The mitochondrial genome of eukaryotic cells is maintained by a mechanism distinct from that employed in the nucleus. Mitochondrial DNA replication at the leading-strand origin is coupled to transcription through the formation of an RNA-DNA hybrid known as an R-loop. In vivo and in vitro evidence has implicated an RNA processing enzyme, RNase MRP, in primer maturation. In our investigation of mammalian RNase MRP, we have analyzed its specific endoribonuclease activity on model R-loops. We demonstrate here that human RNase MRP cleaves this distinctly configured substrate at virtually all of the major DNA replication sites previously mapped in vivo. We further show that the processed RNA products remain stably base-paired to the template DNA strand and are functional for initiating DNA synthesis on a closed circular plasmid. Thus, in vitro initiation of leading-strand mtDNA synthesis requires only the actions of RNA polymerase and RNase MRP for the generation of replication primers.

**EXPERIMENTAL PROCEDURES**

Construction of Substrates—RNA was synthesized by SP6 transcription generated from pK408SP which was digested with restriction endonuclease FokI. This RNA was radiolabeled with [5-32P]pCp (3000 Ci/mmol, NEN Life Science Products) and T4 RNA ligase as described elsewhere (7). Labeled RNA was purified by urea-polyacrylamide gel electrophoresis and used to assemble the R-loop with the supercoiled pK408SP template by the formamide annealing method described previously (9). For the construction of the RNA-DNA heteroduplex hybrid, an oligonucleotide that is complementary to position 294–314 on pK408SP (oligo HF314, 5'-d(TTGATTCCTGCCTCATCCTAT)) was annealed onto the purified radiolabeled pK408SP-derived RNA by heat denaturation at 95 °C for 5 min and slow cooling to 37 °C over 30 min. The reaction mixture was adjusted to 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM MgCl₂, 2 mM dithiothreitol, 0.2 mM spermidine, and 0.4 mM dATP, dCTP, dGTP, and dTTP (Amersham Pharmacia Biotech). 10 units of AMV reverse transcriptase (Life Technologies, Inc.) were added and incubated at 37 °C for 30 min. The resulting RNA-DNA hybrid was phenol-extracted, concentrated by ethanol precipitation and then resolved in a 4% native polyacrylamide gel in 45 mM Tris borate-EDTA buffer at pH 8.3. Individual species were located by autoradiography, and full-length products were excised and eluted into 0.5 M ammonium acetate, 0.1% SDS, overnight at room temperature. The solution containing the hybrid was ethanol-precipitated, resuspended in water, and passed through a Sephadex G-50 (Amersham Pharmacia Biotech) spin column.

Enzymes and Activity Assays—RNase MRP was purified from human KB cells, and assays were carried out as reported previously (7, 10). We detected no deoxyribonuclease activity or any nonspecific ribonuclease activity in our final RNase MRP preparation. Oligonucleotide LRI, used previously for specific RNase MRP inhibitions (8), contains the following sequence, 5'-d(GTTCGGGGAGCTTC). Murine and bovine endo G preparations were kindly provided by Dr. Michael J. Lieber. Reaction conditions were identical to those in Lee and Clayton (8) except endo G was added at various dilutions ranging from 1:1 to 1:1000. Digestion times at 37 °C were also varied for 15–120 min. Reactions were quenched by phenol extraction and ethanol precipitation, and the products were resolved as described in Lee and Clayton (8). Micrococcal nuclease treatment of the endo G fractions had no effect on its nonspecific cleavage activity on the R-loop and RNA-DNA substrates. No reproducible specific cleavage products were observed on either substrate. Sequencing-grade ribonucleases T1 and U2 were purchased sized for primer RNA 3'-end maturation. RNase MRP was originally identified as an activity in mitochondrial extracts that fulfilled at least one of the criteria for a specific primer maturation endonuclease (7). We have recently reported that mouse RNase MRP has the capacity to process a model R-loop substrate containing the O₁ (8). In this report, we extend these studies to examine this unusual RNA processing activity in the human mitochondrial system and further demonstrate that such processed RNA primers are capable of initiating DNA replication on a model double-stranded circular mtDNA template in vitro.
from Amersham Pharmacia Biotech. Bacteriophage SP6 RNA polymerase, *Escherichia coli* RNase H, and RNase T2 were purchased from Life Technologies, Inc. Micrococal nuclease and DNase I were purchased from Boehringer Mannheim. Modified bacteriophage T7 DNA polymerase (Sequenase® version 2) was purchased from Amersham Life Sciences.

