In vitro replication of heavy strand DNA in permeabilized human mitochondria

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ABSTRACT

We have characterized some of the experimental conditions that are essential for initiation of human mitochondrial DNA synthesis. Mitochondria were purified from HeLa cells and were permeabilized with Triton X-100. When supplied with rNTPs and dNTPs, the permeabilized mitochondria synthesized nucleic acids that ranged in size from about 600 to 2000 nucleotides. In vitro DNA synthesis occurred on endogenous DNA templates and required a continuous supply of ATP. Analyses of the synthetic products revealed that almost all of them were of heavy-strand sequence and included authentic 7S DNA. Most of the synthetic products had 5′ ends that mapped to similar locations as those previously identified for nascent heavy-strand DNA. Identification of these parameters should facilitate our efforts to achieve *in vitro* replication of heavy-strand mitochondrial DNA.

INTRODUCTION

Replication of animal mitochondrial DNA (mtDNA) is initiated at two distinct origins (reviewed in ref. 1). A cycle of replication begins with synthesis of daughter heavy strands (H strands). In animal cells, the majority of mtDNA molecules contain a displacement-loop (D-loop) structure that is the result of base-pairing of short H-strand DNA to the parental DNA (2, 3). The function of D-loop strands, also called 7S DNA, is not known. However, it is likely that they arise from the same initiation events that yield fully replicated daughter DNA. The origin of H-strand DNA synthesis (O_H) is located downstream from, and in close approximity to, the promoter for transcription of the template light-strand (L-strand) DNA that is being replicated (4–7). Results of experiments that sought to localize 5′ ends of nascent H-strand nucleic acids have led to the suggestion that transcription of L-strand DNA serves the dual functions of providing RNA primers for replication as well as providing transcripts that are processed to yield tRNAs and mRNAs (5, 6). Experimental verification of this model has not been feasible primarily because the replication of mtDNA in animal cells is not amenable to experimental manipulation. Although the replication of light-strand DNA has been studied in an *in vitro* replication system, replication of H-strand DNA has not been achieved *in vitro* (8, 9). As a first step in our attempt to establish an *in vitro* assay for replication of H-strand mtDNA of human cells, we have examined the conditions that are optimal for H-strand DNA replication in permeabilized human mitochondria.

MATERIALS AND METHODS

Materials

Nucleoside triphosphates, ddTTP, and RNase inhibitor were purchased from Pharmacia. [α-^{32}P]TTP was from New England Nuclear. Phosphoenolpyruvate (PEP), pyruvate kinase (PK), aphidicolin were from Sigma. T4 polynucleotide kinase, S1 nuclease, and Moloney MuLV reverse transcriptase were from Bethesda Research Laboratories. RNase-free DNase I was from Boehringer Mannheim.

Isolation of Mitochondria

HeLa cells were grown in suspension culture in Joklik's MEM supplemented with 5% calf serum. Each batch of mitochondria was isolated from approximately 1×10^9 cells as described previously (10). Mitochondria were purified by banding in a discontinuous sucrose density gradient as described and were washed with a buffer that contained 0.25 M sucrose, 20 mM Tris.HCl, pH 7.5, 0.5 mM EDTA. The isolated organelles were pelleted by centrifugation and resuspended in a lysis buffer that contained 10% glycerol, 20 mM Tris.HCl, pH 7.5, 0.2 mM EDTA, 0.1 M NaCl, and 14 mM 2-mercaptoethanol to a concentration of approximately 10 mg protein per ml. Mitochondria were permeabilized by the addition of a stock solution of 10% Triton X-100 to a final concentration of 0.5%. The permeabilized mitochondria were stored in aliquots at −80°C and were not refrozen after they had been thawed once.

In Vitro Assay for mtDNA Synthesis

Each assay was carried out in a total volume of 100 μl and contained 20 mM Tris.HCl, pH 7.5, 10 mM $MgCl_2$, bovine serum albumin at 0.1 mg/ml, 14 mM 2-mercaptoethanol, pyruvate kinase at 10 μg/ml, 5 mM phosphoenolpyruvate, 5 mM ATP, 200 μM each of CTP, GTP, and UTP, 10 μM [α-^{32}P]TTP (10,000 to 40,000 cpm/pmol), 100 μM each of

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dATP, dCTP, and dGTP, and varying amounts of permeabilized mitochondria. Reaction mixtures were incubated at 37°C for 1 h and reaction was terminated by adding sodium dodecylsulfate and proteinase K to 0.5% and 1 mg/ml, respectively. Incubation was continued at 37°C for an additional 30 min. Incorporation of radioactivity was assayed by precipitation of reaction products with 1 N HCl containing 1% (w/v) sodium pyrophosphate as described before (10). In some experiments, the radiolabeled DNA was isolated by two successive extractions with phenol and phenol/chloroform, followed by ethanol precipitation in the presence of 10 μg E. coli tRNA. Nucleic acids were fractionated by electrophoresis in a 4% polyacrylamide gel containing 7 M urea.

