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Nuclear Gene Products that Function in Mitochondrial DNA Replication

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Nuclear gene products that function in mitochondrial DNA replication

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[Plate 1]

Mammalian mitochondrial DNAs replicate unidirectionally from two distinct strand-specific origins. A round of replication begins at the heavy-strand origin (the D-loop) where transcripts from an upstream promoter serve as the primers for DNA synthesis. The transition from RNA to DNA synthesis occurs within short, conserved nucleotide sequence blocks and is mediated by specific endonucleolytic cleavage of the primary transcript. An enzymic component involved in the generation of primer RNA in mouse mitochondria has been identified. It is a sequence-specific endoribonuclease that cleaves single-stranded RNA substrate precisely at one of the transition sites. The other origin, that for light-strand synthesis, is located well apart on the genome and functions only when in a single-stranded template form. This origin has a defined secondary structure that is the most highly conserved sequence element in mammalian mitochondrial DNAs. Initiation of replication at this origin is by the action of a mitochondrial DNA primase, which is capable of synthesizing a short stretch of ribonucleotides before switching to DNA synthesis. Mitochondrial DNA primase appears to have an associated RNA species and the evidence to date suggests that components of both the D-loop endoribonuclease and the DNA primase are nuclear gene products.

Introduction

Mitochondria of eukaryotic cells contain closed circular DNA genomes that are replicated within the confines of the organellar matrix. Although a typical animal cell has less than 1% of its genetic material as mitochondrial DNA (mtDNA), because the genome is of small size (16 kilobases), the resultant copy number is high (10^3–10^4 per cell). Replication appears to be under relaxed control and a portion of the molecules can undergo two replication events before some replicate even once (Flory & Vinogard 1973; Bogenhagen & Clayton 1976, 1977). This means that there is no apparent requirement in animal cells for each individual mtDNA molecule to be duplicated at each doubling of the cell population, although labelling data indicate that the net amount of mtDNA replication is exactly sufficient to maintain a constant number of genomes per cell (Berk & Clayton 1974). Bogenhagen and Clayton (1977) have shown that mtDNA molecules can replicate in any and all phases of the cell cycle in mouse L cells.

The availability of *in vitro* systems for initiating transcription (Walberg & Clayton 1983; Chang & Clayton 1984, 1986*a*–*c*; Fisher & Clayton 1985) and replication (Wong & Clayton 1985*a*, *b*) has permitted investigations of the processes of initiation of replication at the two separate and distinct origins of animal mtDNA replication. I describe here our current understanding of these events, which are clearly different for each origin.

Overall scheme of the replication cycle

The fact that mtDNA exists in a closed circular conformation not only permitted its original isolation in pure form, but also promoted the subsequent characterization of replicative intermediates. Most of the data on replication of animal mtDNA come from studies of the mode of replication in mouse and human cells (figure 1). The initial investigations relied on electron microscopic and centrifugal analyses of mtDNA molecules to develop a hypothetical model of replication. Further characterization and kinetic ordering of *in vivo* replicative intermediates were facilitated by the identification of a mitochondrial thymidine kinase in cells that lacked the major cellular thymidine kinase activity. Such cells provide a means of incorporating radioactive thymidine label into mtDNA to the exclusion of significant incorporation into nuclear DNA, thereby permitting identification and subsequent analysis of mtDNA replicative intermediates (Robberson & Clayton 1972, 1973; Berk & Clayton 1974, 1976).

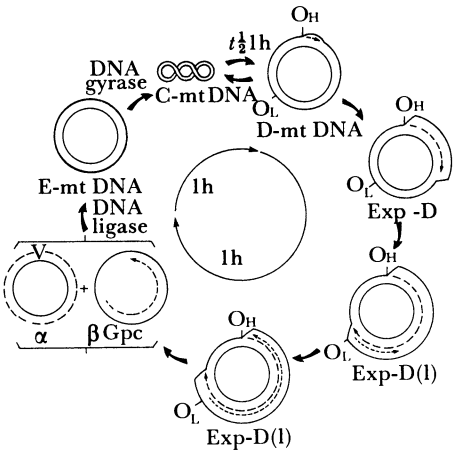

Figure 1. Replication model for mammalian mtDNA. Solid lines: parental strands. Dashed lines: nascent strands. Replication begins by the productive synthesis of an H-strand at the D-loop origin (O_H). After H-strand synthesis is two thirds completed, the origin of L-strand synthesis (O_L) becomes capable of initiation (Exp-D(l) molecules). Daughter molecules require approximately 1 h to be synthesized and are then processed into closed circular forms. D-loop strands are rapidly lost and resynthesized with a half-life, for mouse cells, of approximately 1 h.

