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Symmetrical *In Vivo* Transcription of Mitochondrial DNA in HeLa Cells

(electron microscopy)

Y. ALONI AND G. ATTARDI

Division of Biology, California Institute of Technology, Pasadena, Calif. 91109

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ABSTRACT **RNA·DNA hybridization experiments utilizing separated strands of HeLa mitochondrial DNA and mit-RNA from HeLa cells exposed to short pulses of [5-^{3}H]uridine have shown that the labeled RNA hybridizes with both the light (L) and the heavy (H) strand, though to a different relative extent depending upon the labeling time. Thus, hybridization of pulse-labeled RNA is about equal with the two strands when the pulse is very short (1–5 min), and becomes more and more predominant with the H strand with increasing pulse length. Pulse-labeled fast-sedimenting mit-RNA forms RNase-resistant double-stranded structures up to more than 5 μm long when self-annealed or annealed with an excess of unlabeled mit-RNA. These observations and the previous evidence of complete transcription of the H strand strongly suggest that mit-DNA is transcribed in HeLa cells symmetrically over a considerable portion of its length, with the transcript of the L strand being rapidly degraded or otherwise removed from the mitochondrial fraction.**

RNA·DNA hybridization experiments utilizing separated strands of HeLa mit-DNA and mit-RNA from HeLa cells uniformly labeled with [5-^{3}H]uridine have shown that this RNA contains sequences complementary to the whole or almost whole length of the heavy (H) strand and to a short portion of the light (L) strand (1, 2). These results have been interpreted to indicate that mit-DNA in HeLa cells is completely or almost completely transcribed, and, furthermore, that this transcription occurs almost exclusively from the H strand, as previously reported for rat liver mit-DNA (3). However, these results did not exclude the possibility that the L strand is also transcribed to a considerable extent, if not completely, with the majority of this transcript being subsequently degraded or otherwise removed from the mitochondrial fraction. In the course of investigations on the synthesis and processing of mit-RNA, an analysis of the hybridization properties of RNA from HeLa cells labeled for a short term with [5-^{3}H]uridine has unexpectedly revealed that the above mentioned possibility is indeed true. This paper describes these experiments.

MATERIALS AND METHODS

The methods of growth of HeLa cells in suspension, labeling with [5-^{3}H]uridine, preparation and partial purification of mitochondria by isopycnic centrifugation in sucrose gradient and extraction of mitochondrion-associated RNA have been previously described (4) (the isopycnic centrifugation was performed here for 40 min at 25,000 rpm in the Spinco SW25.1 rotor).

The RNA samples, fractionated on a sucrose gradient, to be used in RNA·DNA or RNA·RNA hybridizations were subjected to DNase digestion and phenol–sodium dodecylsulfate (SDS) extraction and either sedimented again through a sucrose gradient or fractionated on Sephadex G-100 (1).

Hybridization of mit-RNA with H or L mit-DNA strands (which had been separated in an alkaline CsCl density gradient, ref 1) was performed in most cases in 0.5 ml of 0.4 M CsCl –0.01 M Tris buffer pH 8.0 (25°C)–0.01 M EDTA, at 66°C for 4 hr; after rapid cooling, the mixtures were treated with 5 μg/ml of previously heated pancreatic RNase for 20 min at room temperature, then filtered through nitrocellulose membranes, which were washed with 100 ml of 2× SSC (SSC = 0.15 M NaCl–0.015 M Na citrate). Unless otherwise specified, the RNA preparations used in the RNA·DNA hybridizations were denatured by heating at 90°C for 5 min in 1 mM NaCl and quick cooling.

Annealing of the pulse-labeled RNA with an excess of cold mit-RNA or self-annealing was performed in 4× SSC at 66°C for 24 hr. To estimate the RNase-resistant RNA, we brought the reaction mixtures to 2 × SSC, treated them with 10 μg/ml of pancreatic RNase for various times, and precipitated them with cold 10% trichloroacetic acid.

