

**Mapping of nascent light and heavy strand transcripts on the physical map of HeLa cell mitochondrial DNA**

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**ABSTRACT**

The sequences complementary to the nascent RNA molecules isolated from transcription complexes of HeLa cell mtDNA have been mapped on the H and L strands of mtDNA by the S1 protection technique. The distribution of these sequences among different Hpa II restriction fragments was found to reflect the position of these fragments in the Hpa II map of mtDNA. Thus, the S1-resistant hybrids formed with the L strand corresponded almost exclusively to the right half of the genome past the origin of replication in the direction of L strand transcription, and were especially concentrated in the region immediately adjacent to the origin. By contrast, the hybrid duplexes involving the H strand appeared to be localized in the left half of the genome, and in particular in the quadrant of the map adjacent to the origin in the direction of H strand transcription. These results strongly suggest that the region of mtDNA around the origin of replication contains an initiation site for L strand transcription and an initiation site for H strand transcription.

**INTRODUCTION**

One striking feature of the transcription process of HeLa cell mtDNA is its complete symmetry.<sup>1</sup> Both strands are transcribed over their entire length;<sup>2</sup> furthermore, pulse-labeling experiments have suggested that the two strands are transcribed at a comparable rate. However, the transcripts of the H and L strands have a quite different half-life. In particular, the transcripts of the L strand have a very fast turnover and do not accumulate to any great extent.<sup>1</sup> The symmetry of transcription of mtDNA has to be contrasted with the great difference in informational content of the two strands. The H strand codes for the two rRNAs,<sup>3</sup> for most of the tRNAs<sup>4,5</sup> and for most of the poly(A)-containing RNAs.<sup>6</sup> The L strand apparently codes for seven tRNAs<sup>4,5</sup> and for one small poly(A)-containing RNA.<sup>6</sup> The low informational content of this strand makes its complete transcription very intriguing. Understanding of the significance of such a phenomenon would be facilitated if one knew whether and how the complete transcription of the L strand is related to the expression of the few genes situated on this strand, or, possibly, to the expression of the H strand genes. In order to obtain information relevant to these questions, in the present work an investi-

gation has been carried out on the location of the initiation sites of HeLa cell mtDNA transcription. Nascent RNA molecules isolated from transcription complexes<sup>7,8</sup> have been mapped on HeLa cell mtDNA by the S1 protection technique.<sup>9,10</sup> The results obtained strongly suggest that the region of mtDNA around the origin of replication contains an initiation site for L strand transcription and an initiation site for H strand transcription.

### MATERIALS AND METHODS

#### Cell Growth

The S3 strain of HeLa cells was grown in suspension in modified Eagle's medium with 5% calf serum as previously described.<sup>11</sup>

#### In vivo Labeling Conditions

In the pulse labeling experiments, exponentially growing cells were pelleted by centrifugation and resuspended at a concentration of  $10^5$  cells/ml in warm fresh medium containing 5% dialyzed calf serum. After 10 min, actinomycin D was added at 0.04  $\mu\text{g/ml}$  to inhibit the synthesis of cytoplasmic ribosomal RNA, and, 30 min later, the cells were exposed for different lengths of time to  $[5\text{-}^3\text{H}]\text{uridine}$  (1-60  $\mu\text{Ci/ml}$ ; 25-29 Ci/mole). After the pulse, the cells were rapidly collected on crushed frozen isotonic salt solution (0.13 M NaCl, 0.005 M KCl, 0.001 M  $\text{MgCl}_2$ ) in a flask immersed in an ice-salt bath. Long term labeling of mtDNA was carried out by exposing the cells for three days to either  $[2\text{-}^{14}\text{C}]\text{thymidine}$  (0.0125  $\mu\text{Ci/ml}$ ; 50  $\mu\text{Ci}/\mu\text{mole}$ ) in modified Eagle's medium containing 5% dialyzed calf serum, or to  $[^{32}\text{P}]\text{orthophosphate}$  under the conditions previously described.<sup>12</sup>

#### Isolation of mt DNA-RNA Complexes

MtDNA-RNA complexes were isolated as described by Aloni & Attardi<sup>7</sup>, with some modifications. All operations were performed at 4°C, unless specified. The mitochondrial fraction of each cell population was prepared by differential centrifugation and washed in the presence of 0.04 M EDTA, lysed at room temperature with 2% SDS in 0.01 M Tris-HCl, pH 6.7 (25°C), 0.1 M NaCl, 0.001 M EDTA (SDS buffer), and rapidly layered on a 15-30% sucrose gradient in SDS buffer prepared over a 7 ml cushion of 64% sucrose. After centrifugation in an SW27 rotor at 26,000 rpm for 5 h at 20°C and fractionation of the gradient, the fast sedimenting complexes were ethanol-precipitated.

#### Extraction and Fractionation of RNA

The ethanol-precipitated material was dissolved in 0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.001 M EDTA, and treated with 100  $\mu\text{g/ml}$  of pronase (previously digested for 2 h at 37°C at a concentration of 2 mg/ml) for 1 h at room temperature, then extracted with phenol-chloroform-isoamyl alcohol (50:50:1) and ethanol precipitated twice.

The final pellet was dissolved in 0.01 M Tris-HCl, pH 6.7, 0.15 M NaCl, 0.001 M EDTA, and layered on a 0.9 x 50 cm Sephadex G-100 column equilibrated with the same buffer. The material in the void volume was collected and used in the hybridization experiments.

#### Isolation of mtDNA and Strand Separation

The isolation of unlabeled and [2-<sup>14</sup>C]thymidine or [<sup>32</sup>P]orthophosphate labeled mtDNA and the separation of heavy (H) and light (L) strands were carried out as previously described.<sup>12,13</sup>

#### Restriction Enzyme Digestion of mtDNA

Digestion with the restriction enzyme Hpa II of closed-circular mtDNA and fractionation of the restriction fragments by electrophoresis through a polyacrylamide slab gel were carried out as previously described.<sup>12</sup> In order to determine the concentration of individual restriction fragments, a sample of in vivo [<sup>32</sup>P]orthophosphate labeled mtDNA of known specific activity was added to a known amount of unlabeled DNA, and the specific activity of the mixture was used to determine the amount of each electroeluted fragment.

