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Replication priming and transcription initiate from precisely the same site in mouse mitochondrial DNA

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Mammalian mitochondrial DNA maintains a novel displacement-loop region containing the major sites of transcriptional initiation and the origin of heavy strand DNA replication. Because the exact map positions of the 5′ termini of nascent mouse displacement-loop strands are known, it is possible to examine directly a potential relationship between replication priming and transcription. Analyses of *in vivo* nucleic acids complementary to the displacement-loop region reveal two species with identical 5′ ends at map position 16 183. One is entirely RNA and the other is RNA covalently linked to DNA. In the latter the transition from RNA to DNA is sharp, occurring near or within a series of previously identified conserved sequences 74–163 nucleotides downstream from the transcriptional initiation site. These data suggest that the initial events in replication priming and transcription are the same and that the decision to synthesize DNA or RNA is a downstream event under the control of short, conserved displacement-loop template sequences.

Key words: displacement loop/promoter/regulatory sequences/replication origin/RNA processing

Introduction

The mammalian mitochondrial genome is a closed circular DNA of ~16 kb. The only extensive non-coding area is a 1-kb region bounded by the structural genes for $tRNA^{Phe}$ and $tRNA^{Pro}$, where heavy strand (H-strand) DNAs of 500–700 nucleotides (DH-DNA) displace the parental H-strand creating a triple-stranded displacement-loop (D-loop) (Clayton, 1982). The major regulatory sequences located within the D-loop region (Clayton, 1984) include promoters for transcription of each mitochondrial DNA (mtDNA) strand (Chang and Clayton, 1984) and the origin of H-strand mtDNA synthesis. A similar proximity of promoters and an origin of DNA synthesis in other systems has suggested that some products of transcription may be involved in the priming of replication (Itoh and Tomizawa, 1981; Romano *et al.*, 1981; Baldacci *et al.*, 1984). Because the template map positions of DH-DNA termini are known with precision (Gillum and Clayton, 1979; Doda *et al.*, 1981; Tapper and Clayton, 1981; Walberg and Clayton, 1981), it is possible to test this hypothesis for mammalian mitochondria by examining the relationship between DH-DNA and other *in vivo* nucleic acids whose syntheses are also directed by sequences within the D-loop region.

The data reported here show that at least two types of H-strand nucleic acids are synthesized from the mouse D-loop region. The first is an RNA-primed H-strand DNA in which an RNA species is covalently linked to the 5′ end of DH-DNA. Within any one molecule, the transition from RNA sequence to DNA sequence appears to be sharp and complete. The second species is entirely RNA, with the same 5′-terminal sequence, extending past the template positions of potential DNA synthesis. This suggests that both priming of H strand replication and transcription of light strand (L-strand) genes begin at the same sequence in the genome and that the transition to DNA synthesis is a later event modulated by internal control sequences.

Results

D-loop H-strand RNA (DH-RNA)

In vivo H-strand RNA complementary to the D-loop region was initially mapped relative to the origin of H-strand replication by blot analyses. Total nucleic acid from purified mitochondria was fractionated by denaturing gel electrophoresis and immobilized on nitrocellulose filters. These filters were then used for hybridization with radiolabeled L-strand sequences specific either for a short region upstream from known DH-DNA 5′ termini (nucleotides 16 127–16 216) or for DH-DNA (nucleotides 15 497–15 973) (Figure 1A). The upstream probe hybridizes to two abundant species of ~140–155 and 80–120 nucleotides in length (Figure 1B, lane A). Both species are RNA as judged by their resistance to DNase I and susceptibility to RNase T1 (lanes B and C, respectively). A number of discrete, longer RNA species ranging in size from 250 to >2000 nucleotides are less visible. Because each strand of the mammalian mitochondrial genome is expressed as polycistronic precursor RNAs originating from the D-loop region and subsequently cleaved to appropriate mRNAs, tRNAs and rRNAs (Clayton, 1984), these longer transcripts most likely represent processing intermediates of the primary L-strand transcript. In summary, these data demonstrate the presence of a significant quantity of relatively short D-loop H-strand RNAs all of which contain sequences immediately upstream from DH-DNA.

The downstream limit of major H-strand RNA species was assessed by a similar hybridization experiment using the DH-DNA-specific probe (Figure 1C). As expected, this probe hybridizes preferentially to the major DH-DNAs of 520, 560 and 610 nucleotides in length (Clayton, 1982) which are present in mitochondrial nucleic acid isolates. Significantly, none of the shorter H-strand RNA species hybridizing to the upstream probe are detected with the DH-DNA-specific probe; therefore, we can position these RNAs upstream from the *Xba*I site at nucleotide 15 975. DNase I treatment reveals essentially the same set of minor, longer transcripts seen with the upstream probe, consistent with the concept that they represent processing intermediates of the primary L-strand transcript as discussed above. The predominance of the short RNAs, relative to the longer transcripts, suggests an unusual stability relative to other processing intermediates. Although a functional assignment for these shorter transcripts cannot be made from these data, their relative abun-