S1 Nuclease Protection Analyses

In vitro reaction products that were radiolabeled using [α-^{32}P]TTP were isolated and were co-precipitated with 40 μg pKB741SP DNA that had been digested with EcoR I. The plasmid DNA contained an EcoR I insert that encodes nucleotides 1−741 of human mitochondrial DNA (23). Nucleic acids were resuspended in 30 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 50 mM Pipes, pH 6.5, and 1 mM EDTA). The mixtures were denatured by incubation for 5 min at 85°C and hybridization was carried out at 40°C for 14 h. S1 nuclease digestion was carried out by adding 0.3 ml of S1 nuclease buffer (0.28 M NaCl, 50 mM NaOAc, pH 4.8, and 4.5 mM $ZnSO_4$) containing 1000 units of nuclease S1 and incubation was continued for another 30 min at 40°C. Nuclease digestion was terminated by extracting the mixtures with phenol/chloroform. Reaction products were isolated by ethanol precipitaton after addition of 10 μg yeast tRNA and were analyzed by fractionation in a 6% polyacrylamide gel that contained 7 M urea.

Primer Extension Analyses

In vitro replication reactions were carried out as described above except that the reaction mixtures contained 0.1 mM TTP that was not radiolabeled. Total nucleic acids were isolated after the 1 h incubation and were used as templates for reverse transcription. Two oligonucleotides, one corresponding to nucleotides 76−90 of L-strand sequence (L-strand primer: 5′-CACGCGATAGCATTG-3′) and the other corresponding to nucleotides 725−709 of H-strand sequence (H-strand primer: 5′-GGTGAACTCACTGGAAC-3′) were synthesized and used as primers. The primers were radiolabeled at their 5′ ends using [γ-^{32}P]ATP and T4 polynucleotide kinase. Approximately 10^4 to 10^5 cpm of the radiolabeled primer (0.2 to 0.5 ng) and isolated nucleic acids were hybridized in 30 μl containing 40 mM Tris.HCl, pH 6.8, 1 mM EDTA, 0.4 M NaCl, and 80% formamide by denaturing at 100°C for 10 min and annealing at 37°C overnight. The hybridized nucleic acids were precipitated with ethanol and resuspended in 20 μl of reverse transcriptase buffer containing 50 mM Tris.HCl, pH 8.0, 60 mM KCl, 10 mM $MgCl_2$, 1 mM of each dNTP, 1 unit/μl RNasin, and 50 μg/ml actinomycin D. Reverse transcription was begun by adding 50 units of M-MuLV reverse transcriptase. Reaction mixtures were incubated at 37°C for 2 h and reaction was stopped by adding EDTA to 25 mM. Reaction products were isolated by extraction with phenol/chloroform and precipitation with ethanol. They were then analyzed by fractionation on a 6% polyacrylamide gel containing 7 M urea. In some experiments, synthetic products were treated with DNase I or alkali prior to primer extension. For DNase I treatment, products of *in vitro* synthesis were resuspended in a total volume of 25 μl that contained 20 mM Tris.HCl, pH 7.5, 5 mM $MgCl_2$, 12 units of RNase inhibitor, and 1 unit of RNase-free DNase I. The reaction mixtures were incubated at 37°C for 30 min. Reaction products were deproteinized and isolated by ethanol precipitation. For alkaline hydrolysis to degrade RNA, synthetic products were resuspended in 25 μl 0.3 N NaOH and were incubated at 55°C for 3 h. An equal volume of 0.3 N acetic acid was then added and reaction products were precipitated with ethanol after the addition of 10 μg E. coli tRNA.

RESULTS

Synthesis of mtDNA in permeabilized organelles

Mitochondria were purified from HeLa cells and were permeabilized with 0.5% Triton X-100. Using the crude lysate, we were able to stimulate endogenous DNA synthesis in the presence of dNTPs, rNTPs, Mg^{2+}, and an ATP-regenerating system that utilized phosphoenolpyruvate and pyruvate kinase (PEP/PK) (Fig. 1). The extent of incorporation of dNMPs increased with increasing protein concentration and reached a maximum at about 30 μg/ml. DNA synthesis was highly