#### In vitro Labeling of Hpa II Restriction Fragments of mtDNA and Strand Separation

The Hpa II fragments of mtDNA were labeled in vitro by nick-translation according to the procedure of Rigby et al.<sup>14</sup> modified as described below. In a typical experiment, eight µg of Hpa II digested mtDNA were mixed with 1.6 nmoles of each of the four deoxynucleoside triphosphates containing a total of 250 µCi of radioactivity, in a final volume of 530 µl of 0.05 M Tris-HCl, pH 7.5, 0.005 M MgCl<sub>2</sub>, 0.0025 M DTT, 50 µg/ml BSA. After a preincubation of 5 min at 15°C, the reaction mixture was treated with 10 pg/ml of RNase-free DNase (Boehringer & Mannheim) and 2 units of *E. coli* DNA polymerase I for 45 min at 15°C. The reaction was stopped by addition of 0.01 M EDTA, 1% SDS, 0.1 M NaCl, and the sample was extracted with phenol, precipitated twice with ethanol, and run on a 2 to 25% polyacrylamide gradient gel at 10 v/cm for 6-7 h, as previously described.<sup>12</sup> The bands, visualized by exposing the wet gel to an X-ray film, were cut out and the fragments electroeluted and ethanol-precipitated. The electrophoretic pattern of the Hpa II fragments labeled as described above, as well as the pattern of another Hpa II digest visualized by ethidium bromide staining, are shown in Fig. 1.

For strand separation, the fragments, suspended in 50 µl of 0.001 M Tris-HCl, pH 6.7, 0.001 M EDTA, were subjected to different treatments depending upon the fragment. Thus, Hpa II fragments No. 1 to 5 were treated with 0.02 M NaOH for 5 min at room temperature and then incubated for 2 min at 65°C. After rapid chilling in ice, the samples were run through a 1.4% agarose gel in 0.006 M Tris, 0.012 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.7, 0.002 M EDTA, at 4 v/cm for 8 h at 4°C.<sup>15,16</sup> To separate the strands of the

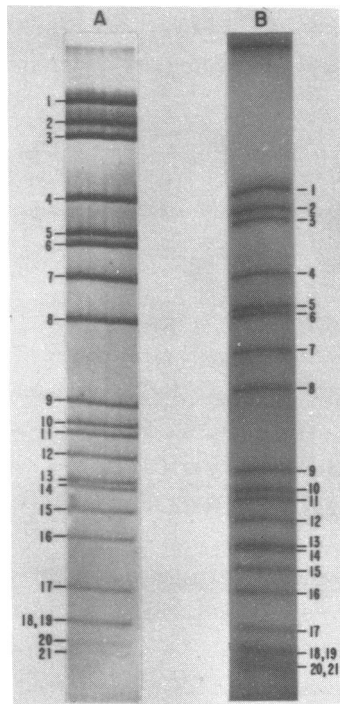


Figure 1. Electrophoretic Pattern of Hpa II Digested HeLa Cell mtDNA. A: Pattern after staining with ethidium bromide. B: Autoradiography of nick-translated Hpa II digested mtDNA.

fragments no. 6 and 8 to 10, the samples, after the denaturing step, consisting of a treatment with 0.3 M NaOH for 10 min at room temperature, were layered on a 4% polyacrylamide gel in 0.05 M Tris-borate, pH 8.3, 0.001 M EDTA, and run at 4 v/cm for 12 h at 4°C. Neither of the two methods described above, when applied to fragment no. 7, gave a good separation of the two strands from each other or from the double-stranded fragment. This separation was achieved, however, by electrophoresis through a 1% agarose gel in the Tris-NaH<sub>2</sub>PO<sub>4</sub>-EDTA buffer mentioned above. After the run, the gels were exposed overnight to an X-ray film, the bands corresponding to the separated strands were cut out, and DNA elution carried out by incubating crushed gel slices either in 0.1% SDS, 0.001 M Tris-HCl, pH 6.7, 0.001 M EDTA (agarose gels), or in 0.5 M ammonium acetate, 0.1% SDS (polyacrylamide gels) (in both cases in presence of 2 µg of yeast tRNA) at 37°C for 24 h in a rotary shaker. After adjusting the elution liquid to 0.5 M ammonium acetate (in the case of agarose gel eluates), the

DNA was ethanol-precipitated and pelleted by centrifugation at 40,000 rpm for 1 h in an SW50.1 Spinco rotor. The pellet was dissolved in 0.001 M Tris-HCl, pH 6.7, 0.001 M EDTA, and used in the hybridization experiments.

#### RNA-DNA and DNA-DNA Hybridization Conditions

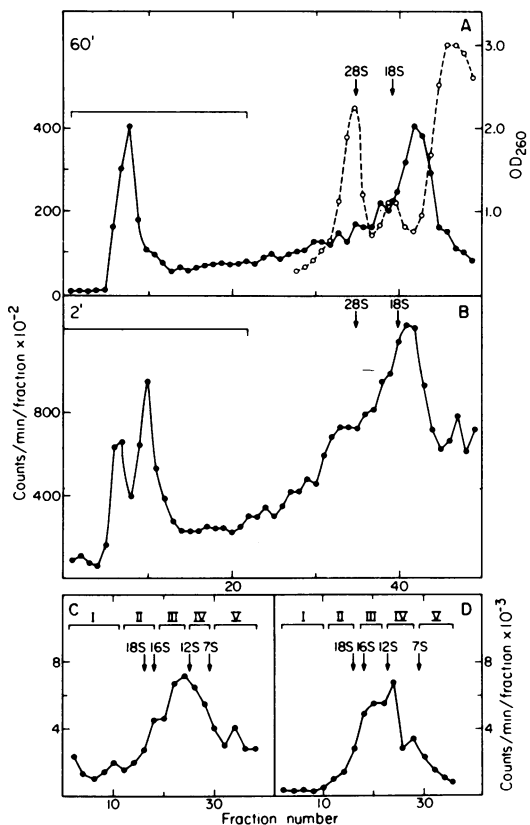
Hybridization between labeled RNA and separated strands of total mtDNA was carried out by mixing appropriate amounts of the DNA and RNA samples in 182  $\mu$ l H<sub>2</sub>O, heat denaturing the mixtures for 5 min at 95°C and finally bringing them to 0.4 M NaCl, 0.01 M Tris-HCl, pH 6.7, in a final volume of 200  $\mu$ l. The samples were incubated at 65°C for 5 h; the amount of radioactivity hybridized was determined by treatment of the incubation mixtures with 10  $\mu$ g/ml of RNase A and 10 units/ml of RNase T1 in 2 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M Na citrate) for 30 min at room temperature, and filtration through nitrocellulose filters, which were then washed at room temperature with 100 ml of warm (55–65°) 2 x SSC each.