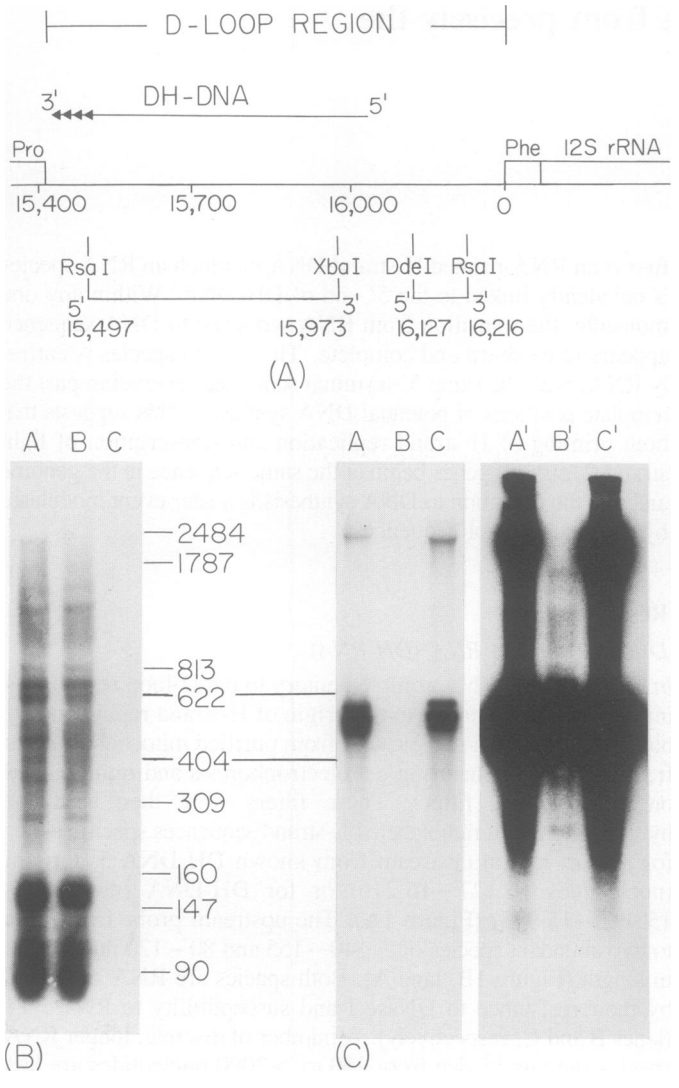

Fig. 1. Organization of the mouse mtDNA D-loop region and identification of DH-RNA. **(A)** The genetic organization of the D-loop is shown, including the 5′ and 3′ termini of major DH-DNA species and the surrounding structural genes for $tRNA^{Pro}$, $tRNA^{Phe}$ and 12S rRNA. Also indicated are the polarity and genomic locations of the DH-DNA specific probe (nucleotides 15 497 – 15 973) and the upstream probe (nucleotides 16 127 – 16 216). Filter hybridization analyses of mitochondrial nucleic acids using the upstream **(B)** or the DH-DNA specific **(C)** probe are also shown. **Lane A,** untreated mitochondrial nucleic acid; **lane B,** DNase I pre-treatment; **lane C,** RNase T1 pre-treatment. After hybridization the blots were exposed for 24 h at −70°C with a Dupont Cronex intensifying screen [**(B)** and right side of **(C)**], or for 30 min at room temperature without an intensifying screen [left side of **(C)**]. The indicated size markers are based on a *Hpa*II digest of 501.1/pBR322 DNA electrophoresed in an adjacent lane for each blot (not shown).

dance, polarity, and map location just upstream from DH-DNA 5′ termini suggest a role in priming H-strand DNA synthesis.

H-strand RNA contains a unique 5′ end and some is covalently linked to H-strand DNA

The existence of H-strand transcripts upstream from DH-DNA makes determination of their exact genome location potentially important in understanding initiation of both transcription and replication. Toward this end, the 5′ termini of these molecules were first mapped by the primer extension technique. A primer located just inside the 5′ end of the major DH-DNA species was constructed from a *Xba*I-*Hph*I fragment (nucleotides 15 975 – 16 000) by 5′ end-labeling the L strand. This oligonucleotide will hybridize to any H-strand species containing 50 nucleotides or more of the 5′ end of the major DH-DNA (Figure 1A). Elongation of all resultant hybrid primer-templates using reverse transcriptase and unlabeled dNTPs will then create fully duplex fragments. Upon denaturation, a family of labeled strands will be released whose lengths accurately map the 5′ termini of all *in vivo* species capable of annealing to the primer.

The products of the primer extension reaction were analyzed on a denaturing gel (Figure 2). To help determine the nature of each visualized band, the mitochondrial nucleic acid preparation was either untreated (lane B), limit DNase I digested (lane C), limit RNase T1 digested (lane D), or treated with alkali prior (lane E) to primer hybridization. Lane B exhibits a complex pattern of products. The major species extending from the primer to nucleotide 16 036 represent the 5′ extent of the major DH-DNA strands reported earlier (Gillum and Clayton, 1979). They are sensitive to DNase I but relatively resistant to RNase T1 or alkali, which suggest the presence of no more than a few ribonucleotides at each 5′ end and no significant frequency of internal ribonucleotides. The small group of bands at 16 057 and the faint family of bands between 16 093 and 16 103 also correspond to previously noted minor DH-DNA species. Gillum and Clayton (1979) reported that 16 057 molecules contain no 5′ ribonucleotides and that all species in the 16 093 – 16 103 group contain 5′-terminal ribonucleotides ranging in length from one to ten. Primer extension analysis confirms these observations because all members of the 16 093 – 16 103 group are shortened to 16 093 upon RNase T1 or alkali pre-treatment, while 16 057 molecules are unaffected. Position 16 093 occurs within a run of guanine residues; thus, as expected, there is no difference between degradation by RNase T1 or alkali treatment.

Three species in lane B of Figure 2 represent molecules not seen by previous analyses of isolated DH-DNA molecules. The most predominant species is an RNA molecule whose 5′ end maps approximately to nucleotide 16 183. This is an entirely RNA species, as evident by its resistance to DNase I pre-treatment (lane C), and the longest molecule detected. As such, it may represent the product of a transcriptional event beginning at or near nucleotide 16 183 and extending at least through nucleotide 15 975. The other new species map approximately at nucleotide 16 092 and at 16 079. Both are minor DNA species not seen in earlier studies, most likely because they do not extend significantly beyond the primer at 15 975 (S1 nuclease analysis, below, which requires all strands to extend at least to 15 851, does not detect these species).

Two aspects of the data in Figure 2 are novel in that they provide evidence for covalent linkage between relatively long tracts of RNA and the 5′ ends of DH-DNA molecules. First, the pattern of bands around 16 029 is dependent on the type of RNA degradative protocol. Relative to the untreated preparation, alkali pre-treatment, which removes all ribonucleotides, gives a pattern of 5′-deoxyribonucleotide ends virtually identical to that analyzed by Gillum and Clayton (1979). However, RNase T1 pre-treatment, which cleaves only after riboguanosine, produces a new major species with its 5′ end at 16 037. This is the position of the first guanine residue upstream from the major DH-DNA 5′ end at 16 029. No cleavage is evident at the next guanine residue (nucleotide 16 028); hence the transition from ribo- to deoxyribonucleotides occurs between 16 029 and 16 037 for most species in this size range. Additionally, because the band at 16 037 generated by RNase T1 is longer than any other species in the untreated sample in this size range, it must have been deriv-