A major form of mtDNA in most animal cells is a covalently closed circle with a displacement loop (D-loop) at the origin of replication of the heavy (H) strand (figure 1). This triplex D-loop structure is formed by the synthesis of a short daughter H-strand that remains associated with the parental closed circle (Bogenhagen & Clayton 1978*a*). Replicative intermediates are defined as those molecules in which H-strand synthesis has extended past the D-loop region (Exp-D). Synthesis of the daughter H-strand continues in a unidirectional manner until completed. Before this, when H-strand synthesis is 67 % complete, the origin of synthesis of the light (L) strand is displaced as a single-stranded template and initiation of L-strand synthesis can and does occur (Exp-D(l); figure 1). This offset in initiation of L-strand synthesis results in the segregation of two types of daughter molecules (α and β), with one consisting of the parental H-strand and a partly synthesized daughter L-strand (βGpc; figure 1). These α and β daughter molecules are then converted to closed circles (E-mtDNA) with few, if any,

superhelical turns (in the case of β daughters, L-strand synthesis must proceed to completion after segregation). Approximately 100 negative superhelical turns are then formed in the closed circular DNA population (Bogenhagen & Clayton 1978*b*). This orthodox superhelical DNA (C-mtDNA) serves as the template for the formation of D-mtDNA (D-loop mtDNA), which completes the cycle. The synthesis of full-length daughter strands requires approximately 1 h, and the entire replication cycle is completed in approximately 2 h. This means that the overall rate of polymerization is less than 300 nucleotides per minute per strand, much slower than estimated rates for nuclear DNA.

Details of initiation at the origin of heavy-strand synthesis

Animal mtDNA in the D-loop form represents a parental template potentially primed for H-strand synthesis. The frequency of D-loop forms (figure 2, plate 1) found in closed circular mtDNA populations ranges from less than 1% in *Drosophila* (Klukas & Dawid 1976; Rubenstein *et al.* 1977; Goddard & Wolstenholme 1978, 1980) to greater than 75% in mouse L cells (Robberson & Clayton 1972) and unfertilized *Xenopus laevis* eggs (Hallberg 1974). The location of the D-loop region in mouse and human mtDNAs was shown to be unique by cleavage of D-mtDNA with restriction endonucleases (Brown & Vinograd 1974; Robberson *et al.* 1974; Tapper & Clayton 1981). It could also be shown that one end of the D-loop region mapped at the same fixed position as one end of the expanded replicative region (Brown & Vinograd 1974; Robberson *et al.* 1974); thus, the 5′ end of the D-loop H strand and the 5′ end of nascent daughter H strands are initiated at the same point on the genome, and replication proceeds unidirectionally from this origin.

Approximately one quarter of the D-loop strands of mouse mtDNA have 5′-ribonucleotide ends (Gillum & Clayton 1978, 1979). In detailed studies of mouse D-loop strands, a distinct species is revealed beginning approximately 70 nucleotides upstream of the major 5′ end of D-loop DNA strands. This larger species is unique in that it exclusively contains from 1 to approximately 12 purine ribonucleotides at its 5′ end (Gillum & Clayton 1979; Chang *et al.* 1985). Other species that map between the 5′ end of this strand and the final downstream commitment to DNA synthesis are found to contain either ribonucleotide or deoxyribonucleotide 5′ ends and encompass the RNA priming region of figure 3.

The interesting issue of whether D-loop strands serve as primers for replication remains unresolved. The alignment of nascent, elongated human H-strands to the origin template suggests the same 5′-end distribution as for D-loop strands (Tapper & Clayton 1981), and D-loop strands in closed circular mtDNA are capable of elongation *in vitro*. These results are consistent with, but do not prove, the proposition that preexisting D-loop strands may serve as primers for *in vivo* H-strand replication. Instead, a productive replication event may involve initiation and synthesis past putative stop signals that otherwise arrest synthesis to form a D-loop (Doda *et al.* 1981). Under this model, one can argue that essentially all strands isolated from D-loop mtDNA represent abortive events with respect to replication. Because the four major mouse D-loop strands are known to have a short half-life of approximately 1 h (Bogenhagen & Clayton 1978*a*), it is an empirical fact that greater than 95% of these D-loop strands are lost by turnover and cannot serve as primers for mtDNA replication.

