

# Identification of initiation sites for heavy-strand and light-strand transcription in human mitochondrial DNA

[*in vitro* capped RNA/poly(A)-containing RNA/mitochondrial DNA transcription complexes/S1 endonuclease]

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**ABSTRACT** The initiation sites for heavy (H) and light (L) strand transcription in HeLa cell mitochondrial DNA have been investigated by mapping experiments utilizing *in vitro* "capped" mitochondrial RNA molecules or nascent RNA chains. Mitochondrial poly(A)-containing RNA molecules were labeled at their 5' ends with [ $\alpha$ - $^{32}$ P]GTP and guanylyltransferase ("capping" enzyme) and mapped on the mitochondrial genome by DNA transfer hybridization and S1 nuclease protection experiments. A mapping site for the capped 5' ends was found on the H strand very near to the 5' terminus of the 12S rRNA gene, and another site was found on the L strand very near to the 5' terminus of the 7S RNA coding sequence. In parallel experiments, the 5' ends of the nascent chains isolated from mitochondrial DNA transcription complexes were similarly mapped very near to the 5' termini of the 12S rRNA gene and of the 7S RNA coding sequence. The *in vitro* capped RNA molecules and the nascent chains thus presumably identify the same transcriptional initiation sites on the H strand and the L strand. The occurrence of a second possible initiation site for H-strand transcription 90–110 nucleotides upstream of that described above—i.e., 20–40 nucleotides upstream of the tRNA<sup>Phe</sup> gene—had been previously indicated by a mapping analysis of the nascent RNA chains and has been confirmed in the present work. The presence of two initiation sites for H-strand transcription can be correlated with other types of evidence that point to two different transcription events leading to the synthesis of a polycistronic molecule corresponding to the almost entire H strand and to the synthesis of the rRNA species.

Previous studies on mtDNA transcription in HeLa cells have shown that both strands are completely or almost completely transcribed (1, 2). This conclusion has been recently confirmed for the heavy (H) strand by the observation that the discrete transcripts of this strand form a continuum extending over its entire length, with the exception of a relatively small region from coordinate 98/100 to coordinate 5/100 [relative to the origin of replication taken as 0/100 (3–5) (Fig. 1)]. In the present work, the initiation sites for heavy (H) and light (L) strand transcription in HeLa cell mtDNA have been investigated by an approach that has been used successfully for identifying the transcriptional initiation sites in yeast mtDNA (8, 9). In particular, the 5' ends of total poly(A)-containing mitochondrial RNA possessing a di- or triphosphate, and therefore presumably resulting from transcriptional initiation, have been identified by taking advantage of the capacity of the guanylyltransferase ("capping" enzyme) to transfer GMP from GTP to di- or triphosphate-terminated polynucleotides (10–12). The 5' ends of mitochondrial RNA labeled *in vitro* with [ $\alpha$ - $^{32}$ P]GTP and guanylyltransferase have then been mapped on the mitochondrial

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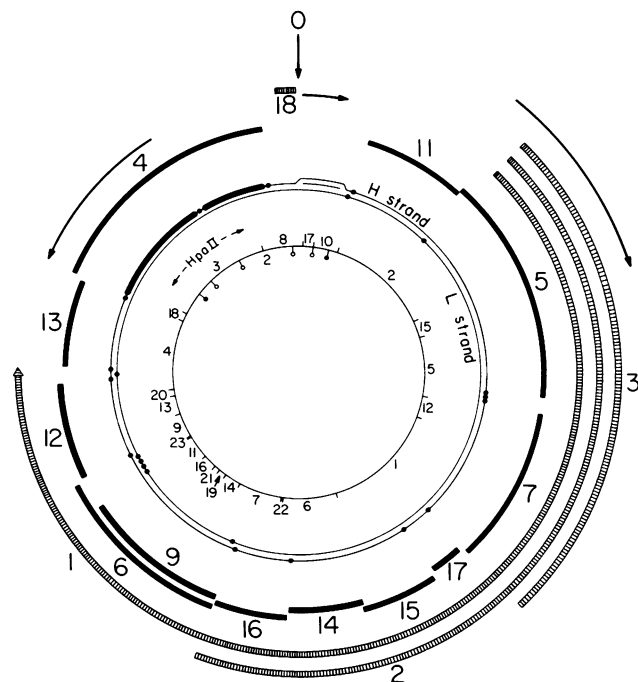