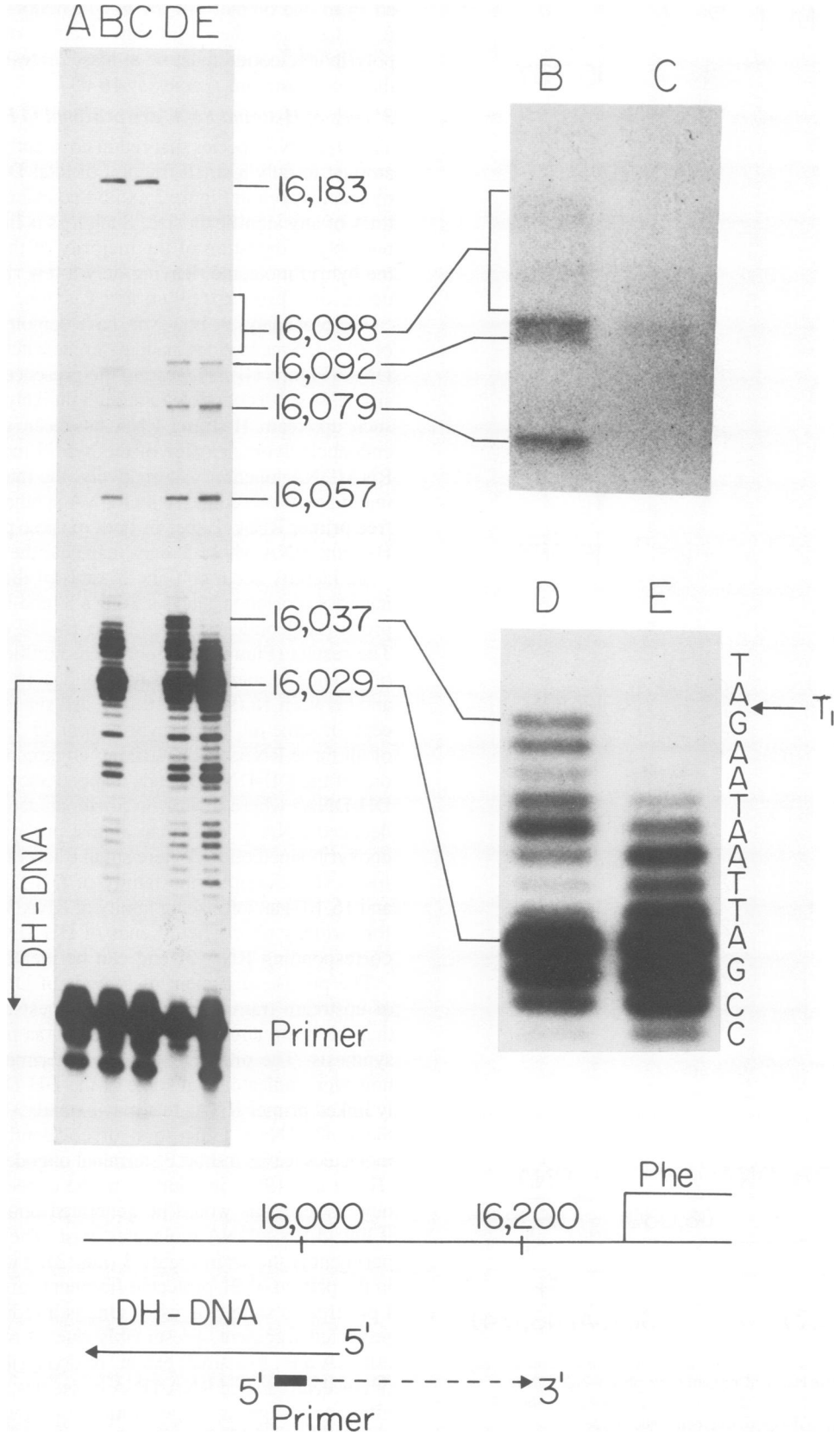

Fig. 2. Location of the 5′ termini of DH-DNA and DH-RNA by primer extension analysis. 10 μg of mitochondrial nucleic acid prepared by hot phenol-chloroform extraction were used in each analysis. The numbers refer to nucleotide positions based on the published mouse mtDNA sequence (Bibb *et al.*, 1981). 5′ end-labeled L strand of a *XbaI-HphI* restriction fragment [nucleotides 15 975(5′) – 16 000(3′)] was used as the primer. **Lane A,** primer-only; **lane B,** total mitochondrial nucleic acid; **lane C,** DNase I pre-treatment; **lane D,** RNase T1 pre-treatment; **lane E,** alkali pre-treatment. The region around 16 100 of **lanes B** and **C** and the region around 16 029 of **lanes D** and **E** are shown enlarged on the right. The nucleotide sequence of the non-coding strand corresponding to the region around 16 029 and the position of RNase T1 cleavage are also shown. The polarity of DH-DNA is indicated on the left.