Structural Probing Assays—1–10 pmol of radiolabeled RNA or R-loops were incubated in RNase MRP reaction buffer for 15 min at 37 °C in a total reaction volume of 25 μL. Serial dilutions ranging from 1:10 to 1:10⁴ of Rnaase T1, T2, V1, and U2 were tested to generate a uniform cleavage pattern. 1 μL of appropriately diluted ribonuclease (between 1:10⁶ and 1:10⁴) was added to the sample and incubated at 37 °C for 10 s. RNase H reactions were carried out using 0.03 unit of enzyme and digested at 37 °C for 1 min. All reactions were quenched by phenol extraction, and samples were recovered by ethanol precipitation. Products were resolved in 6% polyacrylamide gels containing 8 μL urea.

**In Vitro DNA Synthesis Reactions**—General methods to generate R-loops via transcription using bacteriophage SP6 RNAP and supercoiled templates were described in Lee and Clayton (9). Briefly, 0.5–1.5 nmol DNA template, 0.4 μmol complete NTP mixture, and 10 units of SP6 RNAP were mixed in a total reaction volume of 100 μL. When the RNA was to be internally radiolabeled, 100 μCi of [α-32P]UTP (300 Ci/mmol, NEN Life Science Products) were included in the transcription mixture. Reactions were quenched by phenol extraction and ethanol precipitated. Dried pellets were resolved in 50 μL Tris-HCl, pH 8, and applied to a 1.5 cm × 3 cm benzoylated-naphthoylated-DEAE cellulose column as described in Lee and Clayton (9). After the ion-exchange fractionation, the mixture was passed through a 1.5 cm × 6 cm Sepharose CL-4B (Amersham Pharmacia Biotech) column in 20 mM Tris-HCl, pH 8, 50 mM KCl. The substrate concentration was quantitated, aliquoted, and adjusted to standard RNase MRP reaction conditions. RNase MRP digestions were carried out in a total reaction volume of 25 μL; reactions were stopped by the addition of 40 μL oligo LRI. Thereafter, the entire reaction mixture was passed through a Sepharose CL-4B (equilibrated in 20 mM Tris-HCl, pH 8, 50 mM KCl) spin column constructed in a 3-ml syringe barrel. Negative RNase MRP controls were treated in the same manner. Samples were phenol extracted and recovered by ethanol precipitation.

For DNA synthesis, the gel-filtered R-loop sample was treated with RNase MRP or mock-treated (negative control) as above; the RNase MRP reactions were inhibited by the addition of 40 μg of oligo LRI. Thereafter, the reaction mixture was adjusted to 125 μM dATP, dGTP, and dTTP (Pharmacia) and 13 units of modified T7 DNAP was added in a total reaction volume of 100 μL. When the entire reaction mixture was passed through a 1.5 cm × 3-cm Sepharose CL4B column. Thereafter, samples were phenol-extracted, concentrated by ethanol precipitation, and resolved in denaturing gels.

**Results**

**Assembly of R-loops and Structural Analysis**—Model R-loops were reconstituted using a recombinant plasmid containing the human mtDNA O₁ sequence (pK408SP) and RNA synthesized in *vitro* using bacteriophage SP6 RNAP (10). The reconstituted human R-loop exhibited chromatographic and electrophoretic properties (Fig. 1, A and B), consistent with previously observed model R-loops (9). When the R-loop is digested with *E. coli* RNase H, an endoribonuclease specific for the RNA strand

![Image](https://example.com/image.png)