Hybridization between separated strands of Hpa II fragments and total H or L strands was carried out under the conditions described above. After the incubation, the samples were diluted ten times with S1 digestion buffer (0.25 M NaCl, 0.04 M Na-acetate, pH 4.5, 0.003 M ZnCl<sub>2</sub>) containing 10  $\mu$ g/ml of denatured HeLa nuclear DNA, treated with 50 units/ml of the *Aspergillus oryzae* nuclease S1 (Sigma) for 30 min at 45°C, and the amount of trichloroacetic acid precipitable radioactivity determined. Hybridization between nick-translated mtDNA fragments, or their separated strands, and RNA was carried out, in a volume of 40  $\mu$ l, in 80% deionized formamide, 0.7 M NaCl, 0.03 M PIPES, pH 7.0, for 24 h at 48°C; the samples were then diluted with 360  $\mu$ l of S1 digestion buffer containing 10  $\mu$ g/ml of HeLa nuclear DNA, and incubated with 50 units/ml of S1 nuclease for 30 min at 45°C. After addition of 0.01 M EDTA and 1  $\mu$ g of tRNA, the RNA-DNA hybrids were ethanol-precipitated and run through a 5% polyacrylamide gel in 0.09 M Tris-borate, pH 8.3, 0.005 M MgCl<sub>2</sub><sup>17</sup>, for 5 h at 5 v/cm at room temperature; the gel, after drying, was exposed to an X-ray film using a Dupont intensifying screen.

## RESULTS

### Isolation of Transcription Complexes of HeLa Cell mtDNA and Analysis of Strand Homology of the Nascent RNA Chains

Transcription complexes of HeLa cell mtDNA have been previously isolated and characterized in this laboratory.<sup>7,8</sup> These structures have been utilized in the present studies as a source of nascent RNA molecules. When a mitochondrial SDS lysate from cells labeled with [5-<sup>3</sup>H]uridine for 2 min or 60 min is run through a 15–30% sucrose gradient with a 64% sucrose cushion at the bottom, patterns like those shown in Fig. 2 are obtained. In both cases, the radioactivity profile shows a single or double peak at



**Figure 2. Sedimentation Analysis of [5-<sup>3</sup>H]uridine Pulse Labeled Mitochondrial RNA.** A and B: Isolation of mtDNA transcription complexes from cells labeled with [5-<sup>3</sup>H]uridine for 2 min (A) or 60 min (B). The fractions indicated were used for RNA extraction. See text for details. C and D: Centrifugation through sucrose gradients in low salt of 2 min [5-<sup>3</sup>H]uridine pulse labeled RNA molecules isolated from transcription complexes (C) or total mitochondria (D). The RNA dissolved in 400  $\mu$ l of 0.001 M Tris-HCl, pH 6.7, was denatured for 3 min at 80°C, quickly layered on a 5-20% sucrose gradient in 0.001 M Tris-HCl, pH 6.7, 0.001 M NaCl, 0.001 M EDTA, and centrifuged for 22 h in an SW41 rotor at 35,000 rpm at 2°C.

or near the 64% sucrose cushion, a broad peak between 12S and 28S, and abundant heterogeneous material sedimenting throughout the gradient. The peak between 12S and 28S is presumably formed by the numerous discrete RNA components, in the mol. wt. range between  $3$  and  $9 \times 10^5$  daltons, which have been identified in HeLa cell mitochondria,<sup>6</sup> and which include the two rRNA species and many poly(A)-containing RNA species. The fast sedimenting components have been extensively analyzed both by E. M. and biochemically,<sup>7,8</sup> and shown to consist mainly of transcription complexes of mtDNA.

Evidence obtained in the present work has confirmed the nature of these structures, indicating that they contain mostly nascent RNA molecules. Accordingly, the fast sedimenting structures isolated under the conditions described above will be referred to as transcription complexes.

The distribution of radioactivity between H and L strand transcripts after different labeling times was investigated both in the RNA extracted from transcription complexes and in the total mitochondrial RNA by RNA-DNA hybridization experiments carried out in DNA excess. As shown in Table 1, the RNA extracted from the transcription complexes of cells labeled with [5-<sup>3</sup>H]uridine for 2 to 60 min hybridizes to the extent of 75 to 98% with mtDNA; the 1 min labeled RNA shows only a 40% homology with mtDNA, presumably due to a greater contamination of the transcription complexes by nuclear RNA. For all labeling times, the *in vivo* labeled RNA extracted from these complexes hybridizes to a greater extent with the L mtDNA strand than with the H strand. The ratio of radioactivity in the total L and H strand transcripts

Table 1. Strand Homology of [5-<sup>3</sup>H]uridine Pulse-Labeled Mitochondrial RNA

RNA Source	Labeling Time (minutes)	Percent of Radioactivity Hybridized		
		With H Strand	With L Strand	
Transcription Complexes	1	7	34	
	2	I	12	63
		II	7	79
		III	15	76
		IV	21	60
		V	23	58
	5	18	80	
	15	25	55	
	60	25	50	
	Total Mitochondria	2	I	10
II			1	66
III			18	71
IV			17	45
V			18	32
60		61	32	

\*Weighted average

present in transcription complexes appears to decrease with the length of the pulse; it is between 4 and 5 after a 1 to 5 min labeling and about 2 after a 15 min or 60 min labeling. The ratio of radioactivity incorporated in L and H strand transcripts in the total mitochondrial RNA was found to be lower than in the RNA present in transcription complexes, namely, about 3.5 after a 2 min pulse and about 0.5 after a 60 min labeling.

In the lower part of Fig. 2, the size distribution of the 2 min  $^3\text{H}$ -uridine pulse-labeled RNA molecules isolated from transcription complexes (C) or from total mitochondria (D) is shown. Both profiles show a broad peak in the region between 7S and 18S, with faster sedimenting heterogeneous material which is more abundant in the RNA from transcription complexes. The RNA from different regions of the sucrose gradients was tested for sequence homology to separated mtDNA strands. As shown in Table 1, in both RNA samples there is a tendency towards decrease, with the size of the RNA, in the labeling of the L strand transcripts relative to that of the H strand transcripts.