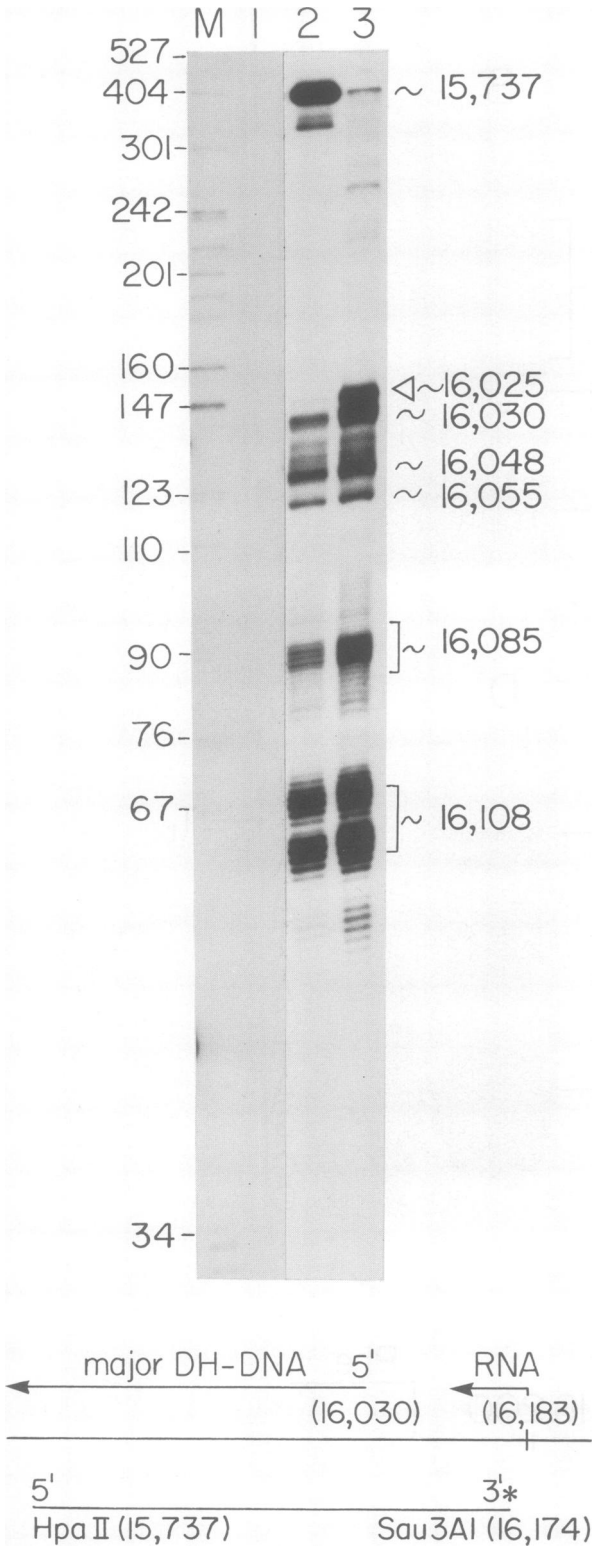

Fig. 3. Location of the 3′ termini of DH-RNA by S1-protection analysis. 10 μg of mitochondrial nucleic acid prepared by phenol-chloroform extraction were used in each analysis. For the S1 protection probe, 3′ end-labeled L strand of a *Sau*3AI-*Hpa*II fragment [nucleotide 16 174(3′) – 15 737(5′)] was used. **Lane M,** *Hpa*II digest of pBR322; **lane 1,** probe DNA only; **lane 2,** total mitochondrial nucleic acid; **lane 3,** DNase I pre-treated sample. The numbers on the right refer to the nucleotide positions (Bibb *et al.*, 1981) of DH-RNA. The 3′ termini uniquely visible following DNase I treatment (see text) are indicated by an open arrowhead. The location of the S1-protection probe, relative to the major DH-DNA species around nucleotide 16 030 and the upstream transcriptional initiation site at nucleotide 16 183, is shown below.

ed from one or more of the longer major bands visible in lane B. Therefore the minimum length of covalently linked polyribonucleotide must be at least 21 residues, the distance to the next upstream species at 16 057.

3′ ends of H-strand RNAs are proximal to H-strand DNA 5′ ends

The DH-DNA species that retain covalently linked primer RNA amount to only a small fraction of total DH-DNA. Most DNA molecules seen in Figure 2 exhibit no evidence of 5′ ribonucleotides of any significant size. Such loss is likely to occur by exonucleolytic digestion of the majority of the 5′ RNA portion of the hybrid molecule, leaving only a few ribonucleotides as evidence of a primer (Gillum and Clayton, 1979).

The blot analyses, however, have demonstrated H-strand RNA of 80 – 155 nucleotides mapping immediately upstream from DH-DNA (Figure 1B), suggesting the presence of abundant primer-size RNAs. It is possible (although unlikely, see Discussion) that such upstream H-strand RNA has been generated by specific endonucleolytic cleavage of the hybrid molecule at or near the RNA/DNA junction. Alternatively, the rate of primer RNA formation may exceed the rate of DNA synthesis, leading to excess free primer RNA. Either or both of these processes would yield H-strand RNA whose 3′ ends map near the 5′ ends of DH-DNA.

To identify accurately the 3′ ends of these transcripts, an S1 nuclease protection analysis using a 3′ end-labeled L-strand DNA probe [nucleotides 16 174 (3′) to 15 737 (5′)] was carried out. The results (Figure 3) demonstrate families of bands indicative of 3′ ends located around nucleotides 16 108, 16 085, 16 055 and between 16 048 and 16 030. All species seen are RNA, except for some full-length hybrids near 15 737. The salient feature of all these RNAs is the striking alignment of each 3′ end with one of the DH-DNA 5′ ends mapped above (Figure 5). In those DH-DNAs where few or no 5′ ribonucleotides were found, the deduced RNA 3′ ends map almost precisely at the 5′-terminal deoxyribonucleotide. Where small tracts of ribonucleotides were found (for example, the family of DH-DNAs between 16 093 and 16 103) an interfacing family of RNA 3′ ends is found. Thus, for virtually all *in vivo* 5′ ends of DH-DNAs mapped above, a corresponding RNA 3′ end can be identified.

The precise alignment of 5′ ends of DH-DNAs and 3′ ends of upstream transcripts strongly suggests, yet does not prove, that the upstream transcripts function as primers for DH-DNA synthesis. The primer extension experiment above (Figure 2), however, indicated that some of the DH-DNAs retain covalently linked primer RNAs that may extend >21 nucleotides. Since the limit DNase I digestion of covalently linked RNA/DNA molecules leaves a short 3′-terminal oligodeoxynucleotide moiety (Hu *et al.*, 1984) in addition to the expected RNA residues, a new 3′ terminus would be generated upon DNase I treatment if linked RNA/DNA molecules exist. The result of such an experiment is shown in Figure 3 (lane 3). Two distinctive changes in the pattern of S1-protected fragments are seen when a DNase I pre-treated sample is used. One is a reduction of the full-size protected fragment, presumably due to removal of full-length mtDNA and, to a small extent, of deoxyribonucleotide residues of covalently linked RNA/DNA molecules. Second, the most significant change is a generation of new 3′ termini around nucleotide 16 025. The fact that the 3′ termini of nucleic acids at this position are only evident after DNase I treatment and that this position extends downstream of other RNA 3′ termini in the untreated sample (lane 2) provide conclusive evidence that it represents limit DNase I digestion products of covalently linked RNA/DNA molecules. Although the exact location of 5′ termini of these molecules cannot be determined from the above ap-