FIG. 1. Genetic, transcription, and physical maps of the HeLa cell mitochondrial genome. The two outer circles show the positions of the two rRNA genes (6) and those of the tRNA genes (●), as derived from the mtDNA sequence (7). Mapping positions of the polyadenylated H-strand transcripts are indicated by the numbered black bars, those of the L-strand transcripts by the hatched bars. Left and right arrows indicate the direction of H- and L-strand transcription, respectively; the vertical arrow (marked O) and the rightward arrow at the top indicate the location of the origin and direction of H-strand synthesis. The inner circle shows the *Hpa* II physical map of HeLa cell mtDNA, the *Kpn* I restriction sites (●) delimiting the fragment cloned in pAD23, and the *Hae* III sites (○) within this fragment (modified from refs. 3 and 6).

genome by DNA transfer hybridization and S1 endonuclease protection experiments. The positions thus found have been compared with the mapping sites of the 5' ends of the nascent RNA molecules isolated from mtDNA transcription complexes on the H and L strands.

## MATERIALS AND METHODS

**Preparation and Fractionation of RNA from Whole Mitochondria and from mtDNA Transcription Complexes.** HeLa cells growing in suspension were harvested, unless otherwise

Abbreviations: H and L strands, heavy and light strands of mitochondrial DNA; nt, nucleotide(s); np, nucleotide pair(s).

specified, towards the end of the exponential phase of growth. Isolation and fractionation of total mitochondrial RNA (13, 14), isolation of mtDNA transcription complexes (15, 16), and extraction of nucleic acids from these (16) were carried out as described, except that, in the latter case, a digestion of the sample with RNase-free pancreatic DNase [20  $\mu\text{g}/\text{ml}$ , in 0.5 ml of 0.05 M Tris-HCl, pH 6.7 (25°C)/0.025 M KCl/0.0025 M  $\text{MgCl}_2$ , for 30 min at room temperature] was performed prior to the final Sephadex G-50 chromatography.

**Guanylyltransferase Reactions.** The isolation of guanylyltransferase from vaccinia virus cores was performed as described (8). Thirty to 80  $\mu\text{g}$  of oligo(dT)-cellulose-bound mitochondrial RNA (from 15–40 g of cells) was dissolved in 3.5–8.75  $\mu\text{l}$  of 0.005 M methylmercuric hydroxide and incubated for 5 min at room temperature to minimize secondary structure. This RNA solution was adjusted to a volume of 20–50  $\mu\text{l}$  containing 0.05 M Tris-HCl (pH 7.5), 0.0025 M  $\text{MgCl}_2$ , 25–45  $\mu\text{M}$  [ $\alpha$ - $^{32}\text{P}$ ]GTP (1,000–3,000 Ci/mmol; 1 Ci =  $3.7 \times 10^{10}$  becquerels), and 0.001 M dithiothreitol (8, 9). The reaction was started with the addition of guanylyltransferase (9–12 units) and continued for 15 min at 37°C. The reaction was stopped with the addition of an equal volume of 1% NaDodSO<sub>4</sub>/0.01 M Tris-HCl, pH 7.5/0.01 M EDTA/proteinase K (EM Laboratories, Elmsford, NY) at 5  $\mu\text{g}/\mu\text{l}$ . After 30-min incubation at 37°C, the mixture was extracted twice with phenol and chromatographed over Sephadex G-50; the fractions containing the peak of radioactivity were pooled and adjusted to 0.3 M sodium acetate and the RNA was precipitated by addition of 3 vol of ethanol. The specific activity of the capped RNA preparations varied between 2,800 and 14,400 cpm/ $\mu\text{g}$ . An oligo(dT)-cellulose-nonbound preparation of HeLa cell mitochondrial RNA was subjected to capping reactions as described above. The specific activity obtained was considerably lower (1,500–2,200 cpm/ $\mu\text{g}$ ).