RESULTS

Sedimentation pattern of pulse-labeled mit-RNA

Fig. 1*a* shows the sedimentation profile of labeled mit-RNA extracted with phenol and SDS from cells exposed to [^{3}H]-uridine for 20 min. The 28S rRNA pertaining to ribosomes of the rough endoplasmic reticulum (5) provides a convenient sedimentation marker. The radioactivity profile shows heterogeneous RNA sedimenting between 4 S and >50 S (6, 7). The radioactivity profile obtained by resedimenting the RNA with $s > 30$ S (including the pelleted material), after DNase treatment and phenol–SDS extraction, is shown in Fig. 1*b*. The DNase digestion did not release RNA components of slower sedimentation rate, and a high proportion of the labeled RNA (~30%) was found in or near the cushion of dense sucrose at the bottom of the tube. Resedimentation for a shorter time (after DNase digestion and phenol–SDS extraction) of the labeled RNA that sedimented to the cushion in a parallel experiment (Fig. 1*c*, *insert*) allowed a further fractionation of this material (Fig. 1*c*).

This is article VII in the series *Expression of the Mitochondrial Genome in HeLa Cells*. Paper VI is ref. 10.

Abbreviations: mit-DNA, mitochondrial DNA; mit-RNA, mitochondrial RNA; H, heavy; L, light; SDS, sodium dodecyl sulfate.

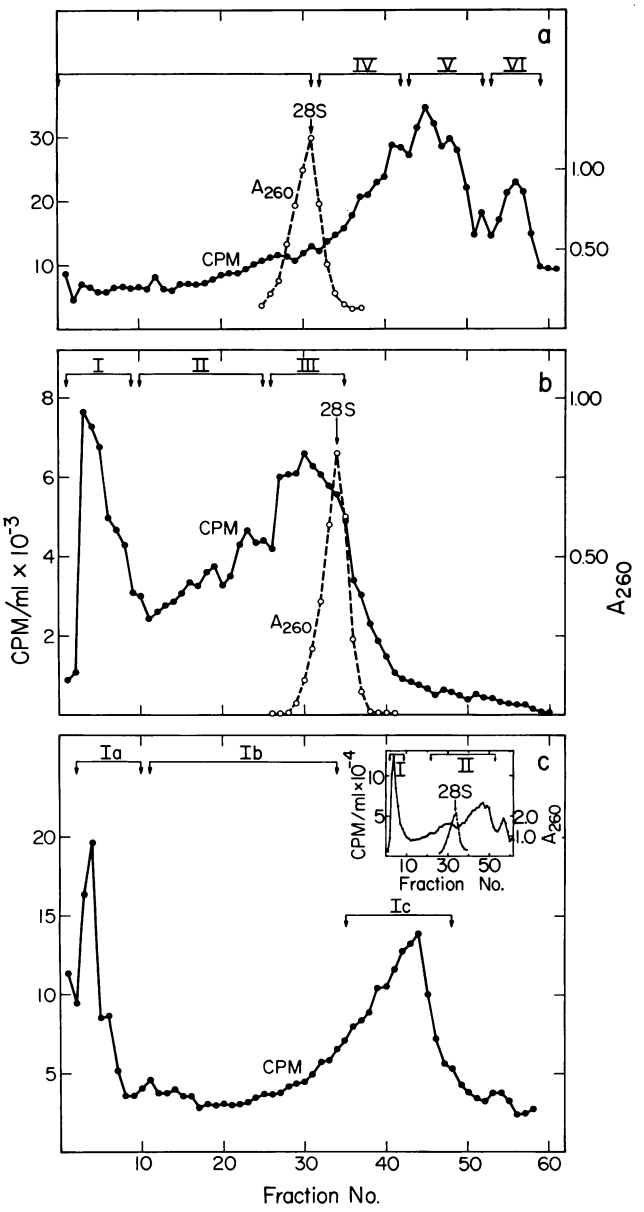

FIG. 1. Sedimentation patterns of mit-RNA from 3 × 10^8 HeLa cells exposed for 20 min to [5-^{3}H]uridine (10 μCi/ml, 25 Ci/mmol) in the presence of 0.04 μg/ml of actinomycin D. 15–30% sucrose gradients in SDS buffer (in *b* and *c*, over 1.5 ml of 64% sucrose), Spinco SW27 (1.59 × 10.16 cm buckets) 12 hr, 26,000 rpm (*a*, *b*, and *c insert*) or 3 hr, 25,000 rpm (*c*).

