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Identification of Discrete Polyadenylate-containing RNA Components Transcribed from HeLa Cell Mitochondrial DNA*

[actinomycin D/denaturation/poly(dT)–cellulose/polyacrylamide gel/separated strands/RNA·DNA hybridization]

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ABSTRACT **Polyacrylamide gel electrophoresis and sedimentation analysis under denaturing conditions of poly(A)-containing RNA from the polysome region of the sedimentation pattern of a HeLa-cell mitochondrial lysate has revealed the occurrence of a discrete RNA component, which sediments in the native state with a sedimentation constant of about 7 S. From the sedimentation behavior under native and denaturing conditions and the poly(A) content, a molecular weight of about 9×10^4 has been estimated for this component. RNA·DNA hybridization experiments have indicated that this component is coded for by the light strand of mitochondrial DNA. Evidence for the occurrence of a poly(A)-containing RNA component sedimenting at about 9 S and coded for by the heavy strand has also been obtained.**

Poly(A) sequences of a size corresponding to 60–80 nucleotides occur in mitochondrial RNA from HeLa cells (1, 2); a poly(A)-synthesizing enzyme has been identified in rat-liver mitochondria (3, 4). In HeLa cells, the majority of mitochondrial poly(A) is covalently linked to mitochondrial DNA (mit-DNA)-coded RNA (2). Inasmuch as poly(A) in eukaryotic cells appears to be associated only with cytoplasmic messenger RNA (mRNA) and its presumptive nuclear precursors (5–10), and with viral or viral-directed RNA with messenger function (5, 11–15), the above evidence strongly suggests that at least a portion of the mRNA translated by mitochondrial polysomes is coded for by mit-DNA.

Previously (2), no clear-cut evidence of discrete components was observed in the sedimentation patterns of poly(A)-containing RNA from HeLa-cell mitochondria. The main source of difficulty was probably the tendency of poly(A)-containing molecules to aggregate and sediment with heavier molecules. Thus, RNA from the polysome region of the sedimentation pattern of a mitochondrial lysate, selected for poly(A) by two passages through poly(dT)–cellulose and analyzed in sucrose gradient in the native state, showed a fairly uniform distribution of material in the region from 6 S to about 16 S, with only a hint of a 7S component emerging over a background of heterogeneous RNA. After formaldehyde denaturation and sedimentation in the presence of formaldehyde, most of the poly-(A)-containing RNA sedimented more slowly than 12 S RNA, and a pronounced sharp peak sedimenting about 20% faster than denatured 5S RNA and 40% slower than denatured 12S RNA was observed.

In the present work, the nature of the material of the above-mentioned peak was further investigated. Evidence was obtained that it represents a reproducible, discrete poly(A)-containing RNA component with a sedimentation constant of about 7 S in the native state, coded for by the light (L) strand of mit-DNA. Evidence is also presented for the occurrence of another poly(A)-containing RNA component sedimenting at about 9 S and coded for by the heavy (H) mit-DNA strand.

MATERIALS AND METHODS

Growth and Labeling of Cells. Conditions for growth of HeLa cells have been described (16). Mitochondrial RNA was selectively labeled by exposing cells for 2 hr to [8-^{3}H]adenosine (18.3 mCi/μmol; 3.75 μCi/ml), or [8-^{14}C]adenosine (52 μCi/μmol; 0.18 μCi/ml), or [5-^{3}H]uridine (29 mCi/μmol; 2.5 μCi/ml) in the presence of 0.1 μg/ml of actinomycin D [to inhibit cytoplasmic ribosomal RNA synthesis (17–19)]. Long-term labeling of mit-DNA with [2-^{14}C]thymidine was as described (20).