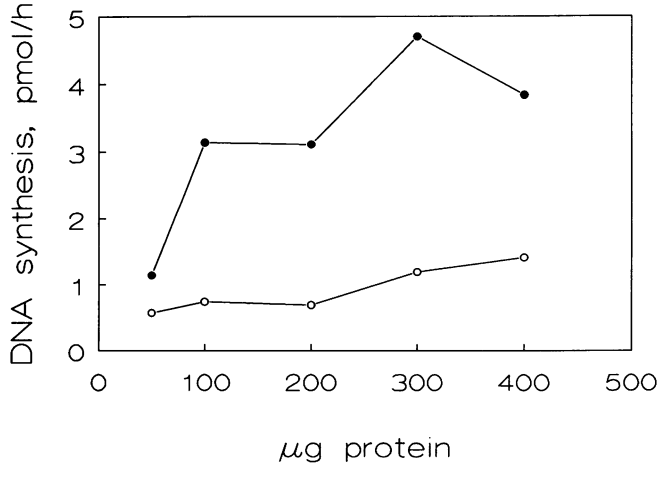

Figure 1. DNA synthesis in permeabilized mitochondria isolated from HeLa cells. Mitochondria were permeabilized and incubated in the absence (open circles) or presence (closed circles) of added rNTPs. Each reaction mixture was in a total volume of 0.1 ml and was incubated at 37°C for 1 h. DNA synthesis was assayed by measuring incorporation of [α-^{32}P]-TMP into acid-insoluble materials.

Table I. Requirements for DNA synthesis in permeabilized mitochondria.

	[α-^{32}P]TMP incorporation, %
Complete	100
Omit rNTP	23
Omit CTP, GTP, UTP	95
Omit ATP	48
Omit PEP/PK	52
Add ddTTP (0.1 μM)	24
ddTTP (1 μM)	6
aphidicolin (20 μg/ml)	89

DNA replication assays were carried out as described in Materials and Methods. Complete reaction contained 5 mM ATP, 200 μM each of CTP, GTP, and UTP, 5 mM phosphoenolpyruvate (PEP), pyruvate kinase (PK) at 10 μg/ml, 100 μM each of dATP, dCTP, and dGTP, 10 μM [α-^{32}P]TTP, and mitochondrial protein at 20 μg/ml.

dependent on the presence of rNTPs in the reaction mixture. With different preparations of mitochondria, the extent of rNTP-dependent incorporation varied between 4- to 6-fold that observed in the absence of added rNTPs. The conditions required for optimal incorporation of dNMPs were further examined by omitting various components from the reaction mixture. It was found that while omission of all rNTPs resulted in a significant reduction in TMP incorporation, omitting ATP or the ATP-regenerating system also reduced incorporation by 50% (Table I). In contrast, there was almost no apparent difference in incorporation efficiency when ATP was the only added rNTP in the reaction mixture. We also examined the effect of inhibitors of DNA polymerases on the *in vitro* DNA synthesis using permeabilized mitochondria. Incorporation of TMP was found to be extremely sensitive to the chain terminator ddTTP, whereas the presence of aphidicolin in the reaction mixture had no significant effect on the *in vitro* reaction (Table I).

Analyses of nucleic acids synthesized *in vitro*

In order to examine the sizes of the *in vitro* synthetic products, we isolated ^{32}P-labeled reaction products and fractionated them on a denaturing gel. As shown in Fig. 2, most of the reaction products were heterogeneous in size and a substantial fraction of the incorporated radioactivity was detectable in species greater than 2000 nucleotides (nt) in size. However, a number of species of discrete sizes could also be detected (lane 2). These nucleic acids had approximate sizes of 1200, 640, 600, and 550 nt. In contrast, the reaction products that were isolated from a reaction mixture that did not contain added rNTPs and PEP/PK were mostly small fragments that had been run off the gel in this experiment (lane 1). The rNTP-dependence demonstrated in the synthetic reactions suggest that there may have been *de novo* synthesis of daughter DNA in an RNA-primed mechanism.

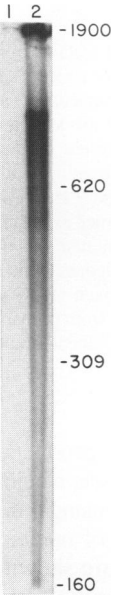

Figure 2. Size fractionation of *in vitro* synthetic products. Permeabilized mitochondria (at a protein concentration of 30 μg/ml) were incubated in the absence (lane 1) or presence (lane 2) of exogenous rNTPs and PEP/PK. Reaction mixtures were deproteinized and products were isolated by precipitation with ethanol. Reaction products were analyzed by electrophoresis on a 4% polyacrylamide gel containing 7 M urea. Numbers on the right side indicate sizes of markers in nucleotides.

