidence for slipped mispairing in mammalian mitochondria

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ABSTRACT **Slipped mispairing between repeated sequences during DNA replication is an important mutagenic event. It is one of several suggested mechanisms thought to be responsible for generating polymorphic regions and large-scale deletions found in mammalian mitochondrial DNA. In the porcine mitochondrial genome, a domain carrying a 10-bp tandemly repeated sequence displays a unique *in vivo* pattern of repeat copy number polymorphs. Upon passage in *Escherichia coli*, a recombinant plasmid containing this domain also displays a unique polymorphic pattern that is different from that seen in the animal. To test the hypothesis that these polymorphisms were slippage induced and that the different polymorphic patterns reflected differences in modes of replication, we performed a series of *in vitro* primer extension reactions. By utilizing either single- or double-stranded templates containing the repeat domain we were able to correlate *in vitro* generated repeat polymorphism patterns with those seen in the mitochondria or the bacteria, respectively, thus providing experimental evidence that slippage replication is responsible for a major class of mammalian mutations.**

Although the existence of tandemly repeated sequences in the mammalian nuclear genome has been known for some time (1), their presence in the mammalian mitochondrial genome is a relatively recent discovery (2, 3). Domains containing tandemly repeated DNA sequences are often highly polymorphic in length due to the propensity of repeat sequences to undergo addition and deletion events. Slipped mispairing between adjacent or nearby repeat sequences during replication is one of several mechanisms by which these types of mutations are thought to occur (4). Slipped mispairing between distant repeats has also been suggested to be responsible for larger-scale mitochondrial deletions associated with a variety of neuromuscular disorders in humans (5, 6). Deletion mechanisms have received widespread experimental attention in prokaryotes (7–9) and to a lesser extent in lower eukaryotes (10) but are, to date, not as well understood in higher organisms or in the mitochondrial organelle.

To investigate mechanisms responsible for DNA plasticity in mitochondria, we analyzed a repeat domain present within the porcine mitochondrial genome. This domain, located at the 5′ end of the displacement loop (D loop) between conserved sequence blocks 1 and 2, is composed of 14–29 copies of a 10-base pair, self-complementary, tandemly repeated sequence CGTGCGTACA (11). During our initial characterization of this region, we noted that repeat domains isolated from a single animal contained a disproportionate abundance of repeat copy number polymorphs that differed in length by multiples of 20 bp (two repeat units). In contrast, upon passage in *Escherichia coli*, plasmid DNAs containing this repeat domain generated polymorphs that differed in length by multiples of 10 bp (one repeat unit). These distinct differences between *in vivo* mitochondrial and bacterial replication products suggested that regeneration of each pattern *in vitro* would yield insight into the mechanism of deletion formation in mammalian mitochondrial DNA (mtDNA).

MATERIALS AND METHODS

Isolation of mtDNA and Cloning of the Repeat Domain. mtDNA was prepared from individual porcine livers as described (12). As diagramed in Fig. 1*A*, a 700-bp *Hpa* II fragment [nucleotides 732–1431; position numbering is according to Mackay *et al.* (12)] containing a repeat domain composed of 17 units was isolated, ligated into the *Acc* I site of pBS(+) (Stratagene), and used to transform *E. coli* DH5-α cells. Exonuclease BAL-31 was used to generate a series of deletion clones, two of which were used in this study, pMR17 (nucleotides 732–1003) and pMR17(+100) (nucleotides 732–1103).

Primer Extension Template Preparation. Plasmid DNAs were digested with either *Hin*dIII and *Pvu* II, *Eco*RI and *Pvu* II, or *Pvu* II only (Fig. 1*A*), and the fragments were resolved on 5% polyacrylamide gels. After staining with ethidium bromide, multiple polymorphic bands were visible; the main band containing the parental number of 17 repeat units was excised and eluted from the gel. This purified homogeneous fragment was then subjected to electrophoresis on a strand separation gel (13) to generate single-stranded DNAs used in the primer extension reactions.