### Mapping of Nascent Mitochondrial RNA Molecules on the Physical Map of mtDNA by the S1 Protection Technique

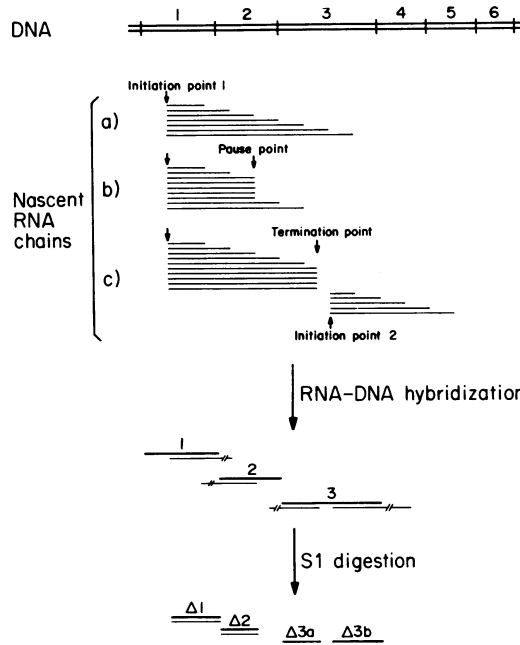
#### (i) The Approach

The previous evidence indicating that the nascent L strand transcripts consist mainly of large molecules<sup>1,6</sup> suggested an approach for the identification of their initiation site(s).

This approach is based on an application of the method developed by Berk and Sharp<sup>9,10</sup> for mapping RNA species, and is illustrated schematically in Fig. 3. In a steady state population of nascent RNA molecules one can in principle recognize subsets characterized by a common 5'- and/or a common 3'-end. A common 5'-end is represented by a fixed initiation point, or processing point, while a common 3'-end is either a nonrandom pause point in the progression of the RNA polymerase or a fixed termination point. The occurrence of such common ends in the nascent molecules makes it possible to use the S1 protection technique<sup>9,10</sup> for the analysis of the RNA-DNA hybrids formed between different restriction fragments and the nascent molecules. If the restriction fragments are small relative to the average length of the nascent chains, it should be possible to identify from the length of the protected DNA segments the positions of the initiation or processing points, pause points or termination points in the nascent RNA molecules.

In the present work, transcription complexes of mtDNA, isolated as described in Materials and Methods from cells labeled for 60 min with [5- $^3\text{H}$ ]uridine, were used as a source of nascent RNA molecules. For the purpose of comparison, the RNA extracted from the slower sedimenting components (12S-28S) in the SDS-sucrose gradient fractionation of the mitochondrial lysate (Fig. 2), which comprises the bulk of mitochondrial





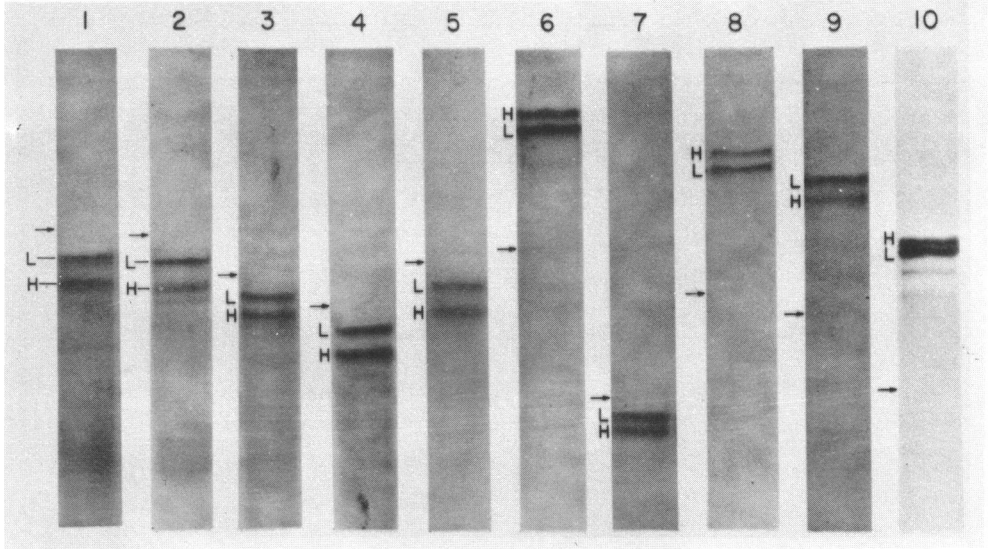
**Figure 3. Diagram Illustrating the Rationale of the Experiments Designed to Map mtDNA Nascent Transcripts.**

RNA, was also utilized in the RNA-DNA hybridization experiments.

(ii) **In vitro Labeling of Hpa II Restriction Fragments of mtDNA and Strand Separation**

The approach described above demanded a high sensitivity of detection in view of the low amount of nascent RNA molecules present in the transcription complexes, and therefore required the use of mtDNA fragments labeled in vitro to a high specific activity by nick translation. On the other hand, the need of obtaining intact strands of the fragments for their separation on gel posed some limits on their degree of in vitro labeling. It was found that an adequate specific activity ( $1$  to  $3 \times 10^6$  counts/min/ $\mu\text{g}$ ), compatible with an acceptable yield of intact strands, could be obtained by labeling the Hpa II fragments of mtDNA according to the procedure by Rigby et al.<sup>14</sup> modified as detailed in Materials and Methods.

Strand separation of Hpa II fragments no. 1 to 10 of HeLa mtDNA was achieved under different conditions for the different fragments, as specified in Materials and Methods. As shown in Fig. 4, in each case, there was no detectable radioactivity at the position where the double stranded fragment was expected to migrate, as deter-



**Figure 4. Strand Separation of Hpa II mtDNA Restriction Fragments.** The arrows indicate the positions of migration of double stranded fragments, as determined in control experiments.

mined in control experiments. It should be noticed that the separate strands migrate ahead of the undenatured fragment in the agarose gel (fragments no. 1 to 5 and 7), and behind in the polyacrylamide gel (fragments no. 6 and no. 8 to 10).

To determine the strand specificity and the purity of the separated strands of fragments no. 1 to 10, hybridization experiments with an excess of total mtDNA H and L strand were carried out. As reported in Table 2, in all cases, except for fragments no. 6, 8 and 10, the slower moving strand (U) hybridized predominantly with the total H strand, and therefore is presumably part of the intact L strand. The slower moving strand of fragments no. 6, 8 and 10 hybridized mainly with the total L strand, and therefore is presumably part of the intact H strand. The lack of complete hybridization to mtDNA of the separated strands of many of the fragments may reflect some nibbling of the ends of the hybrids by S1 nuclease. It is in fact probable that a part of the *in vitro* labeling of the Hpa II fragments in the nick translation experiments is indeed end-labeling, and end-labeled molecules would be expected to be enriched among the intact strands isolated after denaturation of the Hpa II fragments. The lower band (L) of most of the fragments showed some contamination by upper strand material, probably represented by broken strands migrating faster than intact ones.