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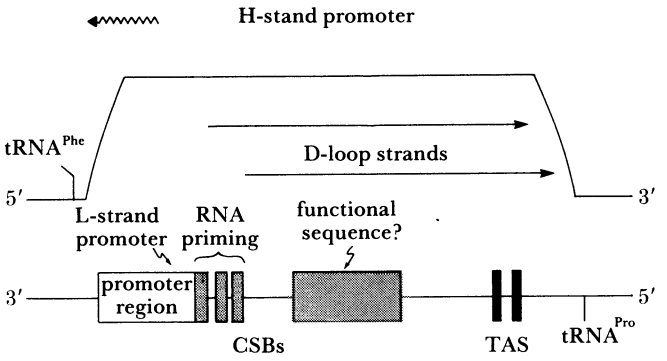

Figure 3. Prototypic D-loop region showing functional landmarks. The D-loop region is defined as that sequence of mtDNA bounded by the genes for $tRNA^{Phe}$ and $tRNA^{Pro}$. D-loop strands (DH-DNA) exist in a variety of species-specific families and, of course, a given D-loop has only one member of a family at any one time. The H-strand promoter has been implicated in transcription of genes located on the H-strand; it has no known role in DNA replication. In contrast, the L-strand promoter is the site of transcriptional initiation for L-strand genes as well as being the priming site for DH-DNA synthesis and extended synthesis of H-strands. As described in the text, cleavage of RNA sequence at the conserved sequence blocks (CSBs) may play a fundamental role in D-loop metabolism. There is an additional, larger region of conserved sequence in the middle of the D-loop that has no known functional significance at this time. TAS: termination associated sequence, see Doda *et al.* (1981).

Map positions of D-loop RNA and DNA

To understand the possible modes of replication initiation it was necessary to map the nucleic acid species synthesized from the D-loop region of human (Chang & Clayton 1985) and mouse (Chang *et al.* 1985) mtDNAs, by using current technologies. The discussion that follows is relevant to that of mouse mtDNA synthesis.

The 5′ ends of D-loop H strands (DH-DNA) mapped by primer extension and S1 nuclease protection were identical to the DH-DNA 5′ termini seen earlier by end-group analysis of isolated D-loop strands (Gillum & Clayton 1979). Mapping of H-strand RNA molecules resulted in two new significant insights. First, only one predominant 5′ RNA end was detected, suggesting that most RNA from the D-loop region may initiate at a singular site. Secondly, the 3′ ends of H-strand RNA aligned remarkably well with DH-DNA 5′ ends, and most importantly, some of this same H-strand RNA was covalently linked to DH-DNA. These observations indicated that DH-DNA is primed by RNA of significant size. Finally, all transitions from primer RNA synthesis to DNA synthesis occur within a 90-nucleotide region encompassing three previously identified sequence blocks (CSB-I, CSB-II, and CSB-III) conserved in vertebrate mtDNA (figure 3) (Walberg & Clayton 1981). Also, 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 & 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 synthesis to DNA synthesis. As such, these sequences might function as recognition sites for endonucleases responsible for primer RNA generation.

Figure 4 depicts a model for initiation of H-strand DNA synthesis in vertebrate mtDNA consistent with the available data. Primer RNA synthesis begins at a major promoter that is also involved in gene expression and the transition from primer RNA synthesis to DNA

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Figure 2. Unicircular dimeric mtDNA with two D-loops. This molecule is from a mouse L-cell tissue culture line that maintains its mtDNA in a head-to-tail dimeric form. It is 32.6 kilobases in size and, as seen, the two D-loops are positioned 180 ° relative to each other.

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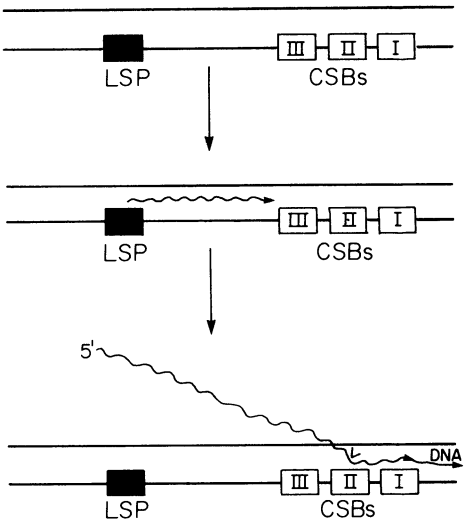