Primer Extension Reactions. *Single-stranded template reactions.* Three nanograms of ^{32}P 5′ end-labeled oligonucleotide primer (complementary to either the T7 or T3 promoter sequences) and ≈400 ng of single-stranded template DNA, isolated from either gel-purified *Pvu* II–*Hin*dIII (containing the T7 promoter) or *Pvu* II–*Eco*RI (containing the T3 promoter) fragments of clone pMR17, were mixed in a reaction buffer of 50 mM Tris·HCl, pH 7.5/50 mM NaCl/10 mM $MgCl_2$ and heated to 80°C. After cooling to room temperature over 2 hr the samples were chilled on ice, and the four dNTPs (300 μM each) along with 2 units of Klenow DNA polymerase were added. The samples were incubated for 1 hr at 37°C, and the products were resolved by electrophoresis on a 6% denaturing polyacrylamide gel alongside a corresponding dideoxy sequencing reaction utilizing the same primer oligonucleotide.

Single-stranded templates with extended 5′ ends. Primer extension reactions were performed as above using longer single-stranded templates as outlined in Fig. 1*A*.

Double-stranded templates. A single-stranded template DNA isolated from a gel-purified *Pvu* II fragment of clone pMR17 was annealed to labeled T7 primer and a partially complementary single-stranded DNA isolated from gel-purified *Pvu* II–*Hin*dIII fragments of clone pMR17(+100). Due to the lack of homology between the extra 100 bases of

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porcine mtDNA originating from clone pMR17(+100) and the vector DNA of plasmid pMR17, the annealed construct results in a double-stranded template that contains a 100-bp nonhybridizing tail on the 5′ end of the displaced strand.

RESULTS

Secondary Structure and Electrophoretic Gel Analysis of the Porcine Repeat Domain and Bacterial Plasmid DNA. The porcine repeat domain and flanking regions have been previously sequenced, and the range of repeat copy number variability was determined (refs. 11 and 14; Fig. 1*A*). Because the frequency of slipped mispairing is increased within sequences capable of secondary structural formation (7), we analyzed the porcine repeat sequence using a computer folding analysis (15). As seen in Fig. 1*B* for two representative hairpins composed of three or four repeats, a strong potential exists for the repeat domain to form secondary structures composed of either odd or even numbers of repeat units.

Total porcine mtDNA was isolated from a single animal, and the composition of the repeat length polymorphs was analyzed by restriction digestion (Fig. 1*C*, left). A distinct alternating pattern of band intensities was clearly visible, with length morphs differing by 20 bp (two repeat units) predominating. Similarly, after passage through *E. coli*, a recombinant plasmid containing 17 repeat units (pMR17) also generated a series of length polymorphs (Fig. 1*C*, right). However, unlike the animal, the pattern of replication products in bacteria was composed of a major species corresponding in size to the parental cloned repeat and a series of mostly smaller, submolar 10-bp (single unit) polymorphs; no alternating pattern of 20-bp repeat polymorphs was seen. To confirm that polymorphs were due to deletions of repeat units, a submolar polymorphic fragment that migrated as a species 20 bp shorter than the main polymorph was excised from a gel and directly sequenced. We found it to be the expected two repeat units shorter than the parent (data not shown).

Regeneration of the Porcine and Bacterial Repeat Patterns *in Vitro*. Mammalian mitochondria (16, 17) and the *E. coli* strain used here [DH5-α (BRL)] are considered deficient for recombination. Therefore, the presence of length polymorphs in the animal and the cloned mtDNA are most likely the result of errors incurred during DNA replication. We hypothesized that the presence of repeated sequences led to replication slippage events and that the differences in the deletion patterns reflected the different nature of the template strands