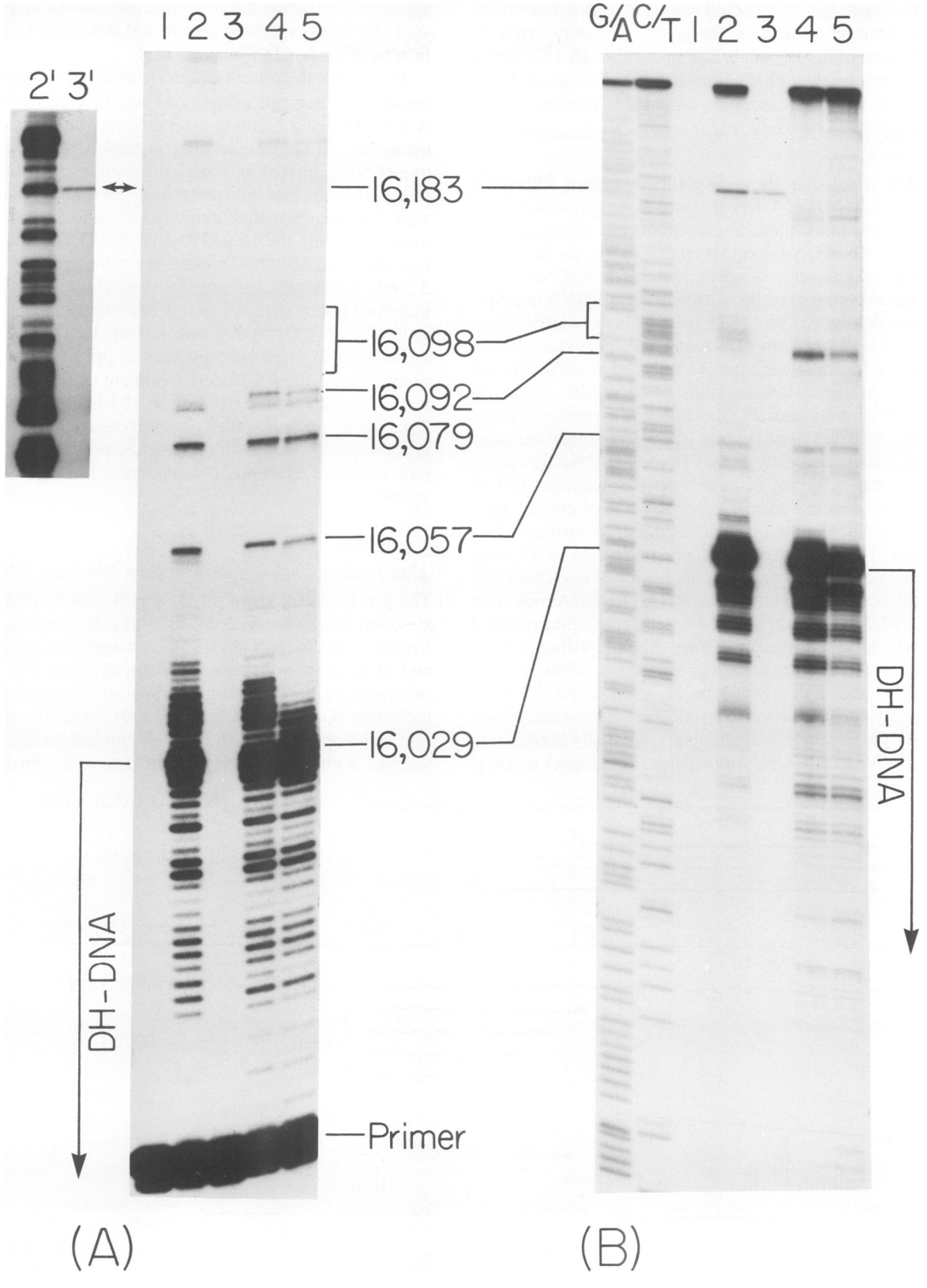

Fig. 4. Location of the 5′ termini of DH-DNA and primer RNA (DHP-RNA) associated with mtDNA by primer extension and S1-protection analyses. 0.5 μg of mitochondrial nucleic acid prepared by ethidium bromide-CsCl buoyant density centrifugation was limit-nuclease treated and analyzed by both the primer extension **(A)** and the S1 nuclease protection technique **(B)**. The numbers refer to the genomic sequence (Bibb *et al.*, 1981). The *Xba*I-*Hph*I restriction fragment as described in the Figure 2 legend was used as the primer. For the S1-protection analyses, 5′ end-labeled L strand of a *Taq*I-*Alu*I fragment [nucleotides 15 851(5′) − 10(3′)] was used as the probe. **Lane 1,** primer only in **(A)** or S1 probe only in **(B); lane 2,** untreated mtDNA; **lane 3,** DNase I pre-treatment; **lane 4,** RNase T1 pre-treatment; **lane 5,** alkali pre-treatment. Lanes G/A and C/T are sequencing ladders generated by partial chemical cleavage of labeled probe. The polarity of major DH-DNA is indicated adjacent to each autoradiograph. A longer exposure of **lanes 2** and **3** in **(A)** around nucleotide 16 183 is shown to the left **(lanes 2′** and **3′)**.

proach, it is evident that they should extend at least to nucleotide 16 174, the position of label on the probe. The only major 5′ end map position of nucleic acids is at nucleotide 16 183; hence these data are consistent with the proposal that all H-strand RNA molecules seen have a common 5′ end at this position.

RNA covalently linked to DNA is harbored in displacement loops in vivo

One question which the above experiment cannot address is whether linked RNA/DH-DNA molecules remain annealed *in vivo* to the whole mitochondrial genome to any significant extent. If hybrid RNA/DNA-loop structures exists, as the above data indicate, they should co-isolate with intact mtDNA. Such structures are expected to be buoyant near form I DNA in a dye-CsCl buoyant density gradient (Gillum and Clayton, 1979; Doda *et al.*, 1981). Therefore form I DNA and molecules buoyant between form I and form II were pooled and used as substrates for an analogous primer extension study. The results, shown in Figure 4A, are almost identical to those seen when total mitochondrial nucleic acids are used (Figure 2). We note that the same feature suggesting covalently linked RNA/DH-DNA is clearly present; i.e., the existence of an RNase T1-generated 16 037 species. Also evident in Figure 4A (and to some extent in Figure 2) is the general increase in the intensity of bands corresponding to the 5′ ends of DH-DNA species around nucleotide 16 030 after RNA degradation (lanes 4 and 5). This feature is also consistent with the presence of covalently linked RNA/DH-DNA molecules as more DH-DNA 5′ ends would be generated upon removal of covalently linked upstream primer RNA. Furthermore, a distinct loss in the 16 183 species is seen upon DNase I treatment (lanes 2′ and 3′) as would be expected if the dye-CsCl buoyant density purification led to an enrichment of hybrid primer RNA/DH-DNA molecules remaining preferentially duplexed to mtDNA, relative to RNA-only molecules. This aspect is not as apparent in Figure 2 due to an over-abundance of the RNA-only 16 183 species compared with hybrid RNA/DH-DNA molecules in total mitochondrial nucleic acid.

To confirm the primer extension analysis, 5′ termini were independently mapped using an S1-nuclease protection protocol. A 5′ end-labeled L strand extending from nucleotide 15 851 to nucleotide 10 (455 nucleotides in total length) was hybridized to mtDNA prepared as above, digested to completion with nuclease S1, and sized on a denaturing gel (Figure 4B). This yields a set of molecules with 5′ termini identical to those seen by primer extension. Only the 16 079 and 16 090 DNA species are missing. As discussed above, these are truncated molecules whose 3′ ends terminate upstream from nucleotide 15 851 and would therefore not be detected in this experiment. The DNA species at 16 029, 16 057, 16 093 – 16 103 and the RNA at 16 183 have the identical 5′ termini to those seen by primer extension analyses. Significantly, upon DNase I treatment, a distinctive reduction in the intensity of the band at 16 183 is also present (lane 3). Thus the S1-nuclease protection analysis confirms the primer extension results, and both approaches provide evidence consistent with the existence of covalently linked RNA/DH-DNA species.