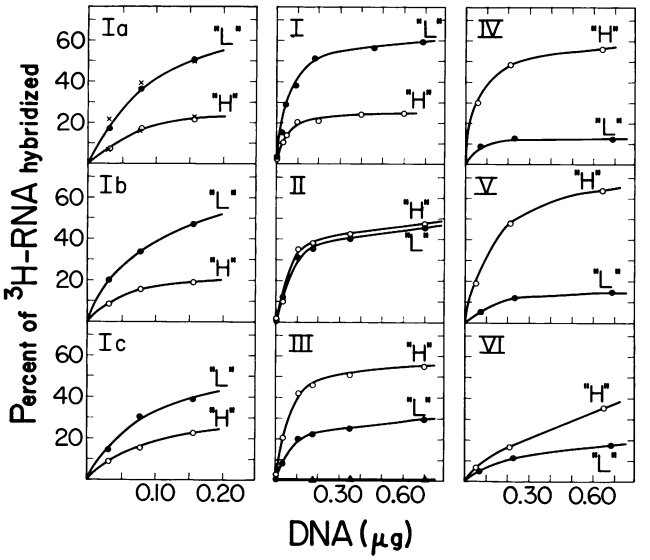

FIG. 2. RNA·DNA hybridization between [^{3}H]RNA (100–300 cpm) from different cuts of the patterns of Fig. 1 and various amounts of L or H mit-DNA strands. The hybridization values have been corrected for the background obtained without DNA (<1% of input). ▲—▲, total denatured HeLa DNA. (I*a*): unheated RNA is indicated by points, x.

Relative proportion of pulse-labeled RNA hybridized with the H and L mit-DNA strands in different portions of the sucrose gradient

Cuts of the sedimentation patterns of [^{3}H]mit-RNA shown in Fig. 1*a*, *b*, and *c* were pooled as indicated, and the material in each cut was collected by ethanol precipitation and centrifugation, and treated with DNase (1). The plateau levels obtained in hybridization tests between a constant amount of these RNA fractions and increasing amounts of separated strands of mit-DNA allowed the estimation of the relative proportion of the *labeled* (i.e., newly synthesized) RNA homologous to the H and L mit-DNA strands. [It should be noticed that because of the 1.4 times higher dT content of the H mit-DNA strand (1), the potential transcripts of the L strands would be, on the average, more labeled with [5-^{3}H]-uridine, up to 1.4 times, than the transcripts of the H strands.] About twice as many cpm of the labeled RNA hybridized with the L strand, as compared to those hybridized with the H strand, when the RNA from the cushion of the sucrose gradient was used (Fig. 2); the proportion of cpm of the labeled RNA that hybridized with the H strand increased when RNA of slower sedimentation rate was used, so that with the labeled RNA sedimenting in the range 10–25 S, four to five times as much hybridization was observed with the H strand as with the L strand.

In the exhaustion experiments of Fig. 2, if we assume that different sequences hybridized with the L and the H mit-DNA strands, between 65 and 95% of the original labeled RNA formed complexes with mit-DNA. Under the conditions of hybridization used here, a certain fraction of complementary sequences of RNA were expected to form RNase-resistant duplex structures (see below); these were presumably in most part lost in the filtration through nitrocellulose membranes.

Relationship between labeling time and the proportion of radioactive RNA hybridized with the H and L mit-DNA strands

Table 1 shows the ratio of [^{3}H]mit-RNA hybridized with the L and H mit-DNA strands in hybridization–exhaustion experiments utilizing mit-RNA extracted with phenol and SDS from cells labeled with [^{3}H]uridine for different periods of time. Almost equal amounts of the [^{3}H]RNA (in cpm) extracted from cells labeled for 1 min hybridized with the two strands; the proportion of labeled RNA hybridized with the H strand increased with increasing pulse length, which suggests a greater accumulation of the transcripts of this strand.