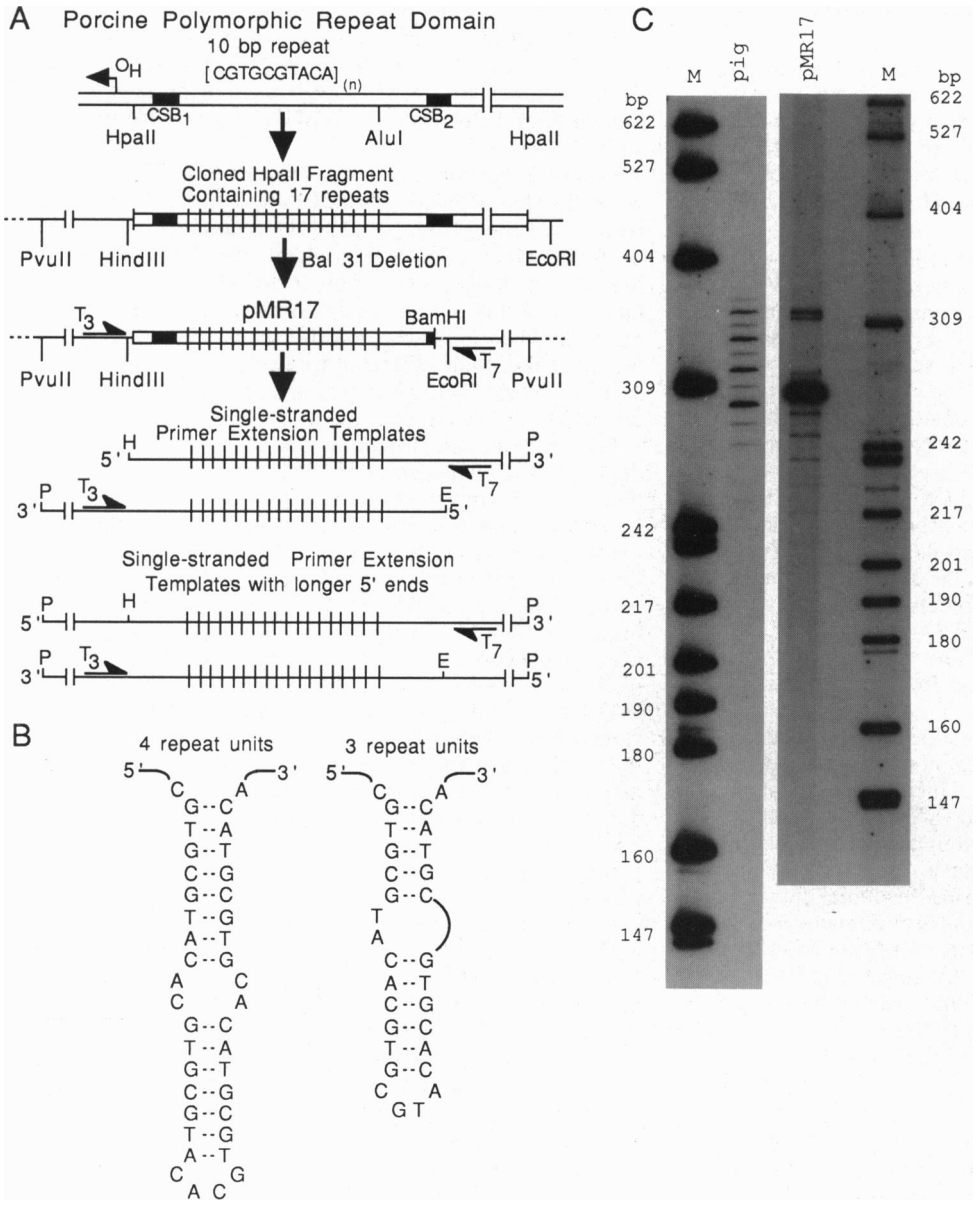

FIG. 1. Deletion-addition patterns of the porcine repeat domain in animal mtDNA and bacterial plasmid DNA. (*A*) Diagram of the polymorphic (14–29 copies) 10-bp tandemly repeated sequence located between conserved sequence blocks 1 and 2 (CSB_1 and CSB_2) and adjacent to the origin of heavy strand DNA synthesis (O_H) in the porcine mitochondria. Also included is a diagram of the restriction sites of deletion clone pMR17 used to generate the primer extension templates and relevant properties of each template. (*B*) Computer folding analysis of single-stranded DNAs containing either four or three repeat units generating stable hairpin structures [−14.0 kcal and −8.8 kcal, respectively (1 kcal = 4.18 kJ)]. (*C*) Left: Total porcine mtDNA was isolated, digested with *Hpa* II, 5′ ^{32}P end-labeled, and run on a 6% polyacrylamide gel. Unresolved polymorphic fragments at about 700 bp containing the repeat region were eluted from the gel, digested with *Alu* I, and resolved on a 6% denaturing polyacrylamide gel to reveal an alternating repeat deletion pattern. Right: After passage through *E. coli*, the repeat domain contained within pMR17 was excised by digestion with *Hin*dIII and *Bam*HI, 5′ end-labeled, and digested with *Alu* I. Electrophoretic gel analysis of the cloned repeat domain reveals the lack of an alternating deletion preference. Lanes "M" contain size markers as indicated.

during replication of the two genomes. Unidirectional replication in mammalian mitochondria creates long stretches of displaced parental DNA single strands (16, 18). The displaced strand, by virtue of maintaining unpaired sequences, would be expected to promote slippage-induced deletions between repeated sequences when copied later in the replication cycle (6). In contrast, bacterial plasmids undergo bidirectional replication (19), in which both parental strands are synthesized coordinately. Thus, the dynamics of slip mispairing on either the leading or lagging strand would be expected to be significantly altered relative to unidirectional replication.