Discussion

Map positions of displacement-loop RNA and DNA

The precise locations of all D-loop H-strand DNA and nearby H-strand RNA termini deduced in this study are summarized in Figure 5. The 5′ ends of DH-DNA mapped by primer extension and S1 nuclease protection are identical to the DH-DNA 5′ termini seen earlier by end-group analysis of isolated '7S DNA' molecules (Gillum and Clayton, 1979). However, the identification and mapping of H-strand RNA molecules are novel observations with several significant features. First, only one

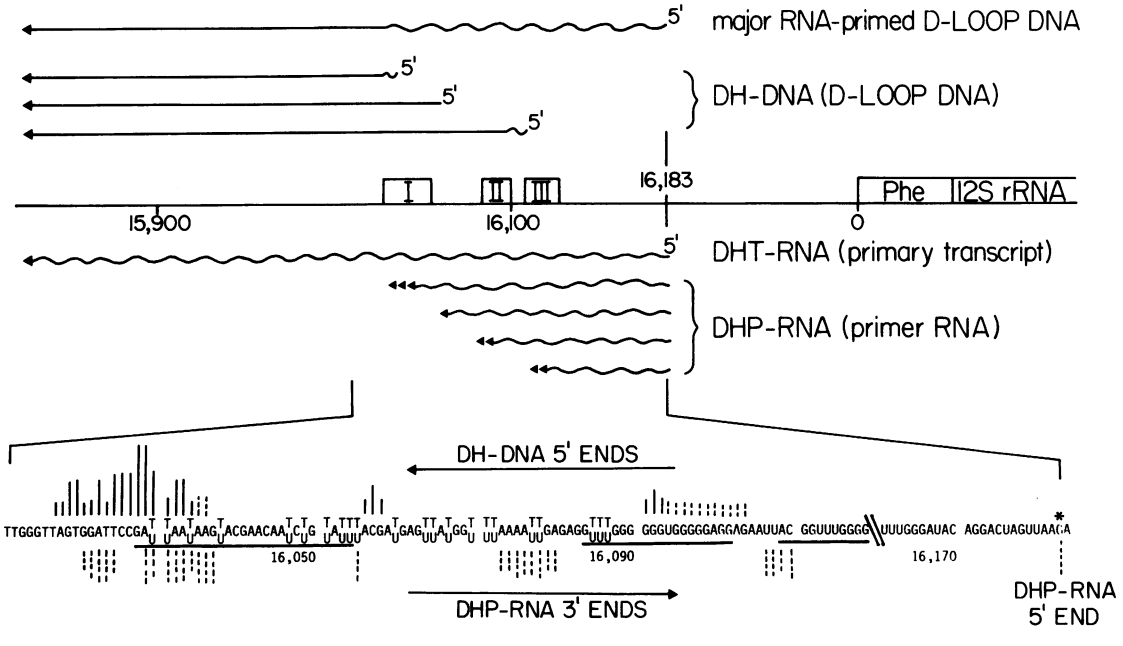

Fig. 5. Schematic diagram showing the DHP-RNA/DH-DNA junctional sites. The D-loop region near the structural gene for $tRNA^{Phe}$ and 12S rRNA is shown. The extent of the DH-DNA species (solid lines) along with their 5′-ribonucleotide residues (wavy lines) and the DHP-RNA species (wavy lines) are also indicated. Multiple solid arrows denote the observed microheterogeneity at the 3′ ends of DHP-RNA. Arrows at the left-hand margin indicate that the species extends beyond the positions shown. The nucleotide sequence of DHP-RNA and the DHP-RNA/DH-DNA junctional sites are shown below. The positions of DH-DNA 5′ termini (above the sequence) and DHP-RNA 3′ termini (below the sequence) are marked by solid lines (deoxyribonucleotide residues) and dotted lines (ribonucleotide residues), respectively. The nucleotide sequence not shown between 16 120 and 16 160 is: UUUUUGUGAU UCUUGAACUU UCUGUUCUGU AUAUUAUAAU UGAUAG. Conserved sequence blocks extending from nucleotide 16 028 to 16 054 (CSB-I), 16 084 to 16 099 (CSB-II), and 16 109 to 16 126 (CSB-III) are boxed in **(A)** and underlined in **(B)**.

predominant RNA 5′ end is detected, suggesting that most RNA from this region may initiate at the same site. Second, the 3′ ends of H-strand RNA align remarkably well with DH-DNA 5′ ends and some of the same H-strand RNA is covalently linked to DH-DNA. These observations indicate that DH-DNA is primed by long stretches of RNA. Finally, all transitions from primer RNA to DNA occur within a 90-nucleotide region encompassing three previously identified sequence blocks (CSB-I, CSB-II, and CSB-III) conserved in vertebrate mtDNA (Walberg and Clayton, 1981; Wong *et al.*, 1983). These sequences may therefore function to ensure a clear transition to DNA synthesis.

Priming of replication

Two general possibilities exist for priming DH-DNA synthesis in closed circular mtDNA. Randomly positioned, very short RNAs (<20 nucleotides) may act as DH-DNA primers in a manner analogous to the priming of Okazaki fragments during bidirectional DNA synthesis (Kornberg, 1980). Each DH-DNA will therefore have different primer RNAs heterogeneous at both 5′ and 3′ termini. Alternatively, the priming event may utilize relatively long transcripts from a unique RNA polymerase initiation site. Processing of the initial transcripts at the formal origin of replication then generates mature primer and leads to subsequent DNA synthesis. Our observations that abundant H-strand RNAs of 80–155 nucleotides in length map immediately upstream from the origin of replication and that some of these molecules are covalently linked to DH-DNA 5′ ends both favor the latter priming mechanism. Furthermore, while these H-strand RNAs have heterogeneous 3′ ends immediately adjacent to the 5′ ends of each DH-DNA species, their 5′ ends are unique, mapping at nucleotide 16 183. This suggests that DH-DNA synthesis is primed by RNA transcribed from one or more promoters at least 155 nucleotides upstream from the major DH-DNA 5′ ends.