Alternatively, the incorporation may have been the result of repair synthesis or elongation of pre-existing molecules in an ATP-dependent manner. Size distribution of the radiolabeled products is in the same range as that observed for 7S DNA (17–20). To examine if the *in vitro* reaction resulted in synthesis of nascent H-strand DNA, we carried out S1 nuclease protection analyses of the radiolabeled products. Synthetic products were hybridized to a DNA fragment that encodes nt 1–741 of human mtDNA. In this region of the organelle genome are located the two major promoters as well as the origin of H-strand DNA synthesis (see Fig. 6 for relative locations of promoters and the origin of H-strand DNA synthesis in this segment of the organelle genome) (4, 5). Protection of synthetic products from nuclease digestion required addition of excess unlabeled plasmid DNA (Fig. 3, compare lanes 2 and 4) and between 60 to 80% of the incorporated radioactivity remained S1-resistant (data not shown). In agreement with results described above, radiolabeled nucleic acids were not detected in those synthetic reactions that were not supplemented with exogenous rNTPs and PEP/PK (Fig. 3, lanes 1 and 3). Synthetic products of a complete reaction that were protected from nuclease digestion showed extensive size

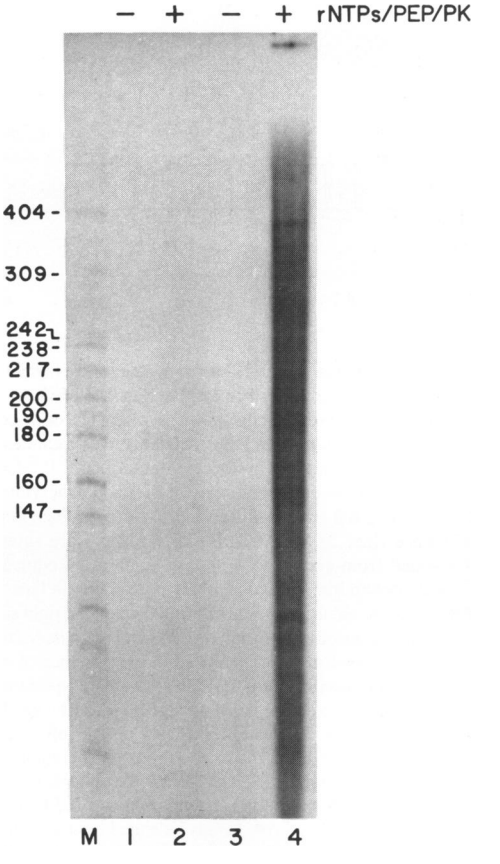

Figure 3. S1 nuclease protection analysis of synthetic products. Radiolabeled products of *in vitro* synthetic reactions were isolated as described for Fig. 2 and were hybridized to 40 μg of a plasmid DNA (pKB741SP) that encoded nucleotides 1–741 of human mtDNA. Hybridization and nuclease digestion were carried out as described in Materials and Methods and digestion products were fractionated in a 6% polyacrylamide gel that contained 7 M urea. In lanes 1 and 2 are shown digestion products obtained without plasmid DNA in the hybridization mixtures, whereas those in lanes 3 and 4 were obtained with the addition of plasmid DNA. Lanes 1 and 3 were from synthetic reactions that did not contain exogenous rNTPs and PEP/PK, whereas lanes 2 and 4 show products obtained from synthetic reactions that contained added rNTPs and PEP/PK.