To test our hypothesis we performed a series of primer extension reactions using as templates either single- or double-stranded DNA fragments containing the repeated region of porcine mtDNA (Figs. 2 and 3). To accurately assess the nature of replication products in this type of assay, it was important that the template DNAs were homogeneous with respect to repeat copy number. Therefore, as described in *Materials and Methods*, care was taken in the purification of template DNA strands, such that all templates contained only 17 repeat units. Primer extension on single-stranded templates yielded the expected full-length product as well as series of lower molecular weight products (Figs. 2*A* and 3*A*). In Fig. 2*A*, consistent with that seen in the animal, a pattern of alternating band intensities was evident, with every other 10-bp polymorph being clearly predominant. The pattern extended for 160 bp below the full-length product, as expected if the repeat domain defined the deletion. Primer extension of the complementary strand produced a similar pattern of deletion polymorphs (Fig. 2*B*). The 20-bp deletion polymorph was favored to such a degree that the intervening band was not as pronounced as in Fig. 2*A*. This deletion pattern extended for 140 bp below the full-length product.

To effectively extend a primer through a double-stranded DNA template without the aid of an added unwinding activity, we constructed a template in which a 100-bp noncomplementary 5′ tail was attached to the displaced strand of the duplex template (Fig. 3*B*). Primer extension of this duplex template generated primarily full-length product along with a series of short polymorphic bands. In this case there was no evidence for an alternating pattern similar to that seen for the single-stranded template. Rather, the polymorphic bands formed a regular pattern of 10-bp deletions similar to that generated during bidirectional replication of plasmid DNA in *E. coli*.

To determine whether pausing rather than repeat-induced deletions accounted for these *in vitro* patterns, we constructed single-stranded templates with longer 5′ ends (Fig. 1*A*). If the alternating patterns observed in Figs. 2 *A* and *B* had been the result of site-specific pausing during DNA synthesis, then, regardless of template length beyond such pausing sites, the submolar pattern of polymorphic DNA products should remain unchanged in size and therefore unshifted on the gel. Conversely, if the alternating pattern resulted from internal deletion events followed by continued synthesis to the end of the template, then lengthening of the template strand past the site of deletion should result in similarly lengthened primer extension products and an upward shift of the alternating pattern on the gel. As shown in Fig. 2*C*, primer extension on the longer templates produced a higher molecular weight version of the same alternating intensity band pattern, coinciding with the expected full-length product. The presence of several bands whose sizes do not correspond with the alternating pattern (mainly products in the T7 reaction located below the alternating pattern) is likely to represent template-specific polymerase pause sites. The lack of corresponding bands of relative size in the shorter