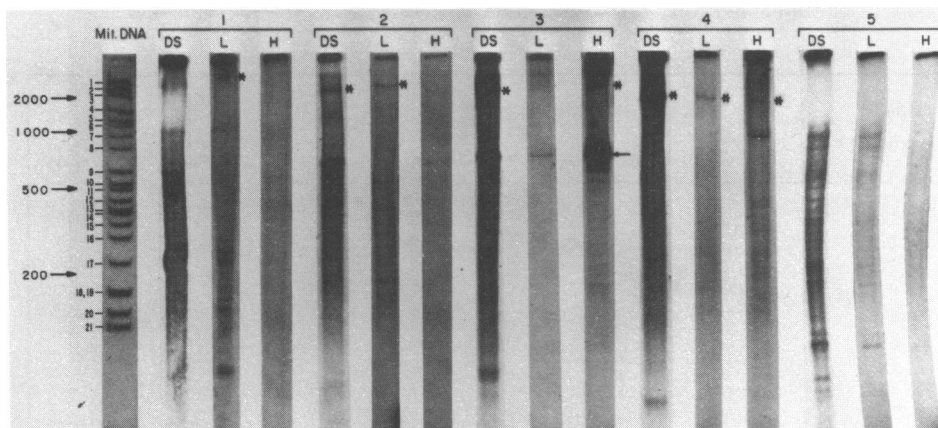
Table 2. Strand Specificity of Separated Strands of mtDNA Hpa II Fragments

Hpa II Fragment*	Percent of Radioactivity Hybridized		Strand Specificity
	With H Strand	With L Strand	
1U	42	2	L
1L	17	49	H
2U	89	6	L
2L	38	62	H
3U	64	23	L
3L	13	40	H
4U	43	1	L
4L	6	37	H
5U	66	1	L
5L	12	89	H
6U	6	83	H
6L	83	11	L
7U	58	2	L
7L	6	65	H
8U	5	83	H
8L	72	28	L
9U	64	1	L
9L	7	55	H
10U	8	72	H
10L	86	12	L

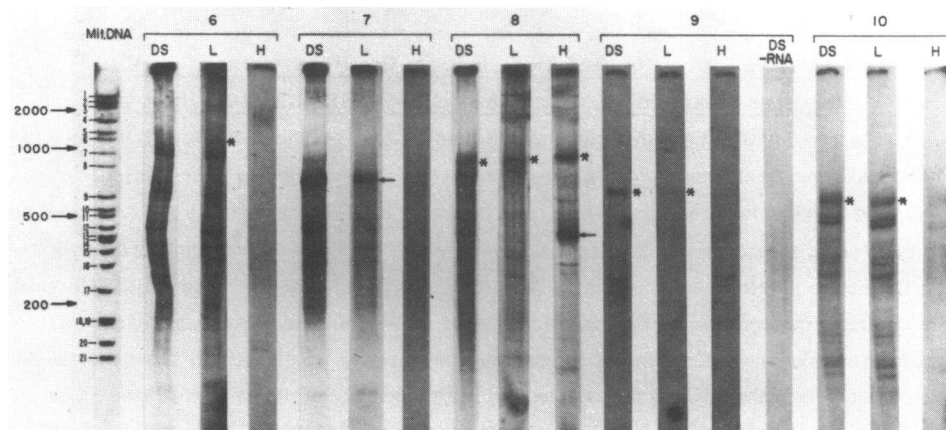
\*U and L refer to the upper and lower strand, respectively, in the separating gel.

(iii) Hybridization of Nascent RNA Chains to Hpa II Fragments of mtDNA or Their Separated Strands and Analysis of the S1-Resistant Hybrids

Figures 5 and 6 show an electrophoretic analysis in polyacrylamide gel, under nondenaturing conditions, of the S1-resistant hybrids formed, in high formamide, between nascent RNA molecules and each of the Hpa II fragments no. 1 to 10. In each case, a cascade of discrete bands is observed, one of which often corresponds to the entire length of the fragment. These full-length S1-resistant segments (indicated by asterisks in Figs. 5 and 6) probably represent renatured Hpa II fragments (formed during the dilution and cooling of the samples prior to the S1 digestion), since they were also observed in the DNA samples annealed in the absence of added RNA; however, a contribution to these bands by RNA-DNA hybrids involving the entire length of the H or L strands of the fragments cannot be excluded, since protected DNA segments of the same size were also frequently observed when one or the other of the separated strands was used for hybridization with the RNA from transcription complexes (see below). Most of the other S1-resistant bands in the hybrid mixtures were not present



**Figure 5. Electrophoretic Analysis of S1-Resistant Hybrids Formed between Nascent Mitochondrial RNA and Hpa II mtDNA Fragments 1 to 5.** Each experiment was carried out using the same amount of the separated strands of individual fragments and an approximately equivalent amount of the double stranded fragment; however, the yield of the hybrids was somewhat variable. An Hpa II digest marker was run with each set of samples. In the figure a representative marker is included. The arrow indicates the position of the strong band involving the H strand of fragment 3, which is referred to in the text.



**Figure 6. Electrophoretic Analysis of S1-Resistant Hybrids Formed between Nascent Mitochondrial RNA and Hpa II mtDNA Fragments 6 to 10.** For explanations, see Fig. 5. The arrows indicate the positions of the strong bands involving the 7L and 8H strands, which are referred to in the text.

in the DNA control samples and, therefore, presumably represent RNA-DNA hybrids.

In order to investigate the role of the two strands of each fragment in hybrid formation, nascent RNA molecules were annealed with the separated strands of Hpa II fragments 1 to 10. The results showed that the majority of the S1-resistant duplexes involve the L strands in the case of fragments 10, 2, 5, 1, 6 and 7, and, by contrast, the H strand in the case of fragments 3, 4 and 9. Fragment 8 is unique in that it produces two different sets of S1-resistant duplexes with its L and H strands. Some bands appear very strong, like a band corresponding to a size of about 700 n.p. and involving the H strand of fragment no. 3, a band corresponding to a size of about 600 n.p. and involving the L strand of fragment no. 7 and a band corresponding to a duplex of about 390 n.p. and involving the H strand of fragment no. 8. A band corresponding to the full size Hpa II fragment (indicated by an asterisk) was observed in most of the hybrid mixtures involving the L strand and in the hybrid mixtures involving the H strand of Hpa II fragments 3 and 8. Since these fully protected DNA segments were in general not observed in the DNA controls, they presumably result from complete protection of the strands by complementary RNA. However, because of variability in yield of S1-resistant segments in different hybridization mixtures, a partial renaturation of the H or L strands by contaminating complementary strands could not rigorously be excluded in every case. For this reason, these fully protected DNA segments were not considered in the analysis described below.