**Isolation and *in Vitro* Labeling of mtDNA Restriction Fragments and Strand Separation.** Isolation of HeLa cell mtDNA *Hpa* II fragment 8 (Fig. 1) was carried out as described (16). For the isolation of *Hae* III fragment 2 (Fig. 1), the plasmid pBHK2, which contains the middle-sized of the three *Kpn* I fragments of human mtDNA (Fig. 1) inserted into pAD23 [pBR322 with *Hind*III fragment D of adenovirus-2 DNA inserted into the *Hind*III site (17)], was used. For some experiments, the restriction fragments were 5'-end labeled with [ $\gamma$ - $^{32}\text{P}$ ]ATP and polynucleotide kinase after dephosphorylation with bacterial alkaline phosphatase (18). Separation and elution of the strands of the *Hpa* II fragment 8 and *Hae* III fragment 2 were performed as detailed elsewhere (16).

**DNA Transfer Hybridization Experiments and S1 Endonuclease Protection Analysis.** DNA blot analysis was carried out as described (19) with *Hpa* II restriction digests (2  $\mu\text{g}$  each) of mtDNA resolved on 2% agarose slab gels and transferred onto nitrocellulose filters (20) and *in vitro* capped oligo(dT)-cellulose-bound RNA (15,000 cpm per filter) or oligo(dT)-cellulose-nonbound RNA (20,000–75,000 cpm per filter). S1 nuclease protection experiments were performed according to a published protocol (19), utilizing high concentrations of formamide for hybridization; the enzyme was used at a concentration of 500 units/ml (for *in vitro* capped RNA) or 50 units/ml (for RNA from transcription complexes) for 30 min at 45°C (unless otherwise specified), and the analysis of the protected DNA segments was carried out under nondenaturing conditions in 5% polyacrylamide slab gels in Tris/borate/ $\text{Mg}^{2+}$  buffer (TBM) (21).

## RESULTS

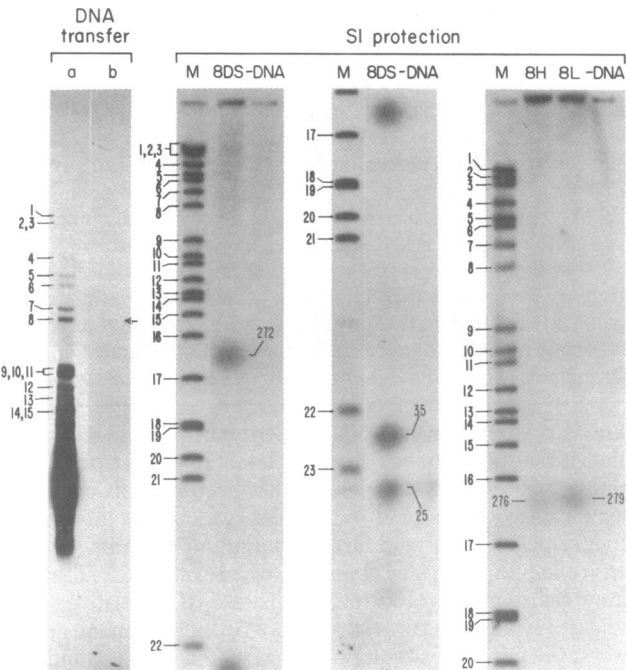
**Mapping of *in Vitro* Capped Mitochondrial RNA on the Physical Map of mtDNA.** An electrophoretic analysis through

an agarose- $\text{CH}_3\text{HgOH}$  slab gel of a sample of *in vitro* capped oligo(dT)-cellulose-bound mitochondrial RNA revealed only a smear of heterogeneous material, whereas, under the same conditions, a sample of *in vivo*  $^{32}\text{P}$ -labeled oligo(dT)-cellulose bound RNA with about one-half the amount of radioactivity exhibited the typical pattern of discrete poly(A)-containing RNA components (14) (data not shown).

A sample of the *in vitro* capped labeled RNA was incubated with a *Hpa* II digest of HeLa cell mtDNA that had been fractionated on an agarose gel and transferred onto a nitrocellulose filter. As shown in Fig. 2, the capped RNA gave a weak but clear hybridization signal with *Hpa* II fragment 8, a fragment that encompasses the origin of mtDNA replication (Fig. 1).