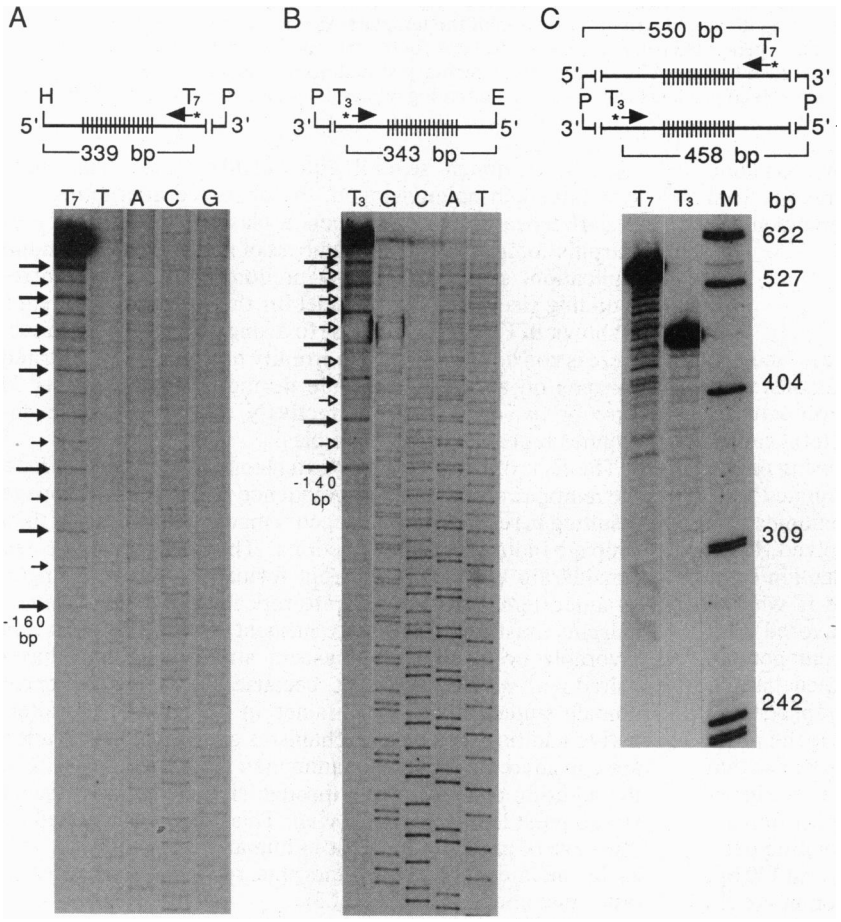

FIG. 2. Primer extension reactions using purified single-stranded templates. (*A*) Primer extension using single-stranded templates generated from *Pvu* II–*Hin*dIII fragments of clone pMR17. An alternating pattern of 10-bp polymorphs (large and small filled arrows) that extends to −160 bp (relative to the full-length product) is easily observable (lane T_7). Lanes T, A, C and G refer to base-specific lanes of a dideoxy sequence of the repeat region that serves as a size marker. (*B*) Primer extension reaction in the opposite direction using complementary single-stranded templates generated from *Pvu* II–*Eco*RI fragments of clone pMR17. A strong pattern of 20-bp polymorphs and faint intervening bands (lane T_3; filled and open arrows, respectively) extending to −140 bp is readily visible. Size marker lanes are as above. (*C*) Primer extension reactions using single-stranded templates with extended 5′ ends generated from *Pvu* II digestion of clone pMR17. The observed shift of primer extension products to higher molecular weights provides evidence that the alternating pattern is due to internal deletion events followed by continued DNA synthesis and not polymerase pausing within the repeat domain.