The distribution of the hybrids formed with the L or H strands appears to reflect the position in the physical map of mtDNA of the fragments involved. This is illustrated in Fig. 7, which summarizes diagrammatically the results of these experiments. It is clear that the hybrids formed with the L strand correspond mainly to the right half of the circular genome (defined relative to the origin of replication set at the noon position, assuming a clockwise direction of L strand transcription). In particular, an examination of Fig. 7 reveals that the bands are especially concentrated in the quadrant region adjacent to the origin of replication in the direction of L strand transcription, with a secondary cluster of bands in fragments 6 and 7, about 180° away from the origin. The bands are very rare in the left half of the genome. By contrast, the hybrid duplexes involving the H strand correspond to the left half of the genome; in particular, they appear to be almost exclusively localized in the quadrant of the map adjacent to the origin in the direction of H strand transcription (counter clockwise), i.e., in Hpa II fragments 8, 3 and 4.

The nature of the duplex of about 390 n.p. formed in the hybridization between RNA from transcription complexes and Hpa II-8 H strand was further analyzed. This duplex could not be due to hybridization with Hpa II-8 H strand of 12S RNA contaminating the transcription complexes; in fact, it is known, both from mapping studies<sup>18,19</sup>

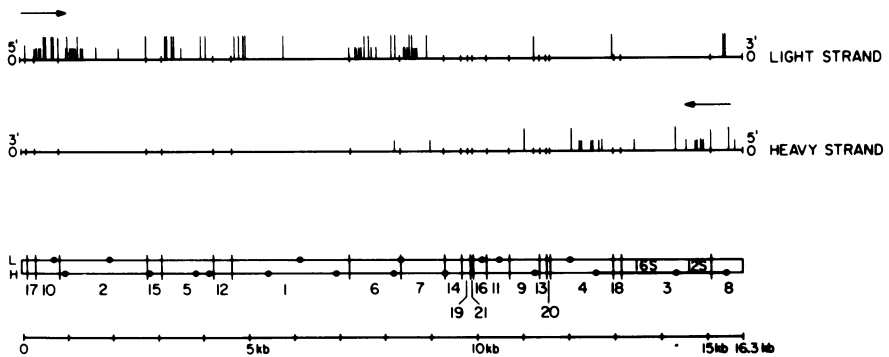
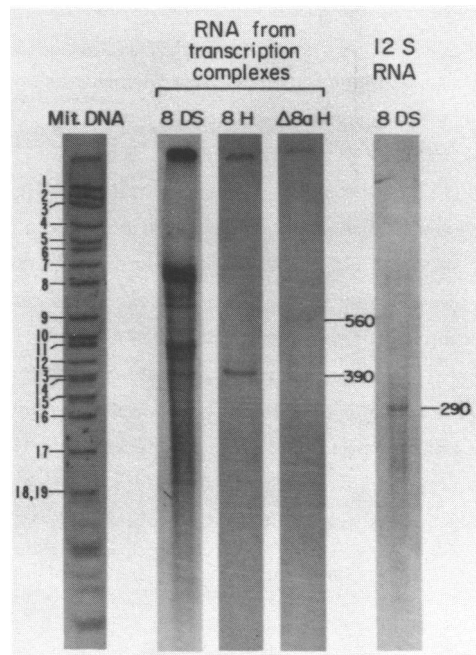


Figure 7. Diagrammatic Representation of the Results Shown in Figs. 5 and 6. In the lower part of the figure, the Hpa II map of HeLa cell mtDNA, linearized from the origin of replication (0), is shown with the positions of the 12S and 16S rRNA genes and of the 4S RNA genes (modified from ref. 12). In the upper part of the figure, the positions in the Hpa II map corresponding to the discrete bands in the gels shown in Figs. 5 and 6 (determined from the length of the protected DNA segments, assuming arbitrarily the origin-proximal end of each fragment in the direction of transcription as a starting point) are indicated by ticks. Long and short ticks refer to relatively intense and faint bands, respectively. The arrows indicate the direction of H and L strand transcription.

and from sequencing data,<sup>20</sup> that the 12S RNA sequence extends into fragment 8 for 286 nucleotides. Indeed, a faint band corresponding to a segment of that size is sometimes visible in the electrophoretic pattern of S1 digests of hybrids formed between Hpa II-8 H strand and RNA from transcription complexes (see Fig. 6, for example). To investigate further the location of the sequences of Hpa II-8 which are involved in the hybridization with RNA from transcription complexes giving rise, after S1 digestion, to the 390 n.p. duplex, hybridization experiments were carried out between this RNA and the H strand of the subfragment  $\Delta 8a^{\text{Hae}}$ : the latter is a 607 n.p. long fragment adjacent to Hpa II-3 which is generated by digestion of Hpa II-8 with the Hae III restriction enzyme.<sup>20,21</sup> As shown in Fig. 8, a band corresponding to a 390 n.p. duplex was again observed. Since the segment of  $\Delta 8a^{\text{Hae}}$  containing sequences of 12S RNA is 286 n.p. long,<sup>18-20</sup> the above result clearly indicates that the sequences of Hpa II-8 involved in the formation of the 390 n.p. duplex must overlap the sequences of the same fragment corresponding to 12S RNA. The most plausible interpretation is that the sequences of the 390 n.p. duplex include the 12S RNA sequences and extend further into Hpa II-8 in the 3' to 5' direction. The 560 n.p. band in the lane  $\Delta 8a\text{H}$  represents full length  $\Delta 8a$  duplex due to full protection of the  $\Delta 8a\text{H}$  strand presumably by complementary RNA (the slightly faster migration than expected from the known length of fragment  $\Delta 8a$ <sup>20</sup> is in agreement with previous observations<sup>21</sup>).