An S1 nuclease protection experiment utilizing *in vitro* capped labeled RNA and the double-stranded *Hpa* II fragment 8 was then performed. As shown in Fig. 2, the electrophoretic pattern of the products of the reaction exhibited a band corresponding to a protected fragment of 272 nucleotide pairs, (np), which was absent from the –DNA control. Furthermore, there were two broad bands at positions corresponding to double-stranded segments of 35 and 25 np. Two very faint bands could be seen at the same positions in the –DNA control; these may have resulted from protection of capped RNA fragments by complementary RNA sequences (3).

S1 nuclease protection experiments were then carried out with separated strands of the *Hpa* II fragment 8 and *in vitro*



**FIG. 2.** Mapping of *in vitro* capped poly(A)-containing RNA in the physical map of HeLa cell mtDNA by DNA transfer hybridization and S1 nuclease protection experiments. In the DNA transfer experiment (first two lanes), a *Hpa* II digest, *in vitro* labeled by reverse transcriptase and [ $\alpha$ - $^{32}\text{P}$ ]dGTP and -dCTP (a), and an unlabeled digest (2  $\mu\text{g}$ ) (b) of HeLa cell mtDNA were fractionated through an agarose gel and transferred onto nitrocellulose filters; the strip containing the unlabeled digest was incubated with 15,000 cpm of *in vitro* capped RNA. In the S1 nuclease protection experiments (lanes 3–12), samples of double-stranded *Hpa* II fragment 8 (8DS) or of the H strand (8H) or L strand (8L) of this fragment were incubated with *in vitro* capped labeled poly(A)-containing RNA. Lanes 3, 4, and 5 and lanes 6, 7, and 8 show the upper and, respectively, lower part of the same autoradiogram. M, marker, a *Hpa* II digest of mtDNA 3'-end labeled with *Escherichia coli* DNA polymerase and [ $\alpha$ - $^{32}\text{P}$ ]dGTP and -dCTP; –DNA, control incubation mixtures lacking DNA.

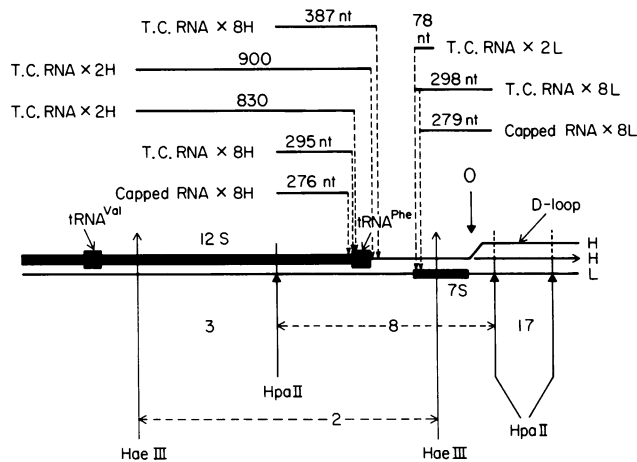


FIG. 3. Summary of the results shown in Figs. 3 and 5. The black bars in the upper part of the diagram represent the segments protected from S1 nuclease. T.C., transcription complexes; x, hybridization; 8H and 8L, H and L strands of *Hpa* II fragment 8; nt, nucleotides.

capped labeled RNA. As shown in Fig. 2, a 276-np protected fragment was observed after hybridization of the capped RNA with the H strand, and a somewhat more pronounced, barely slower-moving protected fragment was observed after hybridization of the RNA with the L strand.

The results of the S1 nuclease protection experiments discussed above are summarized in Fig. 3. Because none of the identified mtDNA-coded RNA species (14) has a size corresponding to that of the protected DNA fragments detected here, it was assumed that these include one or the other of the ends of *Hpa* II fragment 8. It is apparent that the capped RNA molecules encoded in the H strand have a 5' end mapping very close to the 5' end of the 12S rRNA gene, and the L-strand-coded capped RNA molecules have a 5' end mapping close to the 5' end of 7S RNA [polyadenylated RNA 18 (14, 22)].