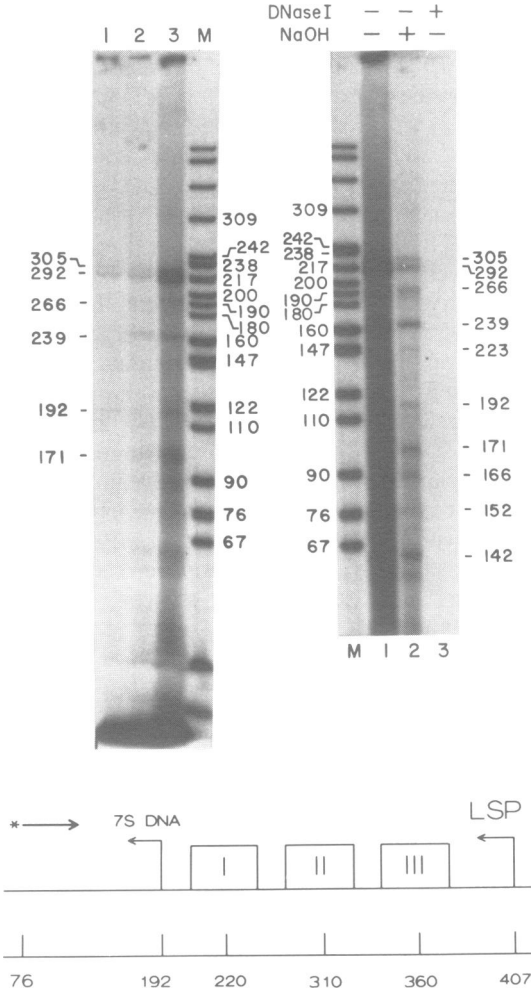

Figure 4. Primer extension analyses of H-strand nucleic acids synthesized *in vitro*. Mitochondria were incubated as described in Materials and Methods and reaction products were isolated by ethanol precipitation. An oligonucleotide corresponding to nucleotides 76 to 90 of L-strand mtDNA sequence was radiolabeled at its 5′ end and used as primer for extension by reverse transcriptase. Products of primer extension were separated by electrophoresis in a 6% polyacrylamide gel that contained 7 M urea. **Top left panel:** Mitochondria were incubated in the absence (lane 2) or prescence (lane 3) of added rNTPs. In lane 1 are shown extension products that resulted from endogenous nucleic acids in an equivalent amount of permeabilized mitochondria without incubation. Markers in lane M are a Hpa II digest of pBR322 DNA and their sizes in nt are shown on the right side. Numbers on the left side are the deduced map locations of 5′ ends of nascent nucleic acids. **Top right panel:** Mitochondria were incubated in the presence of added rNTPs and PEP/PK and reaction products were isolated by ethanol precipitation. They were then subjected to primer extension analysis directly (lane 1), or after treatment with alkali (lane 2) or DNase I (lane 3). Numbers on the left side are sizes in nt of Hpa II fragments of pBR322 DNA and those on the right side are map positions of 5′ ends of *in vitro* synthetic products. **Bottom panel:** Relative locations of the 5′ ends of major 7S DNA and the L-strand promoter (LSP) are represented by the leftward arrows. The three boxed areas indicated the locations of the three conserved sequence blocks (CSBs I, II, and III). The location of the oligonucleotide primer is indicated by the arrow with an asterisk (*). Numbers on the bottom are map locations of human mtDNA sequence.

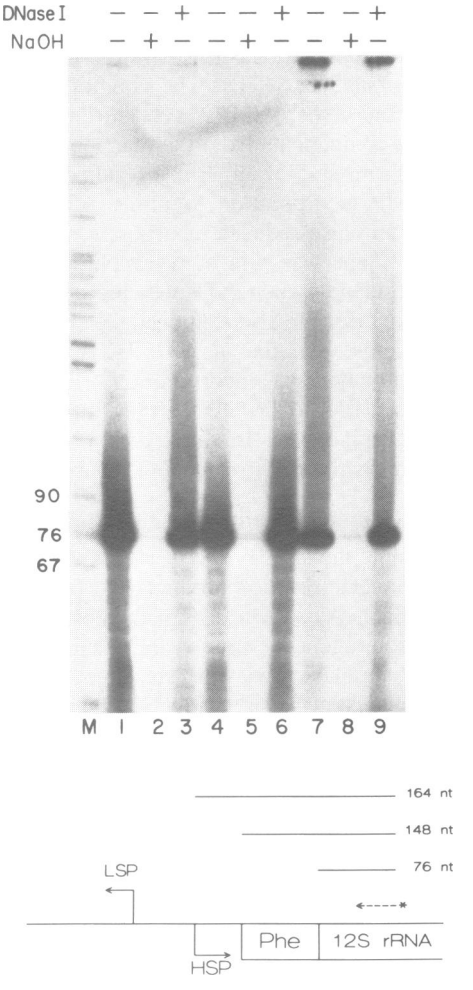

Figure 5. Primer extension analyses of L-strand nucleic acids synthesized *in vitro*. **Top panel:** Reaction products were isolated from incubation of mitochondria with (lanes 1–3) or without (lanes 4–6) added rNTPs and were subjected to primer extension analysis using an oligonucleotide that corresponds to nt 709–725 of H-strand sequence. In lanes 7–9 are shown extension products of equivalent amounts of mitochondria that were not incubated in vitro. In lanes 2, 5, and 8 are shown results obtained by pretreating synthetic products with alkali prior to primer extension. In lanes 3, 6, and 9 are shown results of pretreating synthetic products with DNase I before primer extension. Markers in lane M are Hpa II fragments of pBR322 DNA and numbers on the left side indicate sizes of some fragments. **Bottom panel:** The oligonucleotide primer used in primer extension analysis is represented by the broken arrow with an asterisk (*). Also shown are the expected extension products from primary HSP transcript and from partially processed transcript. The 76-nt fragment would result from extension of mature 12S rRNA.