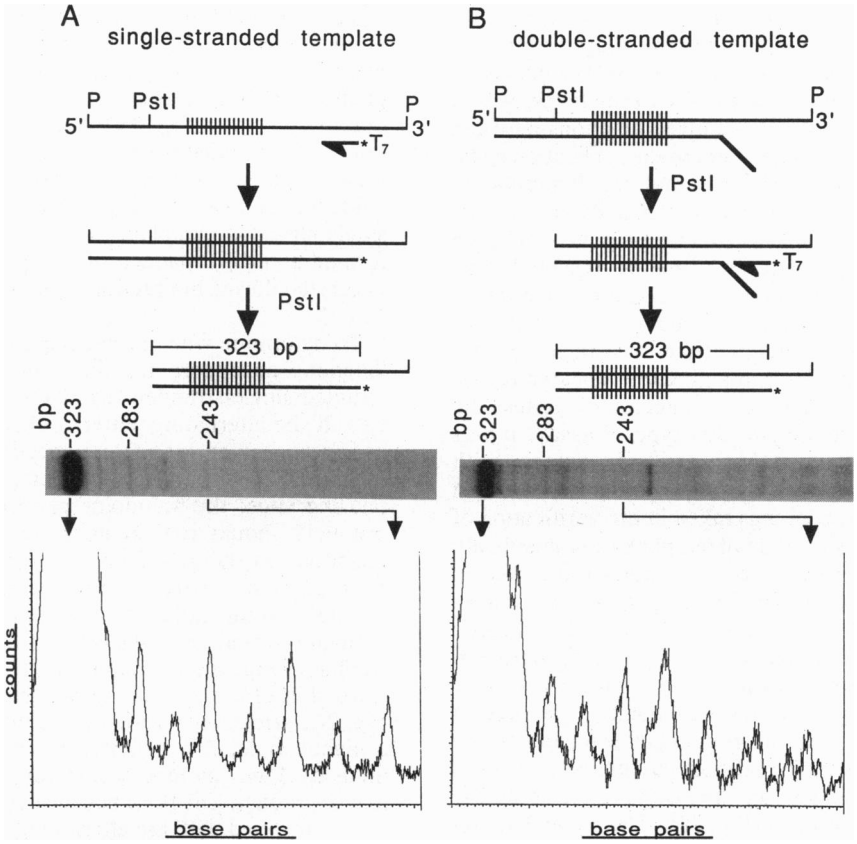

FIG. 3. *In vitro* primer extension reproduction of the two different *in vivo* repeat deletion patterns using single- or double-stranded templates. (*A*) Except for a final *Pst* I digestion, primer extension of the single-stranded template was as described in the legend to Fig. 2*A*. A quantitative radioactivity scan generated from a PhosphorImager analysis (Molecular Dynamics) of the resolved products reveals the "animal-like" alternating deletion pattern. (*B*) Primer extension (using the same reaction conditions as for single-stranded templates) of a double-stranded template containing a 100-bp noncomplementary 5′ tail on the displaced strand. Digestion of the template with *Pst* I prior to primer extension allowed us to discriminate between primer extension products generated from the shorter (323 bp) double-stranded template and those generated from any excess longer (550 bp) single-stranded DNA that failed to anneal with the complementary strand used to make the duplex template. A scan generated from a PhosphorImager analysis of the resolved products reveals a nonalternating repeat deletion pattern similar to that seen in the bacterial plasmid DNA.

template reactions suggests that this is the case. We conclude that the presence of the alternating band pattern results from a repeat-induced deletion mechanism that is dependent on DNA synthesis using a single-stranded template.

DISCUSSION

On the basis of the presence of repeat sequences and the strandedness of the DNA template, we have demonstrated that the major difference between the two deletion patterns seen *in vivo* in mammalian mitochondria and bacteria can be reproduced *in vitro*. Primer extension reactions using only a prokaryotic DNA polymerase cannot be fully equated with the complex process of replicating eukaryotic genomes, yet it is known that regardless of the machinery involved, DNA repeat sequences themselves can influence replication error frequency (20). What remains to be understood is why an alternating pattern of deletion polymorphs is favored with single-stranded templates. The ability of adjacent porcine mtDNA repeat units to form stable self-annealed hairpin structures suggests that, as described for bacteriophage and plasmid genomes (8, 9), hairpin structures increase the probability of deletions in animal mtDNA. Under the premise that hairpin formation promotes replication slippage in single-stranded templates, deletions larger than the repeat domain should not occur. Consistent with this, the alternating deletion pattern produced *in vitro* does not extend beyond 170 bp, the size of the parental clone (pMR17) repeat domain (e.g., Fig. 2). Although several equivalently stable hairpinned structures can be envisioned, the observed alternating pattern of repeat deletions reflects a bias toward formation of hairpins composed of even numbers of repeat units that, upon replication, serve to create a predominance of the corresponding size deletion. A model for this deletion preference is shown in Fig. 4. In contrast to a single-stranded template, there is considerably less opportunity to form template strand hairpins on a duplex molecule during replication either *in vitro* or *in vivo*, thereby effectively eliminating the even-number repeat copy deletion bias.

The lack of visible primer extension products larger than the template strand provides evidence that, *in vitro*, slippage resulting in repeat additions occurs much less frequently than slippage-induced repeat deletions. This is not unexpected considering the ease of hairpin formation within a single-stranded template. To generate repeat additions, however, hairpins must form within the nascent strand (Fig. 4), a less favorable event since the nascent strand is initially base-paired with its template. Yet, because the size of the repeat domain studied here is maintained in the animal, and alternative addition–deletion mechanisms such as recombination are considered unlikely in mammalian mitochondria (16, 17), the addition of repeat units through slippage of the nascent strand most likely occurs *in vivo*. This view is supported by one case of an addition event in human mtDNA (21) as well as by maintenance of polymorphic repeated sequences in other mammalian species (2, 3).