Figure 4. Model for processing RNA synthesized upstream of the origin of H-strand replication. All transcription of the L-strand begins at a single L-strand promoter (LSP) and proceeds into the D-loop region. Candidate sites of RNA processing are located near three previously identified short regions of high sequence conservation between animal species (CSBs). The caret marks the point of cleavage of the currently identified mitochondrial RNA processing enzyme MRP (Chang & Clayton 1987*a*, *b*).

synthesis occurs at distinct sites. A site-specific endoribonuclease, isolated from purified mitochondria, cleaves single-stranded RNA substrates containing the sequence of the O_H region; cleavage occurs at one of the transition sites in a manner consistent with an essential role in genomic mtDNA replication (Chang & Clayton 1987*a*). We have termed this endonuclease RNase MRP (Mitochondrial RNA Processing) and have recently established a requirement for an endogenous nucleic acid component essential for enzymic activity (Chang & Clayton 1987*b*).

Relative location of the cleavage sites

By employing a 3′-end labelled substrate, the exact cleavage site has been mapped to three adjacent nucleotides centred at nucleotide position 16103 of the mouse mtDNA sequence (Bibb *et al.* 1981). Previous studies have shown two types of *in vivo* nucleic acids mapping at this position. One is RNA with heterogeneous 3′ ends centred at nucleotide 16108 (extending from 16102 to 16114) and the other is nascent H-strand DNA with short 5′ oligoribonucleotides of one to ten residues that extend to nucleotide 16104. The boundary for 5′ deoxyribonucleotide residues in this group of nascent H-strand DNAs, as determined by alkaline treatment, maps at nucleotide 16094 (Gillum & Clayton 1979; Chang *et al.* 1985).

The products of cleavage have appropriate 3′ hydroxyl ends to serve as primers to be elongated with deoxyribonucleotide residues. However, based on the above mapping information, it is clear that there are an additional nine ribonucleotide residues between the *in vitro* cleavage site at 16103 and the *in vivo* RNA to DNA transition site at 16094. A possible explanation for this nine nucleotide discrepancy is that the fidelity of the endonuclease is somewhat altered during purification or under the *in vitro* reaction conditions employed. However, the presence of an RNA with a 3′ end at 16102, and the fact that some of the nascent H-strand DNA has 5′ oligoribonucleotides extending to 16104, argue for *in vivo* processing

events at nucleotides 16102, 16103 and 16104. Because these positions are the same as the *in vitro* endonucleolytic cleavage sites, it is likely that the isolated endonuclease is recapitulating an *in vivo* processing event.

A role for RNA processing in DNA replication

What then is the functional role of the RNA processing event at this transition site? An interesting possibility is that the endonucleolytic processing centred at nucleotide 16103 is necessary for a required hybrid to form between the RNA and template DNA. As RNA polymerase transcribes through the replication origin, the free energy available from the superhelicity of a closed circular mtDNA would facilitate the formation of a hybrid. Such a hybrid might soon be displaced unless stabilized against branch migration; the cleavage event could stabilize the hybrid by removing a displaced RNA.

A second view relegates the endonuclease to a role in removing primer RNA from a nascent DNA strand, rather than being principally active in generating primer RNA. A common feature of the completion of DNA replication is the removal of RNA primers before the covalent joining of newly synthesized DNA. Because the O_H of mtDNA uses relatively long RNA primers that might branch-migrate away from the template strand during unidirectional DNA synthesis, the endonuclease activity described here could then be involved in reducing the size of the displaced primer RNA from the nascent H-strand DNA. This might facilitate covalent joining of the ends of the daughter H-strand at the completion of a replication cycle. The development of an *in vitro* replication system capable of initiation at this origin will be required to determine the specific function of this cleavage event in the overall mechanism of mtDNA replication.

Because the overall nucleotide sequences of the O_H regions of mouse and human mtDNA are quite different, it was useful to test the efficacy of cleavage of human substrate with mouse endonuclease, and vice versa. Human RNA substrate was appropriately cleaved by the mouse endonuclease, and vice versa, indicating limited sequence information, common to both mouse and human RNA substrates, is sufficient for the proper recognition of the cleavage site (Chang & Clayton 1987*a*). Apart from the three CSB elements, there are no conserved primary sequences of significant size nor any conserved potential secondary structures in this region. Although the manner in which this mitochondrial endoribonuclease selects its cleavage site is unknown, the fixed distance between the cleavage sites and CSB-II implicates this sequence element in the recognition process. Additional mutational analysis of CSB-II will be required to define the mechanism of cleavage site selection.