Subcellular Fractionation, Extraction, and Analysis of RNA. The mitochondrial fraction was separated by differential centrifugation and sucrose gradient fractionation, lysed with Triton X-100, and centrifuged at $20,000 \times g_{av}$ for 15 min. The supernatant was centrifuged on a sucrose gradient as described (2, 21). RNA was extracted by the sodium dodecyl sulfate–Pronase–phenol method (22) from the material of the polysome region (74–180 S) of the sucrose gradient pattern. Extracted RNA was chromatographed on poly(dT)–cellulose columns for selection of poly(A)-containing molecules, as reported (2). Conditions of sedimentation analysis of RNA samples are described in the figure legends. Electrophoresis was done through 2.7% polyacrylamide gels at 5 mA per 16-cm gel for 3.5 hr (23) in 40 mM Tris buffer, pH 7.4 (25°)–20 mM sodium acetate–2 mM EDTA–0.5% SDS. Electrophoresis of formaldehyde-treated RNA samples [heated to 63° for 15 min in a solution of 3% neutralized formaldehyde in 20 mM Na phosphate buffer, pH 7.8 (24)] through 2.7% polyacrylamide gels in the presence of formaldehyde was performed essentially as described (24) in 20 mM phosphate buffer, pH 7.8–0.5% Na dodecyl sulfate–3% formaldehyde, by running the gels at 5 mA per 16-cm gel for 3 hr. Some of the sucrose gradient and polyacrylamide gel electrophoresis runs involved a small amount of labeled material; however, all sucrose gradient and gel fractions were counted to at least 500 counts.

RNA·DNA Hybridization. Isolation of [2-^{14}C]thymidine-labeled mit-DNA, separation of H and L mit-DNA strands in an alkaline CsCl density gradient, and RNA·DNA hybridization were done as described (2, 22).

Abbreviations: mit-DNA, mitochondrial DNA; H and L strand, heavy and light strand, respectively, of mit-DNA.

* This is article XX in the series "Expression of the Mitochondrial Genome in HeLa Cells." Paper XIX is ref. 2.

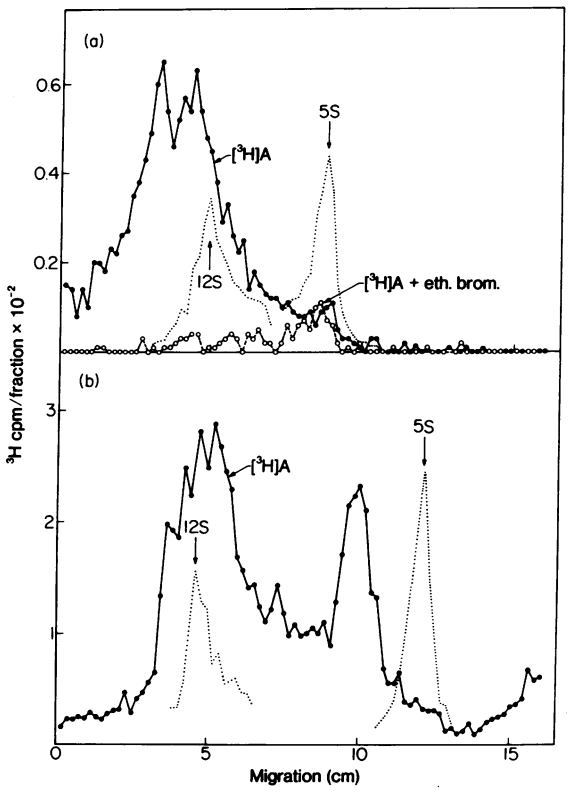

FIG. 1. Polyacrylamide gel electrophoresis in the native state (*a*), or after formaldehyde treatment and in the presence of formaldehyde (*b*), of RNA extracted from the polysome region of the sucrose sedimentation pattern of a mitochondrial lysate from HeLa cells labeled with [*8*-^{3}H]adenosine for 2 hr in the presence of 0.1 μg/ml of actinomycin D and in the absence or presence of 1 μg/ml of ethidium bromide (*eth. brom.*), and passed through poly(dT)–cellulose. The mitochondrial 12S RNA and cytoplasmic 5S RNA were treated as the experimental samples and run in parallel gels.