**Fig. 1. Analysis of reconstituted human mitochondrial R-loop substrate.** A, the assembled RNA-DNA hybrid was resolved by electrophoresis using a 0.8% agarose gel in 45 mM Tris borate, pH 8.3 (TB buffer) and stained with ethidium bromide. The size standard (1 kilobase pair DNA ladder) was resolved in lane 1; the supercoiled pK408SP plasmid used for R-loop assembly in lane 2; and the purified RNA-DNA hybrid in lane 3. The slower migrating complex (labeled hyb) is the R-loop form which is topologically relaxed relative to the free plasmid. B, 32P-end-labeled RNA and the reconstituted R-loop are resolved in an agarose gel, transferred to nitrocellulose, and autoradiographed. Lane 1 is the 32P-labeled size standard; lane 2, the free RNA; and lane 3, the R-loop. C, the reconstituted R-loop is partially digested with *E. coli* RNase H (lane 2), RNase V1 (lane 5), and RNase T2 (lane 8). Partial hydrolysis of the RNA served as markers (lanes 3 and 6). Negative control reactions are shown in lanes 1, 4, and 7. The diagram to the right of the panel shows the relative positions of the CSBs. D, partial RNase T1 digestion of the R-loop substrate denatured in the presence of 5 μM urea (lane 2); the free RNA (lane 3) and the native R-loop (lane 4). Partial RNase U2 digestion of urea-denatured R-loop (lane 5); the free RNA (lane 6); and the native R-loop (lane 7). A mock reaction control is shown in lane 1.
nucleases of various specificities. In an effort to maintain a cleavage frequency of no more than one nick per RNA molecule, ribonuclease reactions were carried out using dilute enzyme concentrations and brief digestion times (1 min for RNase H reactions; 10 s for all others). Because the structure of the RNA may change after an initial cleavage event, it was important to minimize potential experimental artifacts in obtaining structural information.

RNase V1 from cobra venom has been used to probe for regions of potential RNA-RNA base pairing because of its preferential cleavage of RNA in double helical or stacked base conformations (12). Aside from those regions in RNA-DNA pairings as demonstrated by \textit{E. coli} RNase H sensitivity (Fig. 1C, lane 2), much of the full-length of the RNA was digested by RNase V1 in a semiperiodic pattern (Fig. 1C, lane 5), implicating the participation of those residues in RNA-RNA base-pairing. RNase T2 from \textit{Aspergillus oryzae}, a ribonuclease which cleaves after any unpaired residue, cleaved the RNA strand in the R-loop at those sites generally protected from RNase V1 cleavage (Fig. 1C, lane 8), suggesting those regions to be single-stranded. Although the highly reproducible cleavage patterns may reflect a composite of multiple subpopulations of differing RNA conformations, these results indicate that the RNA strand in the R-loop is only partially base-paired to the DNA template.

The surprisingly complex structure of the RNA strand in the R-loop was compared with the free RNA by probing with two highly specific ribonucleases of various specificities. In an effort to maintain a cleavage frequency of no more than one nick per RNA molecule, ribonuclease reactions were carried out using dilute enzyme concentrations and brief digestion times (1 min for RNase H reactions; 10 s for all others). Because the structure of the RNA may change after an initial cleavage event, it was important to minimize potential experimental artifacts in obtaining structural information.