DNA transfer hybridization experiments utilizing a *Hpa* II digest of mtDNA and *in vitro* capped oligo(dT)-unbound RNA were also carried out. Although up to 5 times as much radioactivity was used as in the experiment with the oligo(dT)-bound RNA, no hybridization signal was detected (not shown), possibly as a result of the dilution of the capped molecules by the large excess of unlabeled rRNA and tRNA molecules.

**Isolation of RNA from mtDNA Transcription Complexes.** Previous S1 nuclease protection experiments utilizing nascent RNA chains isolated from transcription complexes of HeLa cell mtDNA and the H strand of *Hpa* II fragment 8 labeled *in vitro* by nick-translation revealed a pronounced band corresponding to a protected fragment of about 390 np; furthermore, a faint band corresponding to a protected fragment of about 286 np was observed (16). In the present work, it seemed important to compare, under the same conditions of analysis, the mapping positions determined for *in vitro* capped RNA, as described in the preceding section, with those determined for nascent RNA chains. Therefore, transcription complexes of mtDNA were isolated by NaDodSO<sub>4</sub>/sucrose gradient fractionation of NaDodSO<sub>4</sub> lysates of HeLa cell mitochondria (15, 16). In agreement with previous observations (16, 23), the electrophoretic pattern, after ethidium bromide staining, of the total RNA extracted from the mtDNA transcription complexes revealed only heterogeneously migrating RNA, with molecular sizes ranging between 300 and 3,000 nt (not shown).

**Mapping of Nascent RNA Chains in the Region of mtDNA near the Origin of Replication.** Fig. 4A (lane 2) shows the S1 nuclease protection pattern generated by hybridization of un-

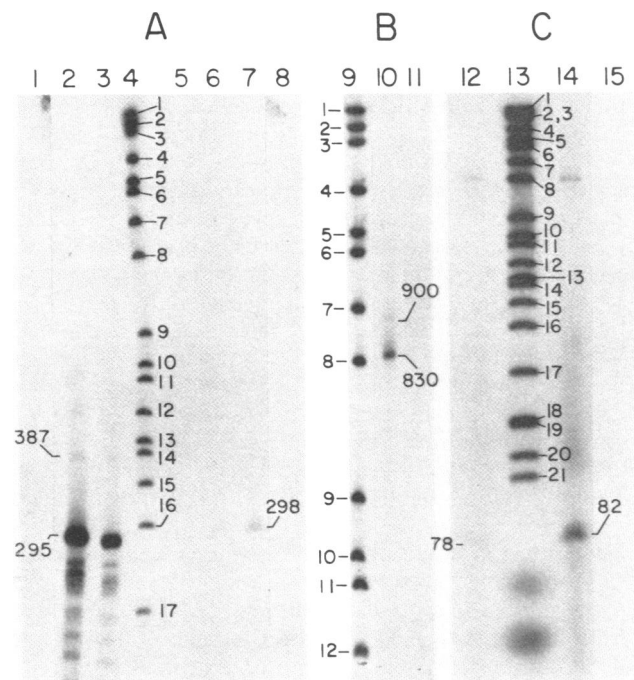


FIG. 4. S1 nuclease mapping of total RNA from mtDNA transcription complexes. In A, equivalent samples of RNA were hybridized with increasing amounts of 5'-end-labeled *Hpa* II fragment 8 H strands (estimated amounts: 5 and 10 ng in samples in lanes 3 and 2, respectively) or *Hpa* II fragment 8 L strands (estimated amounts: 2, 5, and 10 ng in samples run in lanes 5, 6, and 7, respectively); lanes 1 and 8, controls without RNA. In B, a sample of RNA (from exponentially growing cells) was hybridized with 5'-end-labeled *Hae* III fragment 2 H strands (lane 10); lane 11, control without RNA. In C, a sample of RNA from transcription complexes (lane 12) and a sample of poly(A)-containing RNA (lane 14) were hybridized with 5'-end-labeled *Hae* III fragment 2 L strands; lane 15, control without RNA. S1 nuclease digestion conditions were as described in *Materials and Methods*, except for the sample in lane 3, which was digested with 500 units/ml. Lanes 4, 9, and 13, mtDNA *Hpa* II digest markers.