heterogeneity (lane 4). However, a number of products of discrete sizes were also identifiable. These results suggest that the majority of the rNTP-dependent synthesis observed *in vitro* resulted from initiation in the D-loop region. Since the nuclease protection assay detected also nucleic acids that were only partially elongated as well as those that were being degraded, it is not possible to clearly define the nature of the nuclease-resistant products. To further characterize the synthetic products, we carried out primer-extension analyses of the nucleic acids isolated from the reaction mixtures. The advantage of using a primer-extension assay is that it allows both quantitation of nucleic acids as well as mapping of their 5′ ends. In Fig. 4 are shown results of such an analysis using an oligonucleotide probe that is complementary to, and would therefore detect, H-strand sequence. In lane 1 are shown extension products that resulted from endogenous nucleic acids present in the mitochondria. Incubation of the mitochondria in the absence of added rNTPs did not result in any additional new species of nucleic acid except one that was about 160 nt in size (lane 2, left panel). From a reaction mixture that contained added rNTPs as well as PEP/PK, we detected the same species of

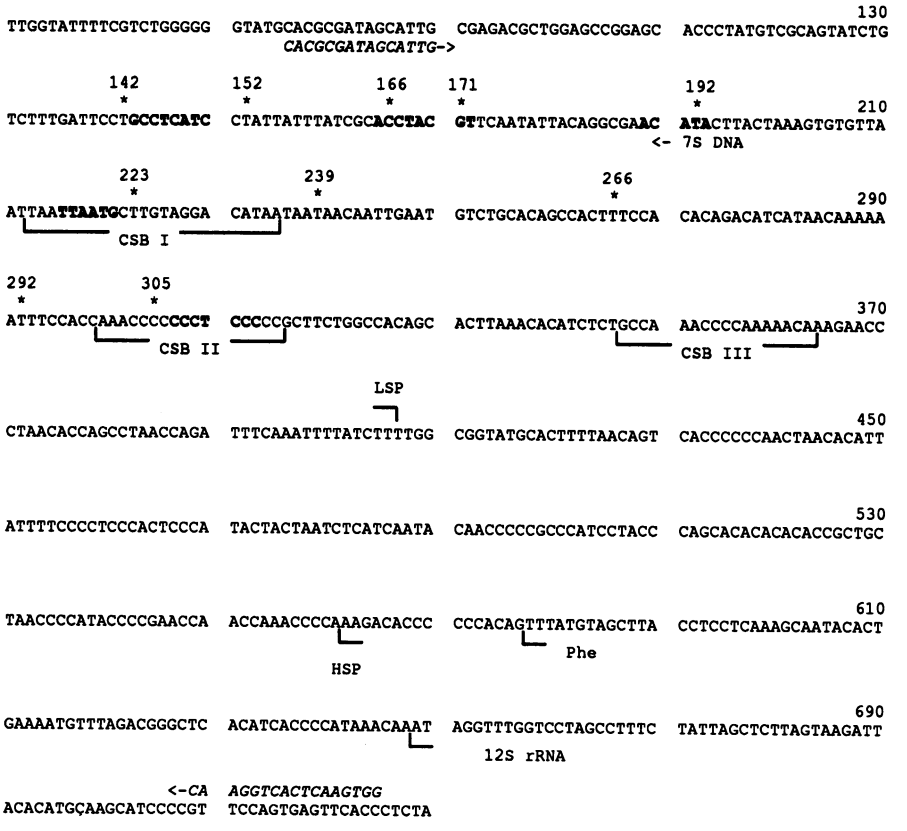

Figure 6. Map locations of products synthesized *in vitro* in permeabilized mitochondria. The L-strand sequence of a portion of human mtDNA is shown. Locations of 5′ ends of products synthesized *in vitro*, as determined by primer extension analyses, are indicated by asterisks (*). Sequences of the oligonucleotide primers are shown in italics and the conserved sequence blocks (CSBs) are marked. Shown in bold-face types are locations of 5′ ends of nascent H-strand DNA as determined by Chang and Clayton (5).

nucleic acids that were already present in the organelles (lane 3, left panel). However, the abundance of most of these species was from 5 to 10 fold greater than in the unincubated mitochondria or in those incubated without added rNTPs. The nature of the nucleic acids synthesized in a complete reaction was determined by treatment of the synthetic products with DNase I or alkali before primer-extension analysis (Fig. 4, right panel). As shown in lane 3, DNase I completely rendered the synthetic products undetectable by primer-extension. Degradation of RNA by a mild alkaline treatment substantially reduced the abundance of the materials that could be detected by primer-extension, but no major change in the sizes of the extension products was observed (lane 2). These results suggest that while almost all of the nascent nucleic acids were DNA, a substantial proportion of them contained ribonucleotides that were incorporated internally in the DNA chains. As a result, treatments that degrade RNA or DNA will both reduce the abundance of reaction products that could be detected by the primer extension assay. Map positions of the 5′ ends of synthetic H-strand nucleic acids are shown to the right of the right-hand panel of Fig. 4. It is apparent that all the nascent strands had 5′ ends that are downstream of the L-strand promoter (LSP).