Extraction of pulse-labeled mit-RNA with cold phenol–SDS is not complete, the yield being about 50% (unpublished observations). An almost complete recovery of pulse-labeled mit-RNA was achieved by SDS–pronase treatment (40 μg/ml,

TABLE 1. *Hybridization of [5-^{3}H]uridine pulse-labeled mit-RNA with separated mit-DNA strands*

RNA extraction procedure	Labeling time (min)	[^{3}H]RNA hybridized with "L" / [^{3}H]RNA hybridized with "H"
SDS–phenol	1	0.98
	5	0.74
	20	0.58
	45	0.25
SDS–pronase–phenol	5	1.43
	5*	1.45

* No actinomycin D was used.

The data for the 5- and 20-min samples (except the sample with no actinomycin D) were derived from the weighted averages of hybridization values obtained from all the different cuts of the sucrose gradient (see, for example, Fig. 1); the other data were obtained from hybridization results with unfractionated mit-RNA subjected to DNase treatment, phenol–SDS extraction, and Sephadex G-100 chromatography.

2 hr at 37°C) followed by phenol extraction. The ratio of hybridization (in cpm) of SDS–pronase–phenol-extracted RNA with the L and H mit-DNA strands was almost twice as high as found with phenol–SDS-extracted RNA after the same labeling time (Table 1); the ratio approached 1.40. If correction is made for the ratio of dT in the H and L mit-DNA strands (~1.40, ref. 1), the initial rate of labeling of the transcripts of the two mit-DNA strands becomes about equal.

In the experiments described above, the cells had been labeled with [5-^{3}H]uridine in the presence of 0.04 μg/ml of actinomycin D to inhibit cytoplasmic ribosomal RNA synthesis. Table 1 shows that omission of this drug did not affect the results.

RNase-resistance tests and hybridization–exhaustion experiments after prior incubation of the RNA

The amount of double-helical structures in the labeled mit-RNA after a 20-min pulse was estimated from a kinetic study of RNA degradation by RNase. The RNase resistance was about 20% in the material from the cushion of the sucrose gradient (cut I of Fig. 1*b*) and decreased in the slower-sedimenting RNA (Table 2). The RNase resistance of the material in the cushion was lost after heating at low ionic strength (Fig. 3*a*).

After self-annealing, the RNase resistance of the RNA increased considerably, up to about 60% for the heavier components (Fig. 3*a*, Table 2).

Hybridization of 20-min-labeled RNA from the cushion of a sucrose gradient with unlabeled RNA sedimenting in the region 10–35 S resulted in 85% of the total radioactivity becoming RNase-resistant (Table 2).

The incomplete RNase resistance of the self-annealed preparations suggested that the amount of sequences complementary to one of the two mit-DNA strands was in excess. In fact, a prior incubation of mit-RNA resulted in an increase in the ratio of cpm hybridized with the H strand to those hybridized with the L strand, from about 1 for cut II and 2.0 for cut III (Fig. 2) to 2.0–2.5 and 5–10, respectively (Fig. 3*b* and *c*). The excess hybridization with the H strand may be underestimated if mit-DNA strands hybridize with nonbase-paired tails of

TABLE 2. *Ribonuclease resistance of newly synthesized mitochondrial RNA*

RNA sample	Ribonuclease resistance (%)			
	Original preparation	After self-annealing		After annealing with unlabeled RNA*
		24 hr	48 hr	
I	20	48	—	85
II	15	45	60	—
III–VI	10	—	—	—
III	—	31	32	—

* 10–35S (~7 μg).

[^{3}H]RNA samples (about 300 cpm) from the indicated cuts in Fig. 1*a* and *b* were tested for RNase resistance as detailed in *Materials and Methods*.