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The surprisingly complex structure of the RNA strand in the R-loop was compared with the free RNA by probing with two highly specific ribonucleases (Fig. 1D). The cleavage patterns generated by RNase T1 from \textit{A. oryzae} (specific for unpaired guanine) and RNase U2 from \textit{Ustilago sphaerogena} (unpaired adenine \& unpaired guanine) revealed that the RNA strand in the R-loop was folded into a structure surprisingly similar to that of the free RNA. For RNase T1, differences between the free RNA and R-loop cleavage patterns were limited to the CSB II-CSB III region, where the RNA is base-paired with the DNA template as judged by the RNase H cleavage pattern. The cleavage sites located at the 3’ boundary of CSB II in the free RNA were reduced in the R-loop; likewise, the relatively efficient cleavage site located between CSB II and CSB III is protected in the R-loop (Fig. 1D, lane 3 versus lane 4). Interestingly, the guanine residues of CSB II are well protected against RNase T1 cleavage even in the free RNA, implying that this region may be configured in a manner that is sterically occluded from the enzyme. For RNase U2, since there is only one adenine residue in CSB II, which is protected in the free RNA, there is little overall difference between the cleavage patterns of the free RNA and R-loop (Fig. 1D, lane 6 versus lane 7), a surprising observation that has been previously reported for the mouse mtRNA sequence (9). Hence, for both ribonucleases used in this assay, there is little discernible difference in the cleavage pattern between the free and R-loop form of the RNA. Although the physiological relevance for the extensively folded RNA structure in the R-loop complex is presently under investigation, our initial experimental results suggest it is likely to be important in RNase MRP substrate recognition.\textsuperscript{2} This similarity in higher order structure may also explain the ability of RNase MRP to cleave the free RNA substrate as previously reported (8, 10).

**Human RNase MRP Cleaves the R-loop Substrate**—Having obtained gross structural information on the R-loop, we then asked if human RNase MRP can cleave this substrate in a physiologically relevant manner. RNase MRP was purified from human KB cells as described previously (7, 10), and a standard amount was added to reaction mixtures containing 0.5–1.5 nM R-loop substrate. Digestions were quenched by phenol extraction, and samples were recovered by ethanol precipitation. Reaction products were resolved in 6% polyacrylamide gels containing 5 M urea and visualized by autoradiography. Addition of the human RNase MRP fraction resulted in multiple cleavage products (Fig. 2A, lane 3). To confirm that RNase MRP catalyzed this reaction, the ribonucleoprotein composition of the holoenzyme was targeted for selective inhibition. Micrococcal nuclease was previously used to digest the RNA subunit of RNase MRP and thereby abolish enzymatic activity (13). A standard amount of the RNase MRP fraction was treated with micrococcal nuclease in the presence of Ca\textsuperscript{2+} (an essential metal cofactor), the digestion was quenched with EGTA (a calcium-specific chelator), and then assayed for R-loop processing activity by the addition of the substrate. Consistent with previous results, the observed R-loop processing activity was abolished (Fig. 2A, lane 7), demonstrating a requirement for RNA in the R-loop cleavage reaction. The requirement for protein subunits in the reaction was tested by heat inactivation or by treatment of the enzyme fraction with proteinase K, a serine protease active on native polypeptides. After digestion of the fraction with proteinase K, all R-loop processing activity was again abolished (Fig. 2A, lane 9). In contrast, inactivated proteinase K had no inhibitory effect (Fig. 2A, lane 8).

The specificity of the RNase MRP RNA subunit involved in the cleavage reaction was previously shown by using an antisense oligonucleotide directed against the 265-nucleotide MRP RNA (13). Addition of the antisense oligonucleotide, oligo LRI (8), inhibited the observed R-loop cleavage reaction in a concentration-dependent manner (Fig. 2B, lanes 3–5). In contrast, an unrelated oligonucleotide of comparable length, oligo 5.8S, had no effect at the concentrations tested (Fig. 2B, lanes 7–10). Since we have previously shown that oligo LRI had no inhibitory effect on the related ribonucleaseprotein endonuclease RNase P, these data suggest that the observed R-loop processing reaction is catalyzed specifically by RNase MRP (8).

Certain antisera from humans with clinical autoimmune syndromes are known to bind particular ribonucleoproteins selectively. The Th/To human autoantisemur was previously shown to immunoprecipitate the RNase MRP ribonucleoprotein and thereby deplete enzymatic activity (14). Crude Th/To serum was adsorbed onto \textit{Staphylococcus aureus} protein A-Sepharose beads (PAS), washed, and incubated with a standard amount of the human enzyme fraction. After centrifugation, the supernatant was assayed for activity. Control experiments were carried out in parallel using serum from a normal subject. While R-loop processing was unaffected by immunoprecipitation with the normal serum (Fig. 2C, lane 2), the Th/To serum depleted the observed R-loop processing activity (Fig. 2C, lane 3).