Ribonucleoprotein properties of the endonuclease

The sensitivity of RNase MRP to either ribonuclease digestion or thermal inactivation demonstrates a requirement for both RNA and protein components for site-specific cleavage by this mitochondrial endoribonuclease (Chang & Clayton 1987*b*). Two lines of evidence argue for the importance of at least one specific RNA. First, the elution profile of a 135 nucleotide RNA from an ion-exchange Mono Q column is identical to the endonucleolytic activity profile, thus providing physical evidence for the association of the 135 nucleotide RNA with the endonuclease. Secondly, the endonucleolytic activity can be inhibited by a specific oligonucleotide complementary to the 135 nucleotide RNA. These data, along with the large sedimentation coefficient of the endonuclease, suggest that RNase MRP is a ribonucleoprotein with at least one RNA component.

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Repeated attempts to identify the protein component of RNase MRP have not yet been successful in establishing any specific polypeptide with the cleavage activity. In regard to the origin of the 135 nucleotide RNA of the endonuclease, there are now persuasive arguments for it being a nuclear gene product. The available RNA sequence information clearly indicates that this RNA is not encoded in mtDNA. No evidence for extragenomic genetic elements exists for mammalian mitochondria, despite numerous and extensive analyses of DNA isolates from this source. Most importantly, a nuclear gene for this RNA species has been identified and sequenced (D. D. Chang, J. N. Topper & D. A. Clayton, in preparation), thereby establishing its genetic identity.

All other known nuclear gene products involved in mitochondrial biogenesis are proteins and translocation of such proteins synthesized on cytoplasmic ribosomes, into mitochondria, occurs post-translationally. RNase MRP was isolated from mitochondria and cleaves a mitochondrial RNA substrate specifically at a previously established *in vivo* processing site (Chang & Clayton 1987*a*). Because the mitochondrial enzyme requires a nucleus-encoded RNA component for its activity, there must be a mechanism for transporting nucleic acid into mitochondria (figure 5). It will be of interest to learn the mode of assembly and transport of this novel mitochondrial endonuclease.

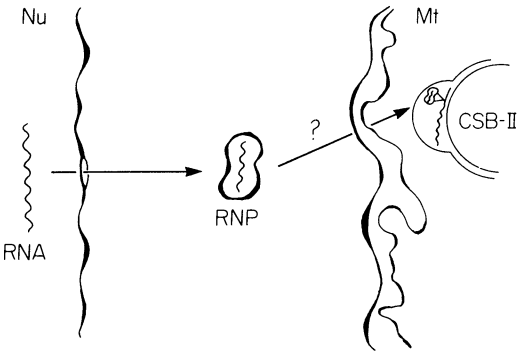

Figure 5. Requirement for RNA import into mitochondria. The gene for the 135 nucleotide RNA of the MRP activity is located in the nucleus. A simple model is that this RNA migrates to the cytoplasm for assembly with protein, as is the case with other known ribonucleoprotein (RNP) activities. Ultimately, the activity must be imported into the mitochondrial matrix space to cleave mitochondrial RNA. The mechanism of importation of this activity is currently unknown.

Origin of L-strand synthesis

As discussed above, H-strand DNA synthesis begins in the D-loop region and is primed by transcription from a major promoter. In contrast, L-strand DNA is synthesized on a single-stranded template and the origin of replication, O_L, is located in a region that has the potential for formation of a stable stem-loop secondary structure (Martens & Clayton 1979; Tapper & Clayton 1981). We have developed a run-off assay for studying *in vitro* replication of the L-strand of mtDNA (Wong & Clayton 1985*a*, *b*). Replication is initiated accurately with synthesis of RNA primers that are approximately 20 nucleotides in size. This *in vitro* system permitted the mapping of primer start sites, as well as the location of the transition from RNA synthesis to DNA synthesis. It has also permitted the identification of a transition zone sequence crucial to efficient synthesis of daughter L-strand DNA (Hixson *et al.* 1986). An mtDNA primase was identified as the enzyme responsible for initiating L-strand DNA replication, which further distinguishes L-strand synthesis from H-strand synthesis.