RESULTS

A Triton X-100 mitochondrial lysate from HeLa cells labeled for 2 hr with [*8*-^{3}H]adenosine in the presence of 0.1 μg/ml of actinomycin D was run through a sucrose gradient, and RNA was extracted from the polysome region (74–180 S) of the gradient (21) and passed through a poly(dT)–cellulose column. It had been shown (1, 2) that poly(dT)–cellulose or poly(U) filters bind mitochondrial poly(A)-containing RNA, while mitochondrial ribosomal and 4S RNA are not retained†. Fig. 1*a* shows the polyacrylamide gel electrophoresis profile of a sample of RNA, in the native state, from the polysome region, which had been retained and eluted from poly(dT)–cellulose. The pattern shows a broad band, partially resolved into two peaks, moving slower than 12S RNA, with smaller amounts of faster moving material extending up to the 5S region, without clearly defined discrete components. The corresponding RNA sample from cells treated with ethidium bromide [a selective inhibitor of mitochondrial RNA synthesis (26)] shows a very low level of radioactivity spread fairly uniformly throughout the proximal half of the gel, indicating that all or about all RNA synthesis in the absence of the drug is from a mit-DNA template. Thermal denaturation of the control RNA in low-ionic-strength buffer, immediately before electrophoresis in a standard gel, resulted in a pattern similar to that of Fig. 1*a*, except that a pronounced shoulder now appeared in the region 6–9 S_E‡. A more drastic change was observed after denaturation in formaldehyde [under conditions that should not introduce any breaks in the RNA (28)] and electrophoresis in the presence of formaldehyde (Fig. 1*b*). The pattern thus obtained exhibits a broad band of radioactivity in the region of 10–13 S_E, and a pronounced peak at about 6.5 S_E. The considerable sharpness of the peak, as compared to that of the 5S RNA and 12S RNA, suggests that it consists of sequences fairly uniform in length.

Fig. 2*a* shows the sedimentation profile in sucrose gradient in low-ionic-strength buffer of a sample of RNA labeled for 2 hr with [*5*-^{3}H]uridine extracted from the polysome region of the sedimentation pattern of a mitochondrial lysate, and passed twice through poly(dT)–cellulose. The sample had been heat denatured (75°, 7 min) in low-ionic-strength buffer (1 mM Tris buffer, pH 7.4–1 mM EDTA) before the rerun through poly(dT)–cellulose to minimize the adventitious retention of nonpoly(A)-containing molecules (2) and again before the sedimentation run. From published rates of thermal degradation of RNA (29) and from previously observed effects of elevated temperatures on high-molecular-weight RNA (28, 30, 31), the two heating steps were not expected to cause any significant amount of thermal scissions in the RNA analyzed. One recognizes in Fig. 2*a* two sharp, partially resolved peaks, one sedimenting somewhat slower than 5S RNA and the other sedimenting between the 5S and 12S RNA markers, which emerge over a background of nonresolved RNA components extending to the heavy side of 12S RNA. The fractions corresponding to each of the two peaks were pooled as indicated, and the material in each cut was run through a sucrose gradient in 10 mM acetate buffer, pH 5.0–0.1 M NaCl. The material of the peak sedimenting slower than 5S RNA in low-ionic-strength buffer (peak II) migrated in 0.1 M NaCl as a fairly sharp peak moving faster than 5S RNA, at about 7S (Fig. 2*b*). Superimposed in Fig. 2*b* is the profile of a discrete 7S peak obtained by running, under the same conditions, the material from the 6–9S_E shoulder of the gel profile of heated, [*8*-^{3}H]adenosine-labeled RNA, which had been further purified by sedimentation in low-ionic-strength buffer. The observed difference in relative sedimentation behavior of the mitochondrial 7S RNA and cytoplasmic 5S RNA marker in the native state and under denaturing conditions is presumably due to the fact that the 5S RNA did not lose all secondary structure in the low-ionic strength buffer and remained more compact than the mitochondrial RNA. A similar incomplete elimination of secondary structure resulting in an anomalously fast sedimentation has been described after formaldehyde treatment for *Escherichia coli* ribosomal RNA (32) and for cytoplasmic 28S RNA from HeLa cells or *Xenopus*

† Under our conditions of poly(dT)–cellulose chromatography, 80–90% of the mitochondrial 16S and 12S RNA purified on sucrose gradients and labeled with [*5*-^{3}H]uridine was not retained in a first passage through a poly(dT)–cellulose column. A major portion, if not all, of the 16S and 12S [^{3}H]RNA retained probably represents contaminant mitochondrial nonribosomal RNA which sediments with the mitochondrial ribosomal RNA, as also suggested by the shape of the DNA saturation curves obtained with these two species (25).