To determine whether the unique structure of the R-loop is required for the cleavage reaction, we assayed a simple RNA-DNA heteroduplex constructed with the same RNA substrate and its cDNA made by reverse transcription (see “Experimen- tal Procedures”). This RNA-DNA hybrid was digested by \textit{E. coli} RNase H in a nonspecific manner, yielding no oligoribonucleotides of significant length (Fig. 2D, lane 2). In contrast, this substrate was not cleaved by RNase MRP (Fig. 2D, lane 3), an observation consistent with previous reports (7, 8). This experiment also demonstrates the absence of nonspecific RNase H activities in the RNase MRP preparation. Interestingly, this type of RNA-cDNA heteroduplex was reported to be a substrate for endo G, an abundant mitochondrial endonuclease proposed to generate primers for mtDNA replication (15). Therefore, in an effort to rule out contamination of our RNase MRP preparations, purified endo G was used to obtain a cleavage pattern on our model substrates. Under our reaction conditions, endo G efficiently degraded the RNA strand as well as the DNA template of our R-loop and heteroduplex in a nonspecific manner,
generating no population of oligoribonucleotides with a distinctive length (data not shown). Thus, the R-loop processing observed with our RNase MRP preparation is not due to the action of endo G. We conclude from these and the above results that the observed R-loop cleavage reaction is an activity of RNase MRP.

Mapping of the cleavage sites on the R-loop was achieved by resolving the RNase MRP reaction products next to RNA sequencing ladders in polyacrylamide-urea gels (Fig. 3A). The cleavage sites, arbitrarily labeled 1 through 7, were then compared with the positions of previously mapped sites of RNA priming and DNA synthesis initiation. Although the human in vivo mapping data are complex with regard to the extensive microheterogeneity of RNA and nascent DNA termini, we observed alignment of the majority of the RNase MRP cleavage sites with a significant number of the in vivo priming sites, most notably the major DNA synthesis initiation site positioned 16–20 bases downstream of CSB I, classically defined as the principal O2 (Fig. 3B, cleavage site 6). Remarkably, RNase MRP cleavage site 6 corresponds precisely with the major H-strand 5′ termini more recently mapped by Kang et al. (5) using a polymerase chain reaction method. By inspection, the cleavage site selection followed no apparent sequence rule. This finding is consistent with previous results in the mouse system, where R-loop cleavage sites do not bear any obvious sequence homology. More importantly, the sites of cleavage for each species, differing in spatial and sequence parameters, reflect the correct placement with respect to mapping of nascent in vivo DNA strands. Conservation of accurate processing across species supports the role of RNase MRP in primer RNA formation.

To address the issue of physiological relevance, we have examined whether the RNase MRP cleavage products are functional as replication primers. Although mtRNA polymerase and mtDNA polymerase (pol γ) activities have been biochemically fractionated from mammalian cell extracts, such preparations are not of sufficient purity to characterize sequential in vitro enzymatic reactions on R-loop substrates reliably. Therefore the well defined activities of recombinant bacteriophage SP6 RNA polymerase and T7 DNA polymerase (pol γ) were employed to follow the fate of the transcription-generated RNA primers as a function of RNase MRP activity.