The origin of L-strand replication is the first DNA regulatory sequence to have been identified in an animal mitochondrial genome. Its assignment was originally based on the 5′-end map position of nascent L-strands and it remains today a distinctive hallmark of mammalian mtDNA. This is due to its striking secondary structure potential, which exceeds that of any other DNA sequence element in these genomes, and its location within a zone of potential secondary structure, being located among five tRNA genes (Bibb *et al.* 1981).

Figure 6 shows the essence of what has been learned by *in vitro* studies. Priming occurs in the T-rich loop sequence by the synthesis of a short stretch of riboadenosines. Primer synthesis continues through the stem sequence until a sharp transition to DNA synthesis occurs on a template sequence near the base of the stem, approximately 20 nucleotides away from the initial priming site. This transition site, 3′-GGCCG-5′, is highly conserved between human and bovine mtDNAs (Wong & Clayton 1985*b*). Considerable sequence variation occurs compared with other species and its exact function remains to be determined.

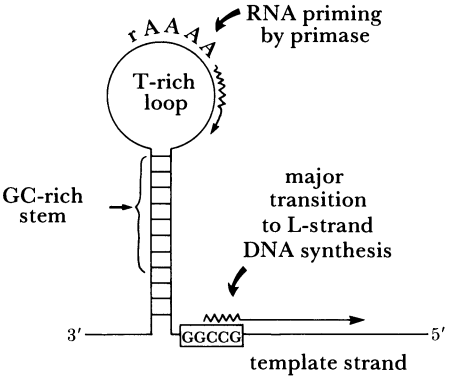

Figure 6. Prototypic origin of L-strand replication. Priming occurs at the T-rich loop (rAAAA) and the major transition to DNA synthesis for human mtDNA is at the template sequence 3′-GGCCG-5′ shown. Common features of this origin in different animal species include the T-rich loop, G + C-rich stem and the ability to form a perfectly base-paired stem structure. The pentanucleotide sequence shown appears critical to human mtDNA synthesis (Hixson *et al.* 1986) but is not conserved in rodent, and presumably other, mtDNAs. It will be of interest to learn the nature of interaction of this part of O_L with any replication proteins.

Properties of human mitochondrial DNA primase

DNA primase isolated from human mitochondria has characteristics that distinguish it from other primases. The mitochondrial enzyme sediments as two species of 30 *S* and 70 *S*. These sedimentation coefficients are substantially greater than those reported for most other DNA primases (less than 10 *S*) and are not likely to be caused entirely by protein aggregation. We have presented evidence that the unusual sedimentation behaviour of mitochondrial primase is a result of its association with RNAs (Wong & Clayton 1986). In addition, these RNAs may play a critical role in the replicative function of the enzyme; degradation of the RNAs with nucleases leads to rapid inactivation of primase activity. Sensitivity of primase activity to RNase treatment is independent of the nature of the DNA template used in the primase assay. It is therefore unlikely that the RNAs in the enzyme preparation are serving as primers for DNA synthesis. Instead, they may be responsible for correct assembly of the multiple polypeptide components or subunits of the active DNA primase.

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Summary and prospects

Animal mtDNA maintains two separate and functionally distinct origins of replication. The origin of H-strand replication, contained in the D-loop, is primed by transcriptional events emanating from the singular upstream promoter located several hundred nucleotides away. RNA processing appears to play a role in cleaving potential primers in the known transition zone to DNA synthesis. There may well be multiple D-loop RNA processing activities involved in priming and perhaps editing or removal of primer RNAs. The first of these to be described is a ribonucleoprotein in which the RNA component is encoded by a nuclear gene (Chang & Clayton 1987*a*, *b*) and the protein components are, most likely, nuclear gene products as well. This observation raises the possibility that ribonucleoprotein transport is a key element in the overall control of organelle biogenesis.

In contrast, the origin of L-strand replication is independent of promoter considerations and appears to utilize an mtDNA primase for initiation purposes. Here, too, RNA involvement in enzymic activity is apparent (Wong & Clayton 1986) and greater purification of the primase activity is required to establish the need for a specific RNA sequence for authentic primase action.

With further identification and physical characterization of activities required for mtDNA synthesis, such as mtDNA polymerase (Wernette & Kaguni 1986), it should be possible to order the events in replication starting from the currently known positions of the first ribonucleoside triphosphates involved in priming to the final switch and commitment to DNA synthesis, and beyond. In that sense it is interesting to recall that the approximately 1 kilobase D-loop region (figure 3) enjoins a microcosm of RNA and DNA synthetic activities; initiation of RNA and DNA synthesis, RNA processing and cessation of DNA synthesis are all occurring with high efficiency *in vivo*.

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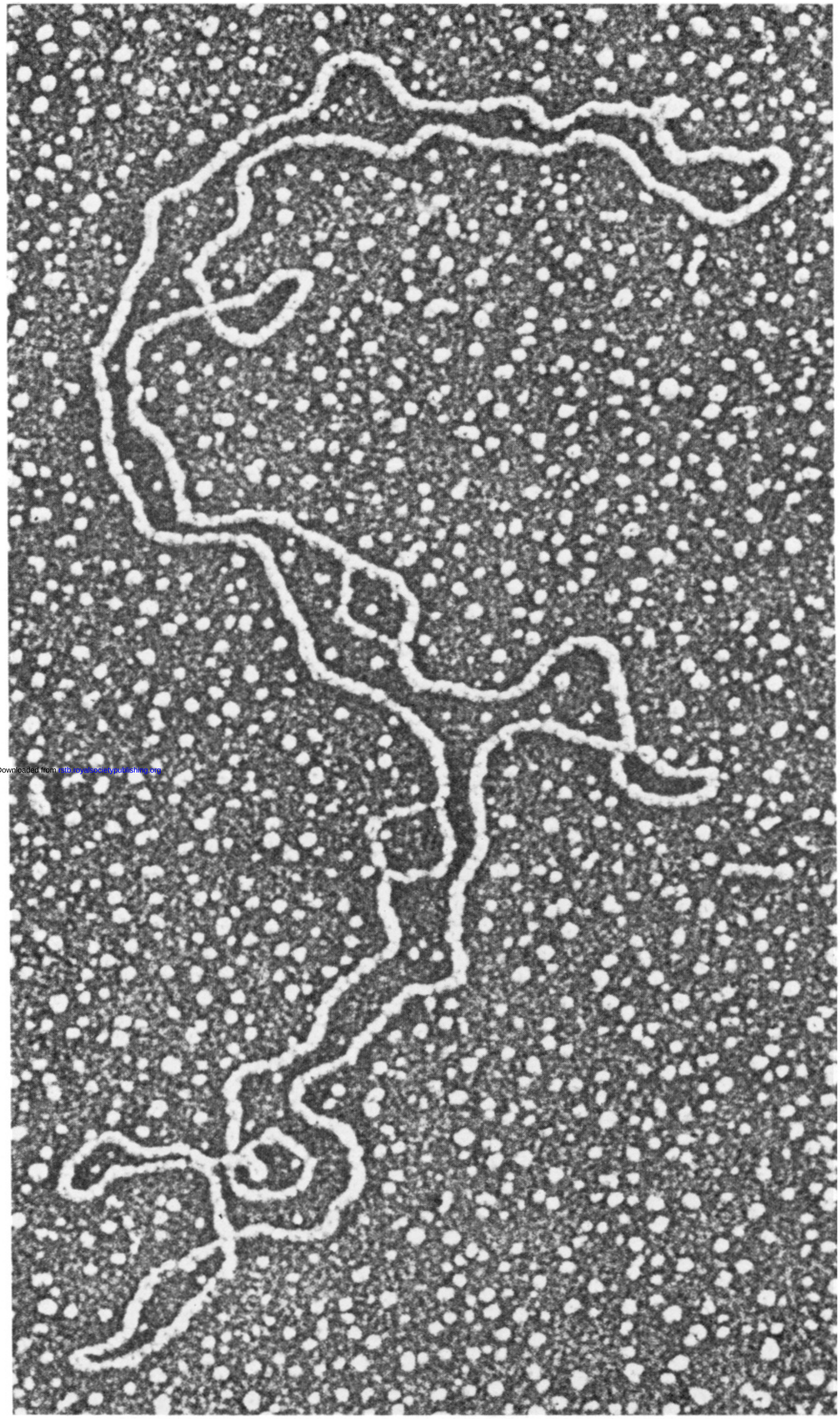
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



igure 2. Unicircular dimeric mtDNA with two D-loops. This molecule is from a mouse L-cell tissue culture line that maintains its mtDNA in a head-to-tail dimeric form. It is 32.6 kilobases in size and, as seen, the two D-loops are positioned 180 ° relative to each other.