labeled total RNA from transcription complexes with the H strand of *Hpa* II fragment 8 labeled at its 5' end with [ $\gamma$ -<sup>32</sup>P]ATP and polynucleotide kinase. One recognizes a pronounced band corresponding to a 295-nt protected H-strand fragment and a much fainter band corresponding to a 387-nt protected fragment. The pattern remained unchanged when 10 times as much S1 nuclease was used (Fig. 4A, lane 3) or when digestion was carried out at 37°C (not shown). The size of the shorter protected fragment would place its 3' end within the tRNA<sup>Phe</sup> sequence very close to the 5' end of the 12S rRNA gene—i.e., near the mapping position determined for the 5' ends of the H-strand-coded capped RNA molecules (Fig. 3). The size of the longer protected fragment is very similar to that of the major S1 nuclease-resistant hybrid previously observed after hybridization of *Hpa* II fragment 8 H and RNA from transcription complexes [390 np (16)]. In addition to the two bands described above, a series of minor bands corresponding to protected fragments smaller than 270 np can be seen (Fig. 4A, lanes 2 and 3).

Hybridization of the RNA from transcription complexes with the L strand of *Hpa* II fragment 8 produced only a faint band, migrating to a position slightly behind that of the 295-np protected fragment obtained with H strand and corresponding to an estimated size of  $\approx$ 298 np (Fig. 4A, lanes 5, 6, and 7). This difference in migration between the two protected fragments was reproducibly observed, in agreement with the results of the S1 nuclease protection experiments utilizing *in vitro* capped

RNA. The difference in apparent size of the protected fragments obtained with the H and L strands strongly suggested that the 298-nt fragment formed with *Hpa* II fragment 8 L strands was significant. However, in order to eliminate completely the possibility that the result obtained with *Hpa* II fragment 8 L strands was due to the presence of a small amount of contaminating H strands in the L-strand preparation, an S1 nuclease protection analysis was carried out with separated strands of *Hae* III fragment 2; these were expected to yield protected fragments of different size from those obtained with the *Hpa* II fragment 8 strands and, most importantly, of different size for the H and L strands. As shown in Fig. 4B, lane 10, with the H strands of *Hae* III fragment 2, two bands, a pronounced one corresponding to a protected H-strand fragment of about 830 nt and a fainter one corresponding to a protected fragment of about 900 nt, were observed; the sizes of these two protected fragments place their 3' ends very close to the positions identified in the experiments using *Hpa* II fragment 8 H strands (Fig. 3).

Hybridization of RNA from transcription complexes with *Hae* III fragment 2 L strands yielded, after S1 nuclease digestion, a faint band corresponding in position to the major protected fragment obtained with the H strands ( $\approx 830$  nt), resulting, presumably, from contaminating H strands (Fig. 4C, lane 12). However, in addition, a faint but clear band could be observed at a position corresponding to a size of the protected fragment of about 78 nt (an identical size of the protected fragment was estimated in another experiment). In the same experiment, hybridization of a poly(A)-containing RNA sample with *Hae* III fragment 2 L strands yielded, besides a faint band corresponding in position to the 830-nt-long H-strand segment protected by RNA from transcription complexes [probably resulting here from protection of contaminating H strands by polyadenylated RNA 4 (3)], a band representing, presumably, a DNA fragment protected by 7S RNA (Fig. 4C, lane 14); the size of this protected fragment was estimated to be 82 nt—i.e., 1–2 nt less than that expected from RNA and DNA sequence data for a segment protected by 7S RNA (22). A size of  $\approx 78$  nt for the segment of *Hae* III fragment 2 L protected by the RNA from transcription complexes would thus place the 3' end of this fragment very close to the mapping position previously determined for the 5' end of the capped RNA molecules complementary to the L strand, in agreement with the results obtained with *Hpa* II fragment 8 L (Fig. 3).