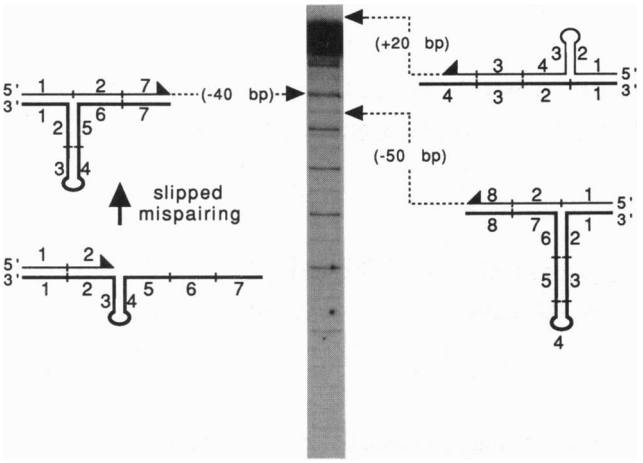

FIG. 4. Model for generation of the alternating repeat deletion pattern. (*Left*) Within a single-stranded repeat domain the smallest stable self-annealed structure is the two-unit repeat hairpin. Once formed, this structure would promote expansion of the hairpin using pairs of flanking repeats, resulting in various sized hairpins composed of even numbers of repeat units (refer to Fig. 1*B*). Slipped mispairing in the nascent strand between repeats during replication produces a deletion corresponding in size to the template hairpin composed of even numbers of repeat units (e.g., −20 bp, −40 bp, −60 bp, etc.). Note that one of the two flanking parental repeat units (no. 6) is lost, analogous to the situation in large human mtDNA deletions. (*Lower Right*) Alternatively, the occurrence of slippage events across hairpins composed of odd numbers of repeat units (refer to Fig. 1*B*) would likewise result in a series of corresponding size deletions (e.g., −30 bp, −50 bp, −70 bp, etc.). A preference for formation of even versus odd repeat copy number hairpins explains the observed alternating pattern of band intensities from single-stranded templates (Figs. 2*A* and 3*A*). This is consistent with the expectation that the most frequent initial event in hairpin formation would be pairing between two adjacent repeat units (the smallest stable hairpin). (*Upper Right*) Although none was evident in the *in vitro* reactions, primer extension products longer than the expected full-length product would require slippage-induced repeat additions through hairpin formation within the nascent strand.

The porcine domain investigated in this study possesses five relevant properties: (*i*) the region contains repeats, (*ii*) the repeats are direct, (*iii*) deletions result in the loss of repeat units, (*iv*) the repeats are tandemly arrayed, and (*v*) the repeats are self-complementary, allowing for hairpin formation within and between repeat units. The first three characteristics have often been associated with changes in mtDNA structure found in humans with mitochondrial diseases. In a large majority of patients affected tissue has been shown to contain mitochondrial genomes with large (2–10 kb) deleted segments that are flanked by direct repeats in normal mitochondria (5, 6, 22). The loss of one of the flanking repeats during these large-scale deletion events is mimicked by the model system studied here and strongly implicates slipped mispairing between repeats as the causative mechanism.

Equating the mechanism of formation of these large-scale deletions in humans, where substantial hairpin formation between direct repeats is not expected, with the smaller deletion events described in this study must take into account differences in their structures, particularly properties *iv* and *v*. Bacteriophage studies demonstrate that hairpins are not an absolute requirement for slipped mispairing but, rather, appear to enhance the rate at which slipped mispairing occurs (9). Thus, the apparent absence of hairpin formation within human mtDNA deleted regions may affect only the frequency at which slipped mispairing occurs. Consistent with this idea, PCR analysis was recently used to demonstrate that large deletions flanked by direct repeat sequences are indeed present at low levels in mtDNAs of phenotypically normal humans (23, 24). Importantly, the single-stranded nature of nontemplate DNA strands during mtDNA replication may allow juxtaposition of direct repeats within replicative intermediates even when separated by great distances, thus enhancing the probability of slipped mispairing. It is interesting to note that almost all deletions found in human mtDNA occur between the origins of heavy and light strand DNA synthesis, a region that may be single-stranded for the greatest period of time during mtDNA replication. Therefore, since both types of deletions appear to rely heavily on the presence of direct repeats, a common mechanism is suggested.

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