RNase-resistant RNA duplexes, which would thus be retained on the filters.

The fact that in RNA fraction analyzed in Fig. 2, II, equal cpm of sequences complementary to the two strands were measured, whereas in Fig. 3*b* there was, after self-annealing, an excess of unannealed labeled sequences complementary to the H strands implies that the "average" specific activity of the transcripts of the L strands is higher than that of the transcripts of the H strands. The same conclusion derives from

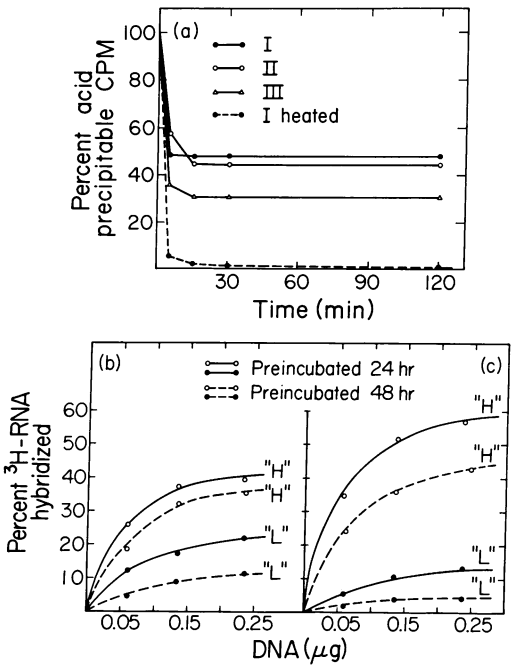

FIG. 3. *a*, Degradation by pancreatic RNase of pulse-labeled mit-RNA after self-annealing. [^{3}H]RNA samples (about 300 cpm) from the indicated cuts of the sucrose gradient patterns of Fig. 1*b* were tested for sensitivity to RNase after incubation for 24 hr. *b* and *c*, Hybridization with mit-DNA strands of self-annealed pulse-labeled RNA. [^{3}H]RNA samples from cuts II (*b*) and III (*c*) of Fig. 1*b* were used for hybridization with mit-DNA strands as in Fig. 2, after incubation for 24 or 48 hr.

In each experiment, the radioactivity of the input RNA was determined by the same procedure (i.e., after trichloroacetic acid precipitation or plating on nitrocellulose membranes) used for the analysis of the experimental results.