**Analysis of Oligo(dT)-Cellulose-Bound and -Nonbound Fractions of RNA from Transcription Complexes.** The finding, discussed above, that the oligo(dT)-cellulose-bound mitochondrial RNA that had been capped *in vitro* with guanylyltransferase and [ $\alpha$ - $^{32}$ P]GTP did not exhibit any detectable discrete species and consisted instead of heterogeneously migrating molecules, was puzzling, because poly(A) addition usually occurs at the 3' end of discrete components. It seemed, therefore, interesting to analyze the possible presence of poly(A) tails in the RNA molecules from transcription complexes that protected discrete segments of the mtDNA H strand near the origin of replication. For this purpose, the RNA from transcription complexes was subjected to repeated cycles of oligo(dT)-cellulose chromatography and resolved at each cycle into a bound and a nonbound fraction, with a RNA denaturation step prior to each run; each fraction was then utilized for mapping the nascent chains by the S1 nuclease protection technique, using an excess of *Hae* III fragment 2 H strands. The results are shown in Fig. 5. It is clear that the majority of the molecules that protect the 830-nt stretch of *Hae* III fragment 2 H are retained on oligo(dT)-cellulose even after three steps of denaturation and therefore are presumably polyadenylated. The implications of these

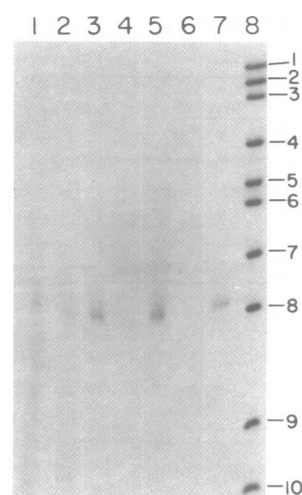


FIG. 5. S1 nuclease mapping of oligo(dT)-cellulose-bound and -nonbound RNA from mtDNA transcription complexes. A sample of RNA was tested either directly (lane 1) or after resolution into an oligo(dT)-cellulose-bound (lane 3) and nonbound (lane 2) fraction. The bound sample was passed again over oligo(dT)-cellulose and resolved into a bound (lane 5) and a nonbound (lane 4) fraction; the twice-bound sample was fractionated a third time into a bound (lane 7) and a nonbound (lane 6) fraction. An equivalent portion of each sample was hybridized with an excess of *Hae* III fragment 2 H strands. Lane 8, mtDNA *Hpa* II digest marker.

results are discussed below. It is interesting that the series of minor bands corresponding to shorter protected fragments (which in Fig. 5 appear to be smeared, for unknown reasons) are found only in the pattern obtained with the first oligo(dT)-cellulose-nonbound RNA fraction (Fig. 5, lane 2).

## DISCUSSION

The present results have fully confirmed the earlier suggestion, based on a mapping analysis of nascent RNA molecules, of the existence in HeLa cell mtDNA of initiation sites for H- and L-strand transcription near the origin of replication (16). It should be noted, however, that although the only detectable hybridization with *in vitro* capped RNA in the DNA transfer experiments was in *Hpa* II fragment 8, the present results do not exclude the presence of transcription initiation sites in other regions of mtDNA. It seems reasonable to interpret the nascent RNA molecules mapping with their 5' ends at or near the 5' terminus of the 12S rRNA gene as being related, and possibly identical, to the fraction of poly(A)-containing RNA molecules that were capped *in vitro* and mapped in the same region. The most plausible interpretation of the present results, therefore, is that the *in vitro* capped molecules and the nascent chains identify the same transcriptional initiation site in DNA very near to the 5' end of the 12S rRNA gene. The absence of any detectable 12S rRNA in the electrophoretic pattern of the capped RNA or of the RNA from transcription complexes, and the observation that the majority of the molecules having their 5' end mapping at the site discussed here were polyadenylated would exclude the possibility that the mapped molecules were 12S rRNA molecules.

A second mapping site for nascent H-strand transcripts 90–110 nt upstream of that discussed above (20–40 nt from the 5' terminus of the tRNA<sup>Phe</sup> gene) had been detected in the earlier mapping analysis of nascent mitochondrial RNA chains (16). Its existence has been confirmed in the present work by the same approach, although the relative number of molecules mapping with their 5' ends at this site has been found to be lower than

in the earlier work for reasons that are not clear. In the present work, no capped RNA molecules were detected as mapping with their 5' ends at this site, presumably due to their very low concentration. It seems possible that the two bands, corresponding to putative protected fragments of 35 and 25 np, observed in the S1 nuclease protection experiments using *in vitro* capped RNA (Fig. 2), result from cleavage occurring *in vivo* and due to processing, at or near the 5' end of the tRNA<sup>Phe</sup> sequence, of the H-strand transcripts mapping at the upstream site considered here.