This priming mechanism for H-strand mtDNA synthesis is a unique eukaryotic example of leading-strand DNA synthesis primed at a limited number of specific sites by relatively long RNA. There is evidence in yeast mitochondria (Baldacci *et al.*, 1984) that RNA initiated at a sequence homologous to sites of gene transcription (Osinga *et al.*, 1984) primes DNA synthesis 10–20 nucleotides downstream. Additionally, *in vitro* replication of a plasmid containing the *Escherichia coli* replication origin is sensitive to rifampicin (Fuller *et al.*, 1981) and proceeds even in the absence of primase if RNA polymerase is present (Kaguni and Kornberg, 1984), indicating a direct role of RNA polymerase in replication priming. However, our data are best compared with two prokaryotic systems, plasmid ColE1 (Itoh and Tomizawa, 1980) and bacteriophage T7 (Romano *et al.*, 1981). In both, *in vitro* leading-strand DNA synthesis is primed at specific locations by transcripts several hundred nucleotides long which themselves initiate from discrete RNA polymerase promoters. In ColE1 and related plasmids, the primer RNA is generated from a transcript initiating 500–600 nucleotides upstream from the replication origin (Itoh and Tomizawa, 1980). The primary transcript forms a hybrid with the template DNA near the origin and is subsequently cleaved by RNase H. The free 3′-hydroxyl end thus generated is suitable for *in vitro* incorporation of deoxyribonucleotides using *E. coli* DNA polymerase I. Although the secondary structures of the primary transcript have been demonstrated to facilitate RNA/DNA duplex formation near the replication origin (Tomizawa and Itoh, 1982; Masukata and Tomizawa, 1984), the precise mechanism of specific RNase H cleavage that leads to unique initiation of DNA synthesis at the origin remains to be established. For T7 DNA replication, only the phage-encoded RNA and DNA polymerases are required for accurate *in vitro* initiation (Romano *et al.*, 1981). RNA synthesis from either of two tandemly located promoters leads to priming of DNA synthesis no more than 100 nucleotides downstream and to covalently linked RNA/DNA molecules (Fuller *et al.*, 1983). The *in vitro* switch from RNA to DNA synthesis occurs at relatively non-specific locations around a 61-bp AT-rich region.

The parallels between the initiation of mouse DH-DNA synthesis and these prokaryotic systems are striking and serve to focus attention on two, as yet unresolved, questions about DH-DNA initiation. First, does primer synthesis begin at a specific location as in the prokaryotic examples? The observation that virtually all RNA molecules which contain sequences upstream from DH-DNA start around nucleotide 16 183 suggests that a unique initiation event occurs at this site. The non-conservation of mitochondrial transcriptional promoter sequence in different species (unpublished observation) does not allow identification of transcriptional initiation sites on a comparative basis. However, a crude preparation of mouse mitochondrial RNA polymerase can initiate specific L-strand transcription around nucleotide 16 183 (Gray, Chang and Clayton, unpublished observation), providing evidence that L-strand transcription begins uniquely at this site.

How is the transition from RNA to DNA synthesis effected?

A second feature common to mouse DH-DNA synthesis and the above prokaryotic replication systems is the specific transition from RNA to DNA at the formal origin of replication. Two observations indicate that, as in priming of ColE1 replication, an RNase H-like activity may cleave nascent transcripts at origins of DH-DNA synthesis to generate primer RNAs. A small but significant amount of H-strand RNA, extending past the 5′ ends of all DH-DNAs, co-isolates with mtDNA in a dye-CsCl buoyant density gradient by virtue of its RNA/DNA duplex nature (Figure 4A). This is expected if hybrid formation between a primary transcript and the parental template DNA precedes specific nicking at the origin and subsequent DH-DNA synthesis. This finding also implies that the priming mechanism does not involve specific transcription termination at the origin. Second, an extremely small amount of H-strand RNA, whose 5′ ends abut the 3′ ends of the primer RNA, is detected by primer extension (data not shown). A processing model involving an RNase H-like activity predicts that such molecules, representing the downstream portion of a cleaved primary transcript, should exist.

The precise mechanism leading to the required sequence-specific cleavage is not known for any system. However, it can be seen in Figure 5 that all RNA-DNA transitions occur between nucleotides 16 027 and 16 110. This region almost exactly encompasses three previously recognized conserved nucleotide sequences (CSB-I, CSB-II and CSB-III). Furthermore, the 3′ termini of D-loop RNA in human mitochondria also map within these sequence blocks, although no covalent linkage to DNA has yet been established (Chang and Clayton, 1985). The association between conserved nucleotide sequences and the switch region for elongation of primer RNAs is more than coincidental and raises the possibility that CSB elements may serve as control sequences involved in the transition from primer RNA to DNA synthesis. As such, these sequences may function as recognition sites for endonucleases responsible for primer RNA generation.

A role for a mitochondrial RNase H-like processing activity

Figure 6 depicts a model for initiation of H-strand DNA synthesis in vertebrate mtDNA consistent with the available data.

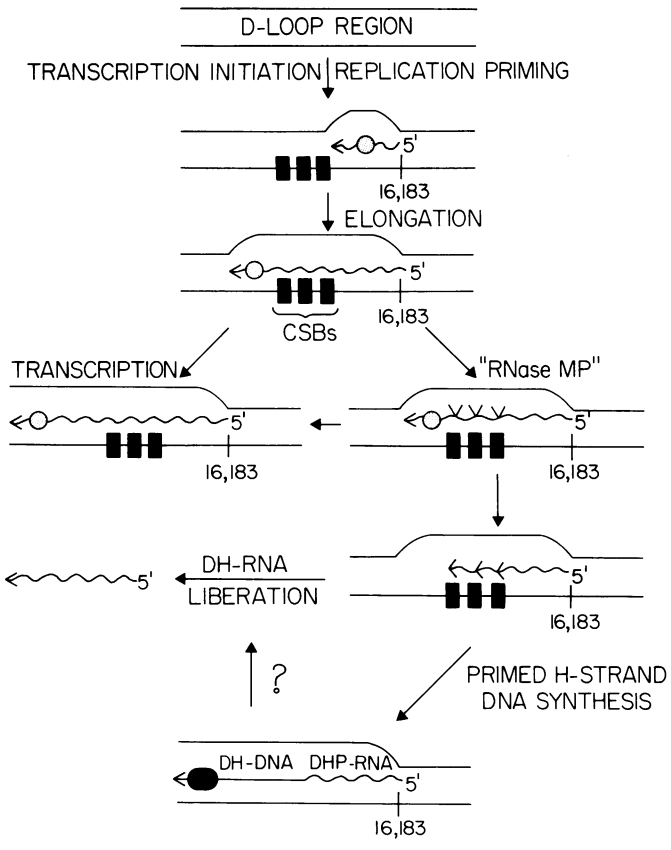

Fig. 6. Model for the generation of D-loop H-strand transcripts (DHT-RNA) and primers for H-strand replication (DHP-RNA) from a common upstream promoter. CSB: conserved sequence block. 'RNase MP': putative mitochondrial processing RNase. Dotted circle: RNA polymerase. Solid oval: DNA polymerase. See Discussion for details.