‡ S_E is used to indicate the S-value estimated from the relative electrophoretic mobility of the RNA (27) by taking as standards cytoplasmic 5S RNA and mitochondrial 12S RNA (with assumed S_E-values of 5 and 12, respectively).

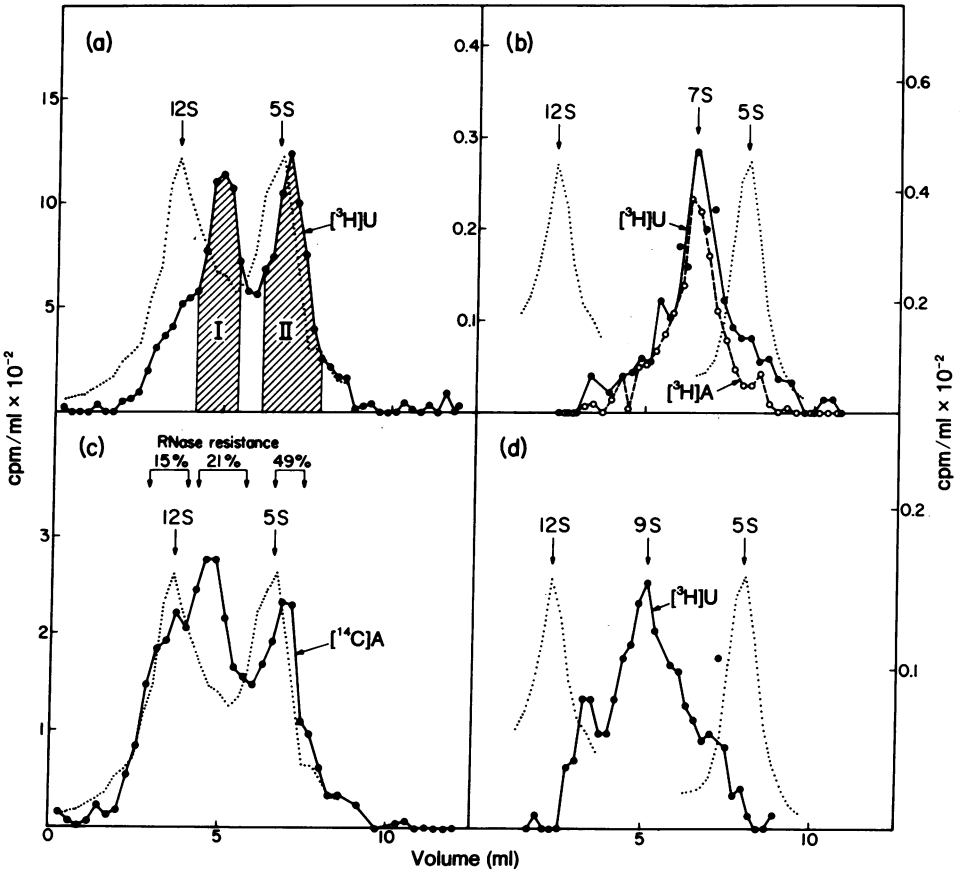

FIG. 2. Sedimentation analysis in low-ionic-strength buffer (*a* and *c*), or in acetate–NaCl (*b* and *d*), of discrete components of poly(A)-containing RNA. RNA labeled with [5-^{3}H]uridine (*a*) or [8-^{14}C]adenosine (*c*) from the mitochondrial polysome region was passed twice through poly(dT)–cellulose, denatured by heating at 70° for 6 min in 1 mM Tris buffer, pH 7.4–0.25 mM EDTA, and fast cooling, and then run through a 5–20% sucrose gradient in the same buffer in the SW 41 rotor at 41,000 rpm for 30 hr. The RNA from the indicated cuts in (*a*) was collected by ethanol precipitation and centrifugation; a portion of each sample was run in a 5–20% sucrose gradient in 10 mM sodium acetate buffer, pH 5.0–0.1 M NaCl in the SW 41 rotor at 41,000 rpm for 18 hr (*b*, cut II, and *d*, cut I); the recovery of ^{3}H label in the reruns was about 81% and 76% in (*b*) and (*d*), respectively. In (*b*), the sedimentation profile of another 7S RNA sample, separated by first running heat-denatured, poly(A)-containing, [8-^{3}H]adenosine-labeled RNA from the polysome region on a gel and then rerunning the material from a 6–9S_E shoulder in the gel pattern in a sucrose gradient in low-ionic-strength buffer, is also shown (O– – –O). The 12S and 5S RNA markers were treated as the experimental samples and run in parallel gradients. The RNA from the indicated cuts in (*c*) was tested for DNase- and RNase-resistant acid-precipitable radioactivity (20 μg/ml of electrophoretically purified DNase, 2 μg/ml of heated pancreatic RNase and 100 units/ml of heated T1 RNase, 30 min at 37° in 10 mM Tris buffer, pH 7.4–1 mM $MgCl_2$–0.25 M NaCl).

laevis (33). The material sedimenting in the region of peak I in Fig. 2*a* showed, in acetate–NaCl buffer, a main peak with an estimated sedimentation coefficient of about 9 S; however, this was only partially resolved from other faster and slower moving components (Fig. 2*d*).