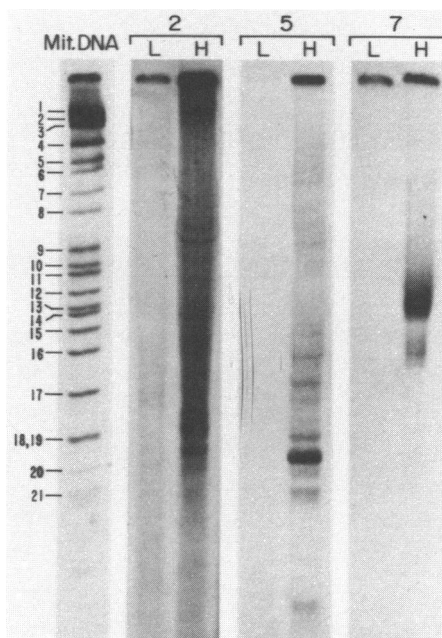


**Figure 8. S1 Protection Pattern Generated by the Hybridization of Nascent Mitochondrial RNA and 12S RNA with the Fragments 8 and  $\Delta 8a$ HaeIII.** The strands of fragment  $\Delta 8a$ HaeIII were separated on a 6% polyacrylamide gel under the conditions described for the fragments 6 and 8 to 10.

#### Hybridization of Mature RNA Species to Hpa II Fragments of mtDNA or Their Separated Strands and Analysis of the S1-Resistant Hybrids

The interpretation of the results described in the previous section depends on the assumption that the DNA segments protected from S1 digestion by the RNA derived from the transcription complexes were indeed hybridized with nascent RNA chains, and not with contaminating mature or partially processed RNA species. The complete or almost complete absence in the gel analysis described above of duplexes of the size expected for hybrids between 12S rRNA and Hpa II fragment 8 (286 n.p.) or between 16S rRNA and Hpa II fragment 3 ( $\sim 1350$  n.p.)<sup>18-20</sup> strongly suggested that the RNA derived from the transcription complexes was substantially free of contaminating mature RNA species. In order to obtain further evidence on this point, S1 protection experiments were carried out by hybridizing RNA isolated from the 12S to 28S region of the sucrose gradients shown in panels A and B of Fig. 2 with the Hpa II fragments. This RNA is known to include most of the high mol. wt. discrete poly(A)-containing and

non-poly(A)-containing components coded for by mtDNA.<sup>6</sup> The analysis by polyacrylamide gel electrophoresis of the DNA segments protected by this RNA gave results strikingly different from those obtained with RNA from transcription complexes. In fact, for all the Hpa II fragments analyzed (no. 1 to 10), a good hybridization was observed with the H strand and very little or none with the L strand. The segments of H strand protected from S1 by hybridization with the mature RNA species constituted a set of discrete bands different and characteristic for each fragment. Representative results are shown in Fig. 9. Many of these discrete duplexes had an electrophoretic mobility corresponding to the hybrids formed between the Hpa II restriction fragments and individual poly(A)-containing RNA species (Ojala, Merkel, Gelfand & Attardi, in preparation). The S1 protection pattern observed with some of the fragments, in particular the longer ones (see, for example, the pattern for fragment no. 2) showed a cascade of bands similar to that described above for the hybrids involving RNA from transcription complexes. The possible nature of these multiple S1-resistant segments will be discussed below. More significant, however, for the problem



**Figure 9. S1 Protection Pattern Generated by the Hybridization of Mature RNA Species (Sedimenting in the 12S to 28S Region of the Gradients Shown in Fig. 2A and 2B) with Some Hpa II Restriction Fragments.**



discussed here is the observation that the DNA segments protected by hybridization with the mature or partially processed RNA species were not protected in the hybridization of the H strand of the Hpa II fragments with RNA from transcription complexes. This strongly argues against an appreciable contamination of the latter RNA by mature or partially processed RNA species.

### DISCUSSION

Previous investigations on mtDNA transcription in HeLa cells, involving analysis of strand homology of mitochondrial RNA labeled after a 5 min [5-<sup>3</sup>H]uridine pulse, had indicated that the two strands of mtDNA are transcribed at a comparable rate.<sup>1</sup> In the present work, using shorter pulse-labeling with [5-<sup>3</sup>H]uridine (2 min), more than three times as much radioactivity was found to be associated with the L strand transcripts present in total mitochondrial RNA as compared to that present in the H strand transcripts. If correction is made for the ratio of dT in the H and L mtDNA strands ( $\approx 1.40$ ),<sup>13</sup> and assuming that the same precursor pools are used for the synthesis of the L and H strand transcripts, the rate of synthesis of the L strand transcripts can be estimated to be two and a half times as high as that of the H strand transcripts. This is a minimum estimate of the relative rate of synthesis of the two types of transcripts, in view of the extremely short half-life of the L strand transcripts.<sup>1,18</sup> The excess of synthesis of L strand transcripts over H strand transcripts makes the complete transcription of the L strand even more intriguing, and reinforces the suspicion that it may serve some function in part at least unrelated to the expression of the L strand genes.

The observation made here that the ratio of radioactivity in L and H strand transcripts is higher in transcription complexes than in total mitochondrial RNA, the difference being especially evident after longer pulses, certainly reflects the much shorter half-life in mitochondria of the L strand transcripts vs. H strand transcripts.<sup>1</sup> On the other hand, the finding that the relative labeling of nascent L strand vs. H strand transcripts in the transcription complexes is greater after very short pulses (1-5 min) than after longer pulses (15-60 min) may be due to the rapid increase in specific activity of the intramitochondrial UTP and CTP pools during the early equilibration period: in fact, this increase would result in a higher specific activity of the nascent transcripts with higher rate of synthesis. Finally, the decrease in the ratio of radioactivity in nascent L strand and H strand transcripts with the size of the nascent RNA, recognizable both in the RNA from transcription complexes and in total RNA after a 2 min [5-<sup>3</sup>H] pulse, is compatible with the interpretation that the nascent H strand transcripts are on the average shorter than the nascent L strand transcripts: this could result from the transcription units of the H strand being on the average shorter than those of the L strand and/or from a faster processing of the nascent H strand transcripts

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while still on the template.

A prerequisite for the approach chosen in the present work to map the main initiation site(s) of mtDNA transcription in HeLa cells was that the populations of nascent RNA molecules used for hybridization with mtDNA were not appreciably contaminated by mature transcripts. The availability of a procedure for the isolation from HeLa cells of transcription complexes of mtDNA provided indeed a convenient source of nascent RNA molecules for the mapping experiments. The substantial purity of these nascent RNA molecules was confirmed here by the complete or almost complete absence of protection by the RNA of the transcription complexes of the DNA segments expected to be protected by the 12S and 16S rRNAs and by the discrete poly(A)-containing RNA species coded for by HeLa cell mtDNA. Recently, an analysis by electrophoresis through an agarose- $\text{CH}_3\text{HgOH}$  gel of RNA from transcription complexes of cells labeled with [ $^{32}\text{P}$ ]orthophosphate has confirmed the absence in this RNA of discrete components, showing only a smear of heterogeneous RNA with traces of 12S and 16S RNA (Gelfand, Ojala & Attardi, in preparation).