**RNase MRP Processing Is Required for Initiating DNA Synthesis in Vitro**—Transcription was carried out in the presence of [α-32P]UTP using SP6 RNA polymerase and the resulting R-loop was purified by ion-exchange chromatography on benzoylated-naphthylated-DEAE cellulose and gel-filtration (see “Experimental Procedures”). These purification steps were required to remove the abundant amount of variable-length free RNAs from those assembled into R-loops. Hybridized RNAs were demonstrated by their persistence after size-exclusion chromatography and sensitivity to E. coli RNase H (Fig. 4, lanes 1 and 2, respectively). The same procedure using the parental vector, pSP65, resulted in
no detectable hybridized RNA (data not shown). Also, R-loop formation via transcription required superhelical tension in the template plasmid as previously reported (9). Consistent with earlier observations (6, 9), the nonspecific smear of radioactivity in Fig. 4, lane 1 contains a group of strong transcriptional pause or termination products of ~130 nt. If we assume that these RNA fragments represent full-length strands initiating from the promoter, their 3' termini would map to the 5' boundary of CSB II. A shorter autoradiographic exposure (data not shown) indicated that this cluster of bands is of the same size as those observed in Fig. 4, lane 4, and, less intensely, in lane 5. We have noticed that this effect was dependent on the NTP and enzyme concentrations as well as reaction times (data not shown), suggesting that multiple rounds of transcription may be blocked at the CSB II site perhaps due to the strong RNA-DNA base pairing in that region. It is also possible that these abortive transcripts may have remained hybridized to the DNA template by base pairing at the CSB III region, an element previously reported to influence greatly the stability of the RNA-DNA interaction (6).
specific radioactivity of the transcript, which was necessary to
detect final products after the multiple reaction and purification
steps, the RNA was unavoidably subject to radiochemical
digestion, contributing to the nonspecific background smear
(Fig. 4, lanes 1 and 3–5).

Using the transcription-generated R-loops, we next assessed
the fate of the hybridized RNA following the action of RNase
MRP. Digestions were carried out as before, but RNase MRP
reactions were quenched by the addition of oligo LRI (40 μg) to
permit rapid handling steps. Because we wished to know
whether the processed RNAs remained base-paired to the DNA
template, the reaction samples were passed over a high molecu-
lar weight exclusion column (Seephase CL-4B). Recovered
samples were then phenol extracted, ethanol-concentrated, and
resolved in denaturing gels as before. As shown in Fig. 4, lane
3, RNase MRP generated only marginally visible discrete-
length RNA fragments against the smear of radioactivity.
However, the relative amount of the ~130-nt cluster of bands
is significantly reduced, indicating that these RNAs were not of
sufficient stability to elute with the DNA template in the sec-
ond gel filtration step.

To address whether the RNAs that remained following the
action of RNase MRP could be extended by a DNAP, the R-loop
substrate was treated with RNase MRP as above and, following
digestion, the mixture was adjusted to 125 μg each of the
four nonradioactive dNTPs and 15 units of modified T7 DNAP
(Sequenase® version 2) in a total reaction volume of 100 μl (see
“Experimental Procedures”). We note that a relatively large
amount of T7 DNAP was required to visualize final products,
perhaps reflecting this enzyme’s low activity on duplex DNA
molecules at their 5’ termini in a configuration also accessible to
RNase H (Fig. 4, lanes 7 and 8, respectively). By comparing the
largest bands in Fig. 4, lane 6, with those of lanes 7 and 8, we can
evaluate the length of the RNA component of the nascent DNA
chains to be ~150 to 190 nt. Interestingly, the RNase H result
demonstrates that the DNAP extension products contain RNA
at their 5’ termini in a configuration also accessible to RNase
T2, a structural presentation similar to those reported by oth-
ers (17, 18). Close scrutiny of the bands in Fig. 4, lanes 7 and 8,
reveals that the RNase H-generated products are larger by 1 or 2
nt relative to those generated by RNase T2; this is also
consistent with the observations of other investigators (17, 18).
We also note that, after the ribonuclease treatments, the total
amount of radioactivity that passes through the gel filtration
column is decreased by 75–90% relative to the untreated
RNase MRP-DNAP reaction, suggesting that the loss of the
DNA template, the reaction samples were passed through the gel filtration column and further processed
in the standard manner. Digestion of these DNA products with
RNase H and RNase T2 each generated smaller fragments
ranging from ~90 to ~150 nt in length, indicating that the
length of the RNA component of the nascent chain is significant
(Fig. 4, lanes 7 and 8). Close scrutiny of the bands in Fig. 4,
lane 7, reveals that the RNase H-generated products are larger by 1 or 2
nt relative to those generated by RNase T2; this is also
consistent with the observations of other investigators (17, 18).
We also note that, after the ribonuclease treatments, the total
amount of radioactivity that passes through the gel filtration
column is decreased by 75–90% relative to the untreated
RNase MRP-DNAP reaction, suggesting that the loss of the
RNA component destabilizes the nascent DNA strand from the
template. These results demonstrate that the processed RNAs
in the R-loops remain on the DNA template and are capable of
providing 3’ termini for initiating DNA synthesis on a closed
circular mtDNA template.