The material of peak II in Fig. 2*a*, when run through a polyacrylamide gel in the presence of formaldehyde, migrated as a sharp peak in coincidence with the 6.5S_E peak appearing in the electrophoretic pattern of formaldehyde-treated, [8-^{14}C]-adenosine-labeled RNA from the mitochondrial polysome region (Fig. 3).

In order to estimate the poly(A) content of the material from the two peaks recognizable after sedimentation under denaturing conditions, RNA labeled with [8-^{14}C]adenosine for 2 hr from the mitochondrial polysome region, which had been passed twice through poly(dT)–cellulose without heat denaturation, was run through a sucrose gradient in low-ionic-strength buffer. The pattern obtained (Fig. 2*c*) is very similar to that of Fig. 2*a*. In this experiment, in order to minimize degradative processes, the mitochondrial lysate had been centrifuged in sucrose gradient to separate the polysomes for only 3 hr (at 27,000 rpm), rather than for 11 hr (at 19,000 rpm), as in the experiment of Fig. 2*a*. The RNA from the indicated fractions in Fig. 2*c* was pooled, heat denatured, and subjected to DNase and pancreatic and T1 RNase digestion, under the conditions normally used for isolation of poly(A) (2). The labeled material corresponding to the region of the slower and the faster sedimenting peak (Fig. 2*c*) was, respectively, about 49% and 21% DNase- and RNase resistant; under the same conditions, 12S RNA was made 94% acid-soluble and [^{3}H]poly(A) was 100% RNase resistant.

In order to investigate the homology to mit-DNA of the two discrete RNA components described above, aliquots of the material from the region of each of the two peaks in Fig. 2*a* were hybridized with increasing amounts of separated, [2-^{14}C]-thymidine-labeled H or L mit-DNA strands. The material from

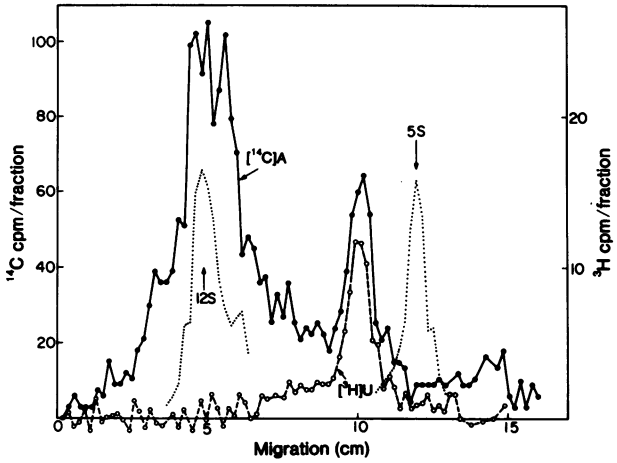

FIG. 3. Polyacrylamide gel electrophoresis after formaldehyde treatment and in the presence of formaldehyde of RNA labeled with [8-^{14}C]adenosine for 2 hr from the mitochondrial polysome region, passed twice through poly(dT)-cellulose. A sample of cut II in the pattern of Fig. 2a was run under the same conditions in a separate gel (O- - -O). Recovery of ^{3}H label was about 100%.