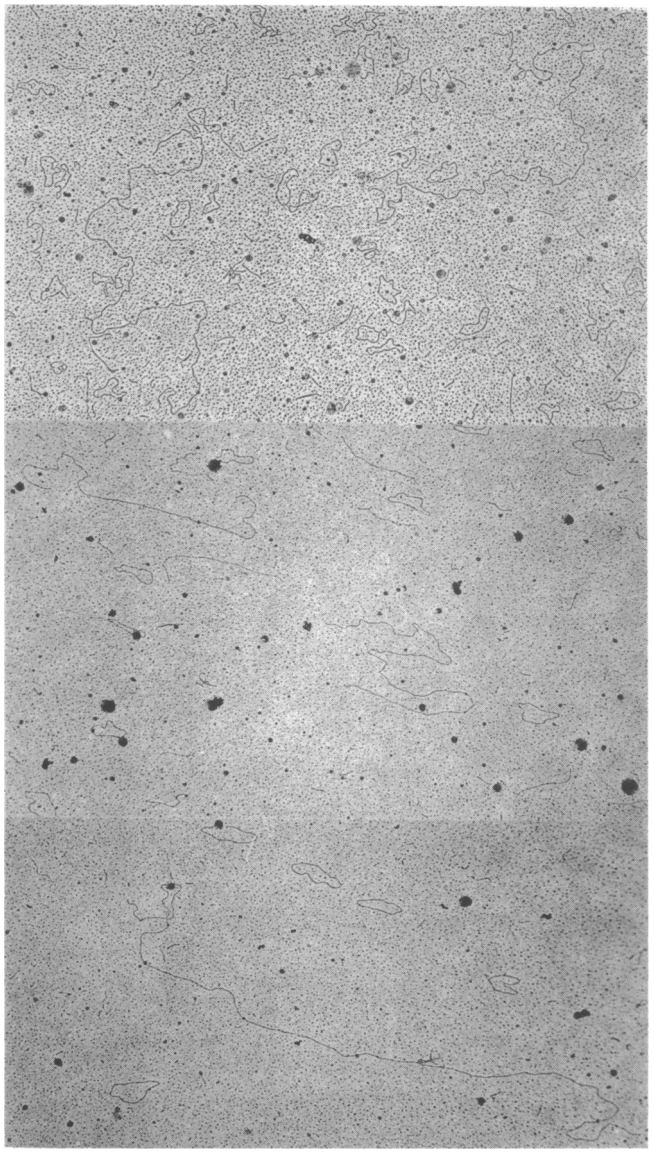

FIG. 4. Duplex nucleic acid structures (stained and shadowed, *upper field,* or simply stained) present in an RNA sample from the cushion of a sucrose gradient (cut I in insert of Fig. 1*c*) after self-annealing and RNase digestion (see text). The circles are of SV40 DNA (~1.7. μm, ref. 12).

the comparison of Figs. 2, III and 3*c*. That this difference in specific activity cannot be solely due to the different uridine content of the transcripts of the two strands is strongly suggested by the previously described decrease in the ratio of labeled L sequences to labeled H sequences with increasing pulse length, which point to a real difference between the two transcripts in the ratio of labeled to unlabeled sequences. Because of the difference in specific activity, the RNA fractions examined in the experiments of Figs. 2, II and 2, III contain fewer copies of L transcripts, relative to the copies of the H transcripts, than indicated by the ratio of radioactivity hybridized with the two strands.

Electron microscopy

The RNA sedimenting to the cushion in a sucrose gradient (see Fig. 1), which was originally about 20% RNase-resistant (Table 2), was pooled, and subjected to extensive DNase digestion, (1) phenol–SDS extraction, and Sephadex G-100 chromatography. After incubation at 60°C for 15 min and RNase digestion, the occurrence of RNase-resistant duplex RNA was investigated in the electron microscope by the basic protein film technique (8–10) and staining with uranyl acetate (11), using circular SV40 DNA, isolated from CsCl-purified particles, as an internal sizing control (~1.7 μm) (12). This examination revealed the exclusive presence of duplex structures in the preparation. Fig. 4 shows typical fields with several duplex nucleic acid molecules. A high proportion of these molecules have a length corresponding to the size of the whole mit-DNA molecules (5 μm) or larger. These duplex molecules presumably represent RNA·RNA hybrids, with the longer ones possibly arising from concatenation of shorter RNA strands.

DISCUSSION

It should first be noticed that, in contrast to the previous work on transcription of mit-DNA (1), the experiments described here have been done with mit-RNA extracted from cells exposed to very short pulses of [5-^{3}H]uridine. Therefore, the sequences measured in these experiments are those present in newly synthesized mit-RNA. Second, the RNA·DNA hybridization experiments performed in this work with separated mit-DNA strands were of the RNA exhaustion type, and measured the amount of RNA complementary to each of the two strands, rather than the fraction of each mit-DNA strand that is homologous to mit-RNA.