A similar strategy was used to analyze and map the 5′-termini of L-strand nucleic acids synthesized in the *in vitro* reaction. A synthetic oligonucleotide complementary to L-strand sequence was used to detect any nucleic acids that may have arisen from near the major H-strand promoter (HSP). As shown in Fig. 5, the major species of nucleic acids had 5′ ends that mapped to the boundary between $tRNA^{Phe}$ and 12S rRNA. DNase I treatment had no apparent effect on the abundance or size of these species, whereas alkaline treatment resulted in virtually complete degradation. Incubation of mitochondria, either in the absence or presence of added rNTPs, resulted in only a minor difference in the abundance of the nucleic acids that could be detected with the primer (compare lanes 1, 4, and 7). These results show that in the *in vitro* reaction there was little initiation events taking place at the H-strand promoter. The major species of nucleic acids that could be detected in the assay most likely represent mature 12S rRNA.

DISCUSSION

Mechanisms of replication initiation have yet to be elucidated for most of the origins that have been identified for animal mtDNA. In the case of human mtDNA, some insights into the mechanism of initiation of L-strand DNA synthesis have been gained through studies that utilized an *in vitro* system (8, 9). However, it has not yet been possible to define features of mtDNA that are essential for initiation of H-strand DNA synthesis. Origins of H-strand DNA replication of animal mtDNA are located in regions of the genome that are highly divergent at the sequence level and do not contain structural features that resemble other replication origins. For some vertebrate mtDNA, three conserved sequence blocks (CSBs) have been identified in the D-loop region (11). However, their function in DNA replication has yet to be demonstrated. Previous results of

mapping 5′ ends of nascent H-strand nucleic acids isolated from human and mouse mitochondria have suggested that transcription from LSP may serve to provide a priming mechanism for H-strand DNA synthesis (5, 6). Earlier studies of *in vitro* mtDNA replication using intact mitochondria isolated from various animal species did not allow detection of initiation events (12 −15). Using permeabilized mitochondria isolated from *Xenopus laevis* oocytes, Dunon-Bluteau et al. demonstrated synthesis of H-strand DNA within the D-loop region. However, the specificity of the latter system could not be evaluated because 5′ ends of the products were not precisely mapped (16). Our ultimate goal in studying mtDNA replication is to be able to reconstitute initiation of DNA replication using purified components. In this report, we have identified some of the experimental conditions that may be necessary for *in vitro* replication of H-strand human mtDNA. We have shown that permeabilized mitochondria of human cells supported DNA synthesis that was dependent on the presence of exogenous rNTPs. That the *in vitro* synthesis was result of action of mitochondrial DNA polymerase was confirmed by its extreme sensitivity to ddTTP and resistance to aphidicolin. A significant extent of DNA synthesis was also observed when ATP was the only exogenous rNTP supplied to the reaction mixture. We believe that we could not observe a stringent rNTP-dependence because of the presence of an endogenous rNTP pool in the isolated organelles. Indeed, we found that freshly isolated mitochondria contained ATP with concentrations in the millimolar range (unpublished results). It is likely that the other rNTPs are also present at significant concentrations. We made several attempts to deplete mitochondrial extracts of NTPs by fractionating the extracts on a gel filtration column or by dialysis. However, the extracts became inactivated and would no longer support DNA synthesis. It is likely that such manipulations depleted some additional factors that are extremely labile or of low molecular weight and clearly more work is needed to identify a way to fractionate the extract without loss of activity. The *in vitro* synthesis in our system is highly dependent on the availability of ATP. Presumably, one of the rate limiting steps in initiation or elongation processes requires a high concentration of ATP. An alternative explanation for the lack of a stringent rNTP-dependence in DNA synthesis is that most of the TMP incorporation was result of repair synthesis or elongation of pre-existing nascent DNA strands, and that these processes also require high concentrations of ATP. However, results of S1 nuclease protection and primer-extension analyses clearly demonstrated that abundant nascent nucleic acids resulted from incubation of mitochondria in the presence of added rNTPs and PEP/PK. It should be noted that the nuclease protection assay yielded products that were highly heterogeneous in size because the assay detected also nascent strands that were only partially elongated as well as those that were being degraded by endogenous nucleases. Bogenhagen and Clayton showed that mouse D-loop strands turned over rapidly and had an apparent half-life of no more than 1 h (24). It is therefore not unexpected that some of the synthetic products were already targeted for turnover by the end of the *in vitro* incubation. It is also noteworthy that one of the prominent synthetic products that were S1-resistant had a size of about 400 nt. These products may represent nascent strands that initiated at LSP. These products were more highly radiolabeled because they were the longest nascent strands that were protected from nuclease digestion. They were not readily detectable in our primer extension assay or in nascent nucleic acids isolated from mitochondria because their relative abundance is quite low (5). The contrast in specific activity of the radiolabeled synthetic products provides additional support to our conclusion that the *in vitro* synthetic reaction represents authentic initiation of H-strand DNA synthesis.