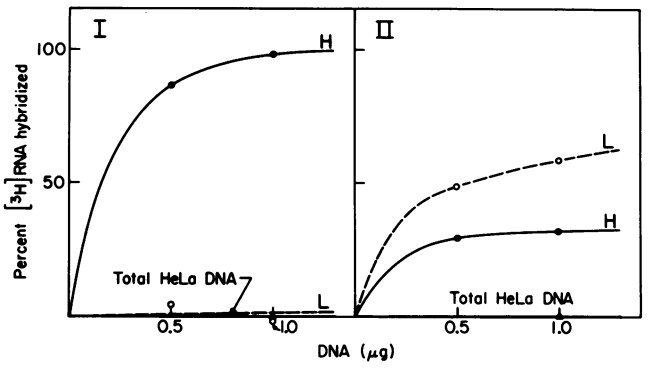

FIG. 4. Homology to separated mit-DNA strands of [5-^{3}H] uridine-labeled RNA from cuts I and II of Fig. 2a. Samples containing 75 (cut *I*) or 95 (cut *II*) cpm were annealed with different amounts of H or L mit-DNA strands or total denatured HeLa DNA.

the region of the faster moving peak hybridized exclusively with the H mit-DNA strand, while about two-thirds of the RNA corresponding to the slower moving peak hybridized with the L strand (Fig. 4). These results strongly suggest that the 9S component is coded for by the H strand, while the 7S component is coded for by the L strand; the minor hybridization with the H strand of the material from the slower moving peak is presumably due to contaminating RNA sedimenting in the region of the 7S component in the sucrose gradient in low-ionic strength buffer.

DISCUSSION

Our results indicate the existence, in RNA isolated from the polysome region of the sedimentation pattern of a mitochondrial lysate from HeLa cells, of two discrete poly(A)-containing components coded for by mit-DNA. The peaks of radioactivity corresponding to the two components, separated by polyacrylamide gel electrophoresis in the presence of formaldehyde or recognizable by sucrose gradient centrifugation, were relatively sharp, suggesting that these components consist of chains fairly uniform in length. It is not known whether they represent individual RNA species. The sharpness of these peaks and the reproducibility of their occurrence and relative amounts speak against the possibility of these components arising from random degradative processes. Besides these two components of poly(A)-containing RNA, the sucrose gradient and gel patterns presented above suggest the probable occurrence of discrete components of higher molecular weight, which are being investigated.§