The approach used here to identify the main transcription initiation sites was in principle only apt to recognize common ends in the nascent RNA molecules and, therefore, not intrinsically capable of distinguishing between initiation and processing points, pause points or termination points in the nascent RNA molecules. However, the use of transcription complexes as a source of nascent RNA molecules probably excluded completed nascent chains from playing a role, since these would not be expected to accumulate on the DNA template on which they are synthesized.

The relatively large number of discrete hybrids formed between the RNA of transcription complexes and one or the other strand of individual restriction fragments was indeed surprising. Not all of these bands could correspond to initiation points, since several of these were found in the middle of 12S rRNA or 16S rRNA cistron. A plausible interpretation is that many, and perhaps the majority, of these bands correspond to nonrandom pause points in the progression of the RNA polymerase. Such nonrandom pauses in chain elongation, possibly related to potential secondary structures in the product or template strand, have been reported to occur with most, if not all, nucleic acid polymerases.<sup>22</sup> It is possible that such pauses in the polymerase progression may be a necessary aspect of attenuation or termination of synthesis during transcription, although each pause would not obligatorily lead to a termination. It is also conceivable that pauses in chain growth may be required for the appropriate processing of nascent RNA molecules to occur. If these putative pauses in chain growth were to lead to frequent premature chain termination at multiple discrete loci as occurs late in adenovirus-2 infection<sup>23</sup>, this could also explain the multiplicity of protected DNA segments detected in the hybridization of mtDNA fragments with the

transcription products sedimenting in the 12S to 28S region of the sucrose gradient. In fact, one would expect that this slower sedimenting RNA would include, besides mature or partially processed RNA species, any discrete, prematurely terminated molecules. Further experiments are needed to clarify this point.

The occurrence of multiple discrete hybrids in the present experiments certainly precluded the recognition of potential initiation site(s) for mtDNA transcription. However, whatever the origin of this multiplicity of protected DNA segments was, it is reasonable to think that the distribution in the mtDNA physical map of the hybrids formed with the nascent chains should reflect the distribution of the RNA sequences involved, and thus may tell something about the location of the main initiation, processing or termination points of the transcripts. In particular, the discrete hybrids formed with the L strand transcripts corresponded almost exclusively to the right half of the genome past the origin of replication in the direction of L strand transcription (clockwise) and were especially concentrated in the region immediately adjacent to the origin: this distribution would be compatible with the idea that these hybrids were formed with a population of nascent L transcripts growing clockwise and having their initiation points in the region close to the origin. The clustering of bands in fragments 6 and 7 suggests that this is perhaps a region of frequent pauses of the RNA polymerase, which may be a prelude to either termination or processing, and that this region possibly contains a secondary initiation site for L strand transcription.

It is interesting to mention that, recently, discrete, giant L strand transcripts mapping in the region of the mtDNA map defined by coordinates 12/100 and 56/100 (relative to the origin taken as 0/100) have been identified (poly(A)-containing RNA species no. 2 in the classification of Amalric et al.<sup>6</sup> (ref. 18 and Gelfand, Ojala & Attardi, in preparation). It seems possible that these are not primary transcripts, but are derived from the primary transcripts by processing at the 5'-end. In any case, the map position of these transcripts is compatible with the tentative assignment made here of a main initiation site of L strand transcription in the region close to the origin of replication.

As concerns the hybrid duplexes between H mtDNA strands and RNA from transcription complexes, their mapping localization in the left half of the genome, and in particular in the quadrant of the map adjacent to the origin of replication (in the direction of H strand transcription (counterclockwise) points to the existence of an initiation point for H strand transcription near the origin, in Hpa II fragment 8 or fragment 17. Several observations strongly suggest that the prominent, 390 n.p. long hybrid duplex formed by the RNA of transcription complexes with the H strand of Hpa II fragment 8 may reflect the position of a promoter or a processing point at about 390 n.p. from the Hpa II site between fragments 3 and 8. Recent work involving

DNA and RNA sequence analysis has in fact positioned the 5'-end of the small rRNA (12S) gene at 286 nucleotides from the Hpa II site between fragments 3 and 8.<sup>20</sup>

Furthermore, a tRNA gene specific for phenylalanine has been identified on the 5'-side of the 12S rRNA gene and immediately adjacent to the latter. In other investigations, aimed at mapping the mtDNA-coded poly(A)-containing RNA species<sup>6</sup> in HeLa cell mtDNA, a species slightly larger than the sum of the two rRNA species has been shown to map in correspondence of the two rRNA genes: this observation and the relatively short half-life of this RNA species<sup>18</sup> strongly suggest that it is probably a precursor of the two mature rRNAs. The 5'-end of this presumptive precursor has been mapped at about 360 n.p. from the Hpa II site between fragments 3 and 8 (Ojala, Merkel, Gelfand & Attardi, in preparation). The close proximity of this site to the position in the map defined by the 390 n.p. hybrid duplex recognized in the present work suggests that the RNA forming these hybrids may be the primary transcript of the rRNA precursor. On the other hand, the full protection of the Hpa II-8H and Hpa II- $\Delta$ 8aH strands observed in the hybridization of these strands with the RNA from transcription complexes suggests the possibility that a promoter for H strand transcription may be located past Hpa II fragment 8 in the clockwise direction, possibly in Hpa II fragment 17. If this were the case, the position at about 390 n.p. from the Hpa II site between fragments 3 and 8 may only represent a partial processing point of a longer transcript. Further work is needed to verify the above mentioned possibilities. The nascent H strand transcripts which in the present work formed discrete hybrids with Hpa II fragments 8 and 3 presumably represent nascent rRNA chains stopped at pause points in the polymerase progression. Among these, the prominent 700 n.p. long hybrid duplex formed with the H strand of Hpa II fragment 3 corresponds exactly in size to the portion of this strand protected by hybridization with the 12S rRNA.<sup>19,20</sup> It seems very unlikely, however, that this hybrid reflects the presence in the RNA from transcription complexes of contaminating 12S rRNA, since no or only marginal amounts of the 286 n.p. hybrid expected to be formed between 12S rRNA and the Hpa II fragment 8 were ever observed. The most likely interpretation is that the 700 n.p. duplex formed by the RNA of transcription complexes represents a pause point in chain elongation, possibly related to the tRNA sequence known to exist at the 3'-end of the 12S rRNA gene (ref. 4, 24 and Barrell & Sanger, personal communication).

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