Other investigators previously analyzed the sizes of D-loop DNA of human mitochondria and identified three major species with sizes between 550 to 670 nt (17−20). In addition, larger fragments (>1000 nt) were also detected but were not further characterized. More precise mapping showed that the major 5′ ends of D-loop DNA mapped to nucleotide 192, but that 5′ end heterogeneity exists and can account for most of the size heterogeneity observed for these nucleic acids (5, 17, 20). Sizes of reaction products that we obtained in the *in vitro* reactions are consistent with the conclusion that they may be D-loop DNA. Interestingly, results of our product analyses suggest that both initiation and arrest of elongation took place precisely in our *in vitro* reaction as were previously inferred to occur in the organelle (25).

Results of primer extension analyses are summarized Fig. 6 and can be compared with previous results of mapping of nascent D-loop nucleic acids. Within the limits of experimental errors, it can be concluded that most of the 5′ ends identified in our system are in excellent agreement with those previously identified for nascent H-strand nucleic acids. Among the ten major 5′ ends that we identified, three (those that mapped to nt 239, 266, and 292) were not noted in previous mapping experiments (5). Another apparent discrepancy is the relative abundance of D-loop strands recovered. Our analyses indicated that D-loop DNA, represented by extension products that map at nt 192, were indeed synthesized in the *in vitro* reaction. However, the abundance of these nucleic acids was much lower than those with 5′ ends that mapped to the conserved sequence blocks. The differences in these data may be a result of the different methods used to isolate nucleic acids. In the experiments described here, total nucleic acids were recovered after deproteinization of the organelles. In previous investigations mitochondrial nucleic acids were isolated using hot phenol extraction or by equilibrium sedimentation in a CsCl density gradient. It is possible that some nucleic acids may have been lost using the other, more selective, methods of isolation. Map locations of 5′ ends of the products synthesized in vitro are consistent with the proposed priming function of transcription from LSP. The inability to detect primary transcripts that emanated from LSP, or nascent DNA with RNA primers at their 5′ ends, point to the possibility that transcript processing and primer removal in human mitochondria are highly efficient. This suggestion was also put forth by other investigators as a result of analyses of 5′ ends of nascent mtDNA (1). It is noteworthy that the 5′ ends of the most abundant synthetic products mapped to CSB II (nt 305). Chang and Clayton identified an RNA processing activity in mouse mitochondria that cleaved an LSP transcript *in vitro* at precisely the same locations (21). It was suggested that the RNase is involved in processing of LSP transcripts for use in priming DNA replication.

It is intriguing that a substantial amount of ribonucleotides were incorporated internally in the nascent nucleic acids, thus rendering the synthetic products sensitive to both DNase I and alkaline treatment. Incorporation of ribonucleotides into the mitchondrial genome was previously demonstrated, thus it is unlikely that the presence of ribonucleotides in the in vitro synthetic products was the result of an *in vitro* artifact (22). At present it is not clear what the cause of this extensive ribonucleotide incorporation may be. It is also apparent from our analyses that there is an imbalance

in the relative abundance of nucleic acids synthesized *in vitro* from the two parental DNA strands. All of the H-strand nucleic acids had 5′ ends that mapped downstream of LSP and could potentially be result of initiation from that promoter. In contrast, no significant initiation from HSP was detected. The apparent bias is consistent with the proposed dual function of LSP and also with the conclusion that de novo synthesis of H-strand DNA took place in the *in vitro* reaction.

In this report we have described *in vitro* synthesis of H-strand mtDNA using endogenous DNA templates. We have not sought to examine if initiation took place also at the L-strand origin. An excellent *in vitro* system for studying L-strand replication was previously described and it is not our intention to duplicate that effort here. We have not been able to detect replication of origin-containing plasmid DNA added to the permeabilized mitochondria (unpublished results). This failure to replicate exogenous template could be a result of the limited availability of essential factors that may have already been sequestered by the endogenous templates. Our next challenge is to be able to fractionate the extract in order to separate and identify components that are required for initiation of H-strand DNA synthesis. The experimental conditions that we have shown to be necessary for DNA replication in permeabilized organelles should facilitate our future efforts to achieve *in vitro* replication of H-strand mtDNA.

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