Because the in vitro DNA synthesis reactions were per-
formed on circular DNA templates, we wished to obtain more
precise positional information on the RNA-primed nascent
DNA strands. Transcription, RNase MRP, and T7 DNAP reac-
tions were performed as before, but in the absence of labeled
nucleotides. RNA-primed DNA products were then analyzed by
primer extensions using AMV reverse transcriptase and a 5’
end-labeled oligonucleotide complementary to the 3’ region
of the furthest RNase MRP cleavage site. Products were resolved
next to dideoxynucleotide-terminated sequencing reactions
using the same radiolabeled oligonucleotide. The experimental
control demonstrates that the SP6 RNAP reaction yields essen-
tially full-length RNA originating from the promoter (Fig. 5,
lane 2). However, when RNase MRP and T7 DNAP are in-
cluded in the reaction, multiple primer extension products are
visible, some minor species extending as far as CSB I (Fig. 5,
lane 3). This result is consistent with that observed with the
internally labeled DNA synthesis reactions described above
(Fig. 4). Although there is a background of multiple primer
extension products due to the presence of the template plasmid
(Fig. 5, compare lanes 1 and 5), the observed cluster of bands is
reproducible and depends on the actions of RNase MRP and T7
DNAP. These products were treated with RNase T2 to deter-
mine the precise locations of the RNA to DNA transitions. As
shown in Fig. 5, lane 4, the transitions map precisely to the
RNase MRP cleavage sites 5, 6, and 7. By comparing the
difference in size of the primer extension products, we deduce
that the RNA component of the nascent DNA chains is approx-
imately 157, 172, and 185 nt. Hence, it appears that only the
In vitro Mitochondrial DNA Replication

In vitro synthesis of nascent DNA chains. R-loop synthesis, RNase MRP processing, and T7 DNAP reactions were carried out as described but in the absence of radiouclides. Products were further processed as described and hybridized to a 5'-end-labeled oligonucleotide complementary to position 294–314 of the H-strand (see “Experimental Procedures”). The oligonucleotide was extended with AMV reverse transcriptase (lanes 1–5). The same primer was used for dideoxyribonucleotide sequencing, lanes G, A, T, and C indicate the respective chain terminator. Lane 1 is the circular DNA template (pK408SP) alone. Lane 2 is the SP6 RNAP reaction alone. Lane 3 is the RNase MRP + T7 DNAP reaction. Lane 4 is the RNase MRP + T7 DNAP product sample treated with RNase T2. Lane 5 is the RNase MRP + T7 DNAP reaction treated with DNase I. Arrowheads indicate the positions of RNA to DNA transitions corresponding to RNase MRP cleavage sites 5, 6, and 7.

RNase MRP-generated primer RNA termini that map downstream of CSB I are extended by T7 DNAP. This result is consistent with the in vivo distribution of RNA to DNA transitions observed in human mitochondria (3). We suggest that our reconstituted heterogeneous DNA replication system correctly recapitulates the in vivo replication mechanism.

DISCUSSION

The results of our studies suggest that the initiation of DNA replication at the mammalian mtDNA O₉₋ involves transcription-coupled synthesis of a structurally complex pre-primer RNA that remains stably hybridized to the DNA template. We have demonstrated the RNA processing activity of human RNase MRP on model R-loops either assembled in vitro or generated by transcription. Since we have found that RNase MRP cleaves at virtually all of the known priming sites, the action of this single enzyme can account for the heterogeneous primer RNA species observed in vivo. We have also demonstrated that the products of RNase MRP cleavage remain stably base paired to the DNA template strand, and these RNA fragments are capable of initiating DNA synthesis in vitro using a heterologous bacteriophage DNAP. Although relatively inefficient, we have shown that in vitro activation of the mtDNA leading-strand origin can be achieved simply by the actions of RNAP and RNase MRP.