A surprising observation was that the majority of the nascent chains that protected mtDNA H-strand segments having their 3' ends at or very close to the 5' terminus of the 12S rRNA gene were bound to oligo(dT)-cellulose. Because the 12S rRNA does not itself bind to oligo(dT)-cellulose, the binding of the nascent chains cannot reflect the presence of A+T-rich stretches; likewise, the design of the experiment would exclude the explanation that this binding is due to aggregation with poly(A)-containing RNA. One must, therefore, conclude that the nascent chains mapping with their 5' ends near the 5' terminus of the 12S rRNA gene are polyadenylated. This observation may reflect the existence, in association with the transcription complexes, as isolated here, of nascent chains arrested at various stages of their growth, which have become polyadenylated by the RNA processing machinery. Preliminary evidence suggests that the high frequency of premature termination and polyadenylation of nascent chains observed in the present experiments may be related to the growth conditions of the cells used, which were harvested towards the end of the exponential phase of growth. In fact, in cells in middle-exponential phase, an approximately equivalent amount of oligo(dT)-cellulose-bound and -nonbound nascent molecules has been found to protect the ≈295-nt stretch of *Hpa* II fragment 8 H (data not shown). The observation that the nonpolyadenylated nascent chains (presumably *bona fide* functional transcripts) map at the same site as the polyadenylated prematurely terminated chains strongly suggests that the transcription initiation site detected here is the correct *in vivo* initiation site. The minor bands corresponding to protected fragments shorter than 270 np, observed in the S1 nuclease protection patterns obtained with oligo(dT)-cellulose-nonbound RNA from transcription complexes, may result from degradation of nascent RNA molecules or, possibly, from infrequent initiation events within the 12S rRNA gene.

The occurrence of two putative initiation sites for H-strand transcription near the origin of mtDNA replication can be correlated with the available evidence, derived from an analysis of the kinetics of labeling and measurements of metabolic stability of the H-strand transcripts (refs. 24 and 25 and unpublished data), which points to the occurrence of two different transcription events, one leading to the synthesis of a polycistronic molecule including RNA 4 and extending over the almost entire H strand and the other limited to the rDNA region and responsible for the bulk of rRNA synthesis (see Fig. 1).

In the L strand of mtDNA, the *in vitro* capped RNA molecules and the nascent chains have been mapped at or very close to the 5' end of the 7S RNA coding sequence. Here too, it seems very likely that the two sets of molecules identify the same transcription initiation site. The size of the L-strand segments protected by capped molecules and the absence of any detectable 7S RNA in the electrophoretic pattern of the RNA from transcription complexes would exclude the possibility that the mapped molecules were mature 7S RNA molecules.

A scanning of the mtDNA sequence in the region between the 5' ends of the 12S and the 7S RNA coding sequences has revealed the occurrence, within the tRNA<sup>Phe</sup> gene, of a 14-nt stretch 21 nt upstream of the 12S rRNA gene, which exhibits a 12/15 homology to another 15-nt stretch 28 nt upstream of

the 7S RNA coding sequence:

L strand

5'... A-A-T-G-T- -T-T-A-G-A-C-G-G-G ...21 nt...12S rRNA

H strand

5'... A-A-T-G-T-G-T-T-A-G-T-T-G-G-G ...28 nt...7S rRNA.

These are the two regions of the H strand and L strand of *Hpa* II fragment 8 that exhibit the greatest homology to each other. Furthermore, these two sequences do not occur anywhere else in the H strand and L strand of the whole mtDNA. Though statistically not significant ( $P \approx 0.2$ ), the homology of the two nucleotide stretches may be meaningful because of their similar position relative to the two transcriptional initiation sites identified in the present work. It is plausible, in fact, that these two sequences are recognition signals related to these initiation sites. The occurrence of a promoter for H-strand synthesis within the tRNA<sup>Phe</sup> gene, although unusual, would not be surprising in a genome having an extreme degree of packing of information as human mitochondrial DNA has.

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