

MITOCHONDRIAL ORIGIN OF MEMBRANE-ASSOCIATED HETEROGENEOUS RNA IN HELA CELLS*

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The occurrence in the cytoplasm of HeLa cells of a membrane-associated heterogeneous RNA distinct in sedimentation properties, base composition, and metabolic behavior from mRNA of free polysomes has been previously reported.¹ The kinetics of appearance of this RNA fraction and its sequence homology with cytoplasmic DNA clearly pointed to a cytoplasmic site of synthesis.

This paper reports the results of experiments aimed at identifying the DNA template involved and the nature of the membrane structures with which this RNA is associated. It was found that the membrane-associated heterogeneous RNA is synthesized at a high rate on mit-DNA; furthermore, the evidence obtained strongly suggests that this RNA is exported in part to the rough ER.

Materials and Methods.—(a) *Cells:* HeLa cells growing in suspension² were used.

(b) *Buffers:* The buffer designations are: T: 0.01 M tris buffer (pH 7.1); TKM: 0.01 M tris buffer (pH 7.1), 0.01 M KCl, 0.00015 M MgCl₂.

(c) *Labeling conditions:* Pulse labeling of RNA was carried out, as detailed below, with H³-5-uridine (17.3–28.8 mc/μM) or C¹⁴-2-uridine (30–50 μc/μM) in modified Eagle's medium (with 5% dialyzed calf serum) or with carrier-free P³²-orthophosphate (37 μc/ml) in medium containing 2 × 10⁻⁷ M phosphate. DNA was labeled by growing cells for 48 hr in the presence of H³-thymidine (1.25 μc/ml; 17.7 mc/μM).

(d) *Preparation of subcellular fractions:* The outline of the procedure utilized has been described previously;¹ TKM was the homogenization medium. Buoyant density fractionation of the 8100 × g membrane components in 1.0–1.7 M sucrose gradient in T buffer (Spinco SW 25.1 rotor, 25 krpm, 18–20 hr) separated a main band containing mitochondria and rough ER from a lighter band of smooth membrane structures.³

(e) *Extraction and analysis of RNA* were carried out as described elsewhere.⁴