(i) Transcription begins at the L-strand promoter region (near or just upstream from nucleotide 16 183 for mouse). (ii) RNA synthesis proceeds through the D-loop region and extends through all L-strand genes. The primary transcript either remains hybridized to or forms a hybrid duplex with the template DNA at the origin of DNA synthesis. The exact topological nature of this structure remains to be determined; however, the presence of RNA co-isolating with mtDNA during dye-CsCl buoyant density centrifugation suggests that such hybrids can form. (iii) A putative RNase H-like activity then cleaves hybrid duplex molecules at specific sites, possibly directed by CSB sequences. (iv) Some cleaved RNA then primes H-strand DNA synthesis leading to both DH-DNA and full-length daughter H-strand molecules. Although in the experiments described above we did not attempt to analyze the small number of molecules in which DNA synthesis had proceeded beyond the D-loop region, it has been shown previously that the 5′ ends of DH-DNA and the 5′ ends of extended nascent H strands are the same in distribution and map position (Brown and Vinograd, 1974; Robberson *et al.*, 1974; Tapper and Clayton, 1981). This model accounts for all DH-DNA and H-strand RNA species detected experimentally, including linked H-strand RNA/DH-DNA molecules. We note that both replication priming and transcription can occur from one RNA synthetic event that, in principle, demands only a single RNA polymerase species.

Are promoters for gene expression commonly used as replication priming sites?

The most obvious comparison to the origin of H-strand synthesis (O_H) is the mitochondrial origin of L-strand synthesis (O_L). This second replication origin is located two-thirds of the way around the genome from the D loop and possesses a characteristic potential dyadic structure (Clayton, 1982). Other than primary sequence, a principle difference between O_H and O_L is the fact that priming at O_H must occur on a closed circular template while priming at O_L occurs on a single-stranded template. Since there are no candidates for putative promoters at O_L, priming may occur near the single-stranded O_L template by a substantially different mechanism than at O_H, suggesting the interesting possibility that each origin demands different enzymatic components in order to function.

For the case of nuclear DNA it is not yet possible to draw inferences or conclusions as to the diversity of initiation events at origin sequences. A DNA primase appears to be associated with the major cellular DNA polymerase and these activities exhibit a strong preference for single-stranded templates *in vitro*. This is consistent with a major role in replicating nuclear DNA already in single-stranded form, a situation relevant once the first initiation event has occurred in any given replicon. In this regard it will be of interest to learn whether any of the nuclear RNA polymerases have a role in providing either an entree for a primase/polymerase complex or an initial primer for subsequent elongation.

Materials and methods

Isolation and limit-nuclease digestion of mitochondrial nucleic acids

Nucleic acids from sucrose step-gradient purified mouse L-cell mitochondria were prepared as previously described (Tapper *et al.*, 1983) either by hot phenol-chloroform extraction (crude mtRNA) or by two cycles of ethidium bromide-CsCl buoyant density centrifugation (mtDNA).

RNase T1 digestion was carried out with 100 units of RNase T1 (BRL) per 10 μg mitochondrial nucleic acids in 50 μl 50 mM Tris-HCl (pH 8.0), 1 mM EDTA at 37°C for 30 min. The reaction was terminated by addition of 5 μl 2% SDS, 100 mM EDTA followed by phenol-chloroform extraction. RNase T1-resistant nucleic acids were recovered by ethanol precipitation with 5 μg carrier *E. coli* tRNA and used in subsequent analyses. DNase I digestion and alkali treatment were performed as described elsewhere (Chang and Clayton, 1984; Gillum and Clayton, 1979).

Filter hybridization

mtRNA samples were fractionated by electrophoresis on 1.4% agarose-1 M formaldehyde gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled RNA probes complementary to the H strand. Baked nitrocellulose filters were pre-incubated overnight at 42°C in 50% formamide, 5 x SSPE, 5 x Denhardt's solution, 0.1% SDS, 180 μg/ml denatured salmon sperm DNA. Filters were hybridized for 24 h in the above buffer containing 2.5% dextran sulfate and 5 ng/ml RNA-probe (sp. act., 6 x 10^8 d.p.m./μg). Filters were rinsed briefly in 2 x SSPE at room temperature to remove excess formamide and washed in 50 mM NaCl, 20 mM sodium phosphate (pH 6.5), 1 mM EDTA, 0.1% SDS at 65°C for 60 min with four changes of the buffer.

RNA probe synthesis

Recombinant plasmids used as templates for RNA-probe synthesis contained a *Rsa*I fragment (nucleotide 15 497 – 16 216) or a *Dde*I-*Rsa*I fragment (nucleotides 16 127 – 16 216) cloned into a vector containing an upstream SP6 promoter (pSP65, Promega). *In vitro* transcription was carried out in a 20 μl reaction mixture containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 4 mM spermidine, 10 mM DTT, 500 μM ATP, GTP, and CTP, 15 μM [α-^{32}P]UTP (400 Ci/mmol), 20 units of RNasin (Promega), 2 μg of linearized template DNA, and 3 units of SP6 RNA polymerase (Promega) at 40°C for 90 min (Green *et al.*, 1983). Following probe synthesis, the DNA template was digested with DNase I and unincoporated ribonucleotides were removed by Sephadex G-50 filtration. The specificity of the RNA-probes was verified by hybridizing each to *Rsa*I or *Dde*I digested 501.1/pBR322 (a pBR322 derivative containing the entire mouse mitochondrial genome at the *Sph*I site, a gift of Dr. R. A. Van Etten).

Labeling and isolation of DNA restriction fragments

DNA restriction fragments were labeled with T4 polynucleotide kinase (BRL) or *E. coli* DNA polymerase, large fragment (Biolabs). Labeled restriction fragments were isolated and strand-separated by acrylamide gel-electrophoresis (Maniatis *et al.*, 1982).

Primer extension analyses

The primer was generated by 5′ end-labeling a *Xba*I-*Hph*I fragment [nucleotides 15 975(5′) – 16 000(3′)] and isolating the L strand by electrophoresis on an 8% acrylamide-7 M urea gel. Primer extension was carried out as previously described (Chang and Clayton, 1984) and the products were analyzed by electrophoresis in an 8% sequencing gel.

Nuclease S1-protection analyses

5′ end-labeled L strand of a *Taq*I-*Alu*I fragment [nucleotides 15 851(5′) – 10(3′)] or 3′ end-labeled L strand of a *Sau*3AI-*Hpa*II fragment [nucleotides 16 174(3′) – 15 737(5′)] was used as the probe in S1 protection experiments. Hybridization was carried out in 20 μl 2 x STE [40 mM Tris-HCl (pH 7.5), 0.3 M NaCl, 2 mM EDTA] at 65°C for 12 h in a glass capillary. S1 nuclease treatment was performed as previously described (Chang and Clayton, 1984).

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