The most plausible interpretation of the observations reported here is that mit-DNA is, at least in part, transcribed symmetrically in HeLa cells. This interpretation is supported by the capacity of the pulse-labeled mit-RNA to hybridize with both L and H mit-DNA strands, and to form double-stranded structures, recognized for their RNase resistance and for their appearance in the electron microscope, when self-annealed or annealed with an excess of unlabeled mit-RNA. The previously obtained evidence that the long-term-labeled mit-RNA hybridizes exclusively with the H mit-DNA strand, and the observations reported above of a higher hybridization capacity of the faster-sedimenting pulse-labeled RNA with the L strand, would exclude that the sequences that hybridize with the L and the H mit-DNA strands in the present experiments are transcribed from self-complementary portions of the H strand. That the symmetric transcription detected here concerns a considerable portion of the mit-DNA molecule is strongly suggested by the observation that the initial rate of labeling of the transcripts of the two mit-DNA strands is about equal (after a correction for the difference in base composition is made), and by the previous evidence of complete transcription of the H strand (1). The position of the L transcripts in the sucrose gradients tends to argue against a small section of the L strand being very actively transcribed as an explanation for the above results. Also, the RNase resistance data and the electron-microscope observations speak in favor of an extensive symmetric transcription of mit-DNA. Work is in progress to establish the actual proportion of the L strand that is transcribed, as well as to determine whether the two strands are transcribed concurrently in the same mit-DNA molecule.

The conclusions by Aaij *et al.* (13) concerning the loss of asymmetry of transcription of mit-DNA in isolated rat liver

mitochondria, and those by Tabak and Borst (14) concerning the erroneous transcription of rat liver mit-DNA by *Escherichia coli* RNA polymerase may have to be revised in the light of the present observations.

The transcripts of the L strand appear to have a shorter half-life in mitochondria than those of the H strand, as judged from the change with time in the relative amount of radioactivity associated with the two transcription products. Furthermore, in contrast to the RNA transcripts of the H strand, there is no substantial accumulation with time of products of transcription of the L strand sedimenting slower than 18 S. In agreement with this conclusion, the base composition of the ^{32}P uniformly labeled 16S and 12S RNA has unequal A + U and C + G (6), and is complementary, as concerns A + U, to that of the H mit-DNA strand (1). The failure to detect in the previous hybridization saturation experiments with uniformly labeled RNA (up to 70S) a substantial hybridization with the L strand (1) can be explained by the low concentration of sequences complementary to this strand in the regions of the sucrose gradient analyzed, and by their rapid annealing in solution with the excess of complementary RNA strands before they reacted to an appreciable extent with the DNA on the filter. An exception to the statement made above that there is no accumulation of transcripts of L strand in mitochondria is represented by some 4S RNA species (1, 15), and there may be other RNA species coded by this strand that accumulate in low concentration. Whether the rapid disappearance from the mitochondrial fraction of the transcripts of the L strand is due to degradation or to export remains to be established.

As concerns the nature of the fast-sedimenting rapidly-labeled heterogeneous RNA (up to 70S and more) (6, 7), its partial RNase resistance, which is almost completely suppressed by heating and fast cooling, points to the existence of aggregates presumably involving RNA transcripts of the two mit-DNA strands. It is likely, however, that free molecules of different size contribute to this heterogeneous RNA. Further work utilizing sedimentation analysis under strongly denaturing conditions and electron-microscope analysis should be able to answer the question of the size of the largest RNA transcripts of the H and L mit-DNA strands. The transcripts of the L strand tend to be more abundant, relative to those of the H strand, among the faster-sedimenting than among the slower-sedimenting components of mit-RNA, so that a partial fractionation of the copies of the two strands is achieved by sucrose gradient centrifugation. This fractionation (see a hint of it also in base composition data, ref. 6) could be due to the rapid processing of the fast-sedimenting transcripts of the H strand to smaller molecules. It is also conceivable that the original transcripts of the L strand are larger than those of the H strand.

A symmetric transcription has been previously reported for portions of the genome of bacteriophages *lambda* (16) and T4 (17); furthermore, the presence of virus-specific double-stranded RNA has been described in vaccinia-infected chick cells (18). The observation reported in the present work constitutes, to our knowledge, the first known instance of symmetric *in vivo* transcription of nonviral DNA. Whether this represents a peculiar property of mit-DNA or whether, on the contrary, the same mode of transcription operates in other DNAs, remains to be established. Likewise, more work is needed to elucidate the detailed mechanism of transcription of the two mit-DNA strands and how, if at all, this process is related to mit-DNA replication. Another important question is whether the transcripts of the L strand contain other informational sequences, besides some 4S species (1, 15), to be utilized inside or outside mitochondria.

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