A critical factor for identification of the two discrete components of poly(A)-containing RNA described here was the use of denaturing conditions for the sedimentation and electrophoretic analyses. RNA extracted from the total mitochondrial fraction of HeLa cells and selected for poly(A) content by binding to poly(U) immobilized on glass-fiber filters, was reported to migrate in polyacrylamide gel electrophoresis very heterogeneously, with mobilities corresponding to sedimentation constants of 10–30 S (1). In agreement with this observation, we found that the RNA extracted from the mitochondrial polysome region, selected for poly(A) content by one passage through a poly(dT)-cellulose column and analyzed in sucrose gradient under native conditions, exhibits mainly heterogeneous components sedimenting in the region between 10 and 20 S, with a substantial amount of faster sedimenting material up to about 35 S (2). A considerable change in the apparent size distribution of this RNA fraction was, however, observed after a heat-denaturation step and a second passage through poly(dT)-cellulose: the retained RNA sedimented now in sucrose gradient heterogeneously in the region from 6 to about 16 S (2). As we report here an even more drastic change in the apparent size distribution of this poly(A)-containing RNA was observed after heat or formaldehyde denaturation and analysis in sucrose gradient or in polyacrylamide gels under denaturing conditions: most of the RNA appeared to be smaller than 12S RNA and the presence of discrete components could be recognized. As described elsewhere (2), a large amount of poly(A)-containing RNA could also be isolated from the heaviest structures (>200 S) of a mitochondrial lysate from HeLa cells. This poly(A)-containing RNA showed, in the native state, a similar heterogeneous distribution to that observed for the poly(A)-containing RNA extracted from the polysome region, and, likewise, exhibited a marked change in apparent size distribution when analyzed under denaturing conditions (2). Since the heat and formaldehyde treatments used for denaturation were not such as to introduce an appreciable amount of breaks of phosphodiester bonds (28–31), it seems reasonable to interpret the apparently large size of the poly (A)-containing mitochondrial RNA analyzed in the native state, found by us and by others (1), as due to the tendency to aggregation of the poly(A)-containing mitochondrial RNA molecules. This behavior may reflect the existence of complementary sequences in these RNA molecules resulting from the symmetric transcription of mit-DNA (22). However, the poly(A) stretch itself may also be involved in this aggregation, possibly due to its lack of secondary structure at neutral pH (34), which makes it available for base-pairing with complementary sequences. This is suggested by the previous finding that free poly(A) also had a tendency to

§ **Note added in proof.** In recent experiments, analysis by polyacrylamide gel electrophoresis under denaturing conditions has allowed the resolution of additional discrete poly(A)-containing RNA components in the region of 11–13 S_E.

aggregate and cosediment with heavier molecules (2). Observations suggesting a tendency to aggregation in sucrose gradient of globin mRNA purified on poly(dT)–cellulose have been reported (35).

The molecular weights of the 7S and 9S components, as estimated from their sedimentation coefficients in sucrose gradient in 0.1 M salt relative to the 5S and 12S RNA markers, by a formula (36) that gives for 16S and 12S mitochondrial RNA molecular weights only slightly lower than those estimated by electron microscopy (37), corresponds to about 8.5×10^4 and 1.5×10^5, respectively. From the sedimentation velocity of the slower component in sucrose gradient in the presence of formaldehyde the estimated molecular weight (28) is about 9.3×10^4. From the electrophoretic mobility in polyacrylamide gel in the presence of formaldehyde (24), with respect to the 12S and 5S markers, of the slower sedimenting component treated with formaldehyde the estimated molecular weight is about 7.5×10^4. However, the unreliability of electrophoresis in polyacrylamide gels for determination of molecular weight of mitochondrial RNA (32, 33) makes the above estimate less valid than the sedimentation estimates. On the basis of the poly(A) content of the slower discrete component, estimated from the RNase resistance data, and assuming a length of 70 nucleotides for the poly(A) stretch and an A content of 32% (23) for the mitochondrial RNA molecules to which it is attached, one can arrive at an estimate of about 9.5×10^4 for the size of this component, in good agreement with the sedimentation estimates.

The two discrete poly(A)-containing components described here probably represent mit-DNA-coded mRNA. The relatively small size of these molecules would agree with this interpretation, in view of the small size of mitochondrial polysomes in HeLa cells, estimated to consist of two to seven monomers (21). The two discrete poly(A)-containing components identified here represent a substantial portion of the RNA from the region of functioning polysomes, selected for poly(A) content by two passages through poly(dT)–cellulose columns. Previous work from this laboratory (38) has shown that the *in vivo* and *in vitro* products of mitochondrial protein synthesis consist of a group of not well-resolved components with molecular weights of 12,000–25,000, and another group, more abundant, with molecular weights of 40,000–55,000. The larger components may be polymeric forms of smaller units, as in yeast. In this organism, the major product of mitochondrial protein synthesis appears to be a protein of molecular weight of about 8000 (39), about the size of the protein that would be expected to be coded for by the 7S mitochondrial RNA described here.

The observation made here that the 7S RNA hybridizes with the L mit-DNA strand adds one, and possibly more, RNA species to the three 4S RNA species already known to be coded for by this strand (40, 41), and amplifies the informational significance of the extensive transcription of this strand (22).

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