Compelling observations support a conserved mechanism among animal mitochondria, the universal proximity of known transcriptional promoters and replication origins, and the ability of RNase MRP from various species to process the respective mtRNAs in all cases where it has been investigated. However, a true in vitro mtDNA replication system awaits the availability of well defined mammalian mitochondrial enzymes. The sequence of the putative human mtRNA polymerase catalytic subunit is now known (19); however, the precise composition of the holoenzyme remains ill defined. Characterizations of the mtDNA polymerase (pol γ) from Drosophila indicate that it is a multimeric holoenzyme with at least two subunits (20, 21), but the mammalian counterparts still remained poorly characterized. Pol γ appears to lack associated 5'-to 3' exonuclease and RNase H activities (22), features that are compatible with the proposed R-loop mechanism since we have shown that RNase H activity destabilizes the RNA-DNA hybrid. This condition presents a potentially important consequence because of the strong RNA-DNA interaction which may impede the complete replication of the H-strand. Therefore we postulate a separate mitochondrial RNase H activity that is involved in primer RNA removal. It is also conceivable that RNase MRP, given its ability to cleave at multiple sites, may be involved in primer RNA excision once DNA synthesis has initiated; this remains to be tested. It is also interesting that the nascent DNAs in our heterologous DNA synthesis system appear to be unstable after ribonuclease treatment (Fig. 4, lanes 7 and 8). These findings suggest that the RNA-DNA hybrid may influence the stability of the nascent DNA chain on the template DNA strand, perhaps by functioning as a tether that resists branch migration. In this way, such an RNA-DNA association may contribute to the stability of nascent H-strands in vivo as manifested in the three-stranded “D-loop” structure of mtDNA (1). A more complete understanding of how initiation is controlled requires further characterization of the mitochondrial transcription and DNA replication machineries.

Our in vitro model system has permitted the clear demonstration of transcription-coupled DNA synthesis that requires a critical RNA processing step. The remarkable correlation of in vitro cleavage sites on the model R-loops with the known RNA to DNA transitions at the mtDNA O₉₋ suggests that the RNase MRP reaction proceeds in vivo. How substrate recognition is achieved by RNase MRP remains unclear, but our current work implies that a specific higher order RNA structure is important. Functionally, the mtDNA initiation mechanism is similar to those of certain prokaryotic systems such as the ColE1 replicon and bacteriophage T7 where RNA-DNA hybrids are critical intermediates of transcription-generated RNA primers. For ColE1, the large (555 nt) mature primer is produced by the action of RNase H (23, 24); while for T7, replication occurs by the sequential actions of phage-encoded RNAP and DNAP presumably via the formation of transient but sufficiently stable RNA-DNA hybrids (25, 26). The mitochondrial system is unique in this regard, requiring a ribonucleoprotein enzyme to generate the mature primer termini. Why such an enzyme has been evolutionarily selected to process the mitochondrial primer RNAs is enigmatic, but the highly organized structure of the preprimer RNA suggests a unique substrate-enzyme interaction.

Phylogenetic sequence comparisons have suggested the presence of tRNA-like structures at known or presumed O₉₋ regions of several mammalian species (27). The presence of these structures is intriguing because of biochemical and antigenic similarity of RNase MRP to the universally found ribonucleoprotein RNase P, which catalyzes the 5'-maturation of pre-tRNAs (28). Our initial R-loop structural studies indicate that the RNA structure is not confined to a canonical RNA-DNA hybrid throughout its length, and it is likely to exist as a specifically
folded entity. We suggest that this folded structure is the substrate feature recognized by RNase MRP. Additional speculation on a common ancestral past between RNase MRP and RNase P is based on the proposed nucleolar function of RNase MRP in rRNA processing in *S. cerevisiae* (29–31). Indeed, it is probably more than coincidental that, for mammalian mtDNA, the two rRNAs are immediately separated and flanked by tRNAs. How the distinct nuclear and mtRNA processing roles of RNase MRP are coordinated and regulated remains a challenging question for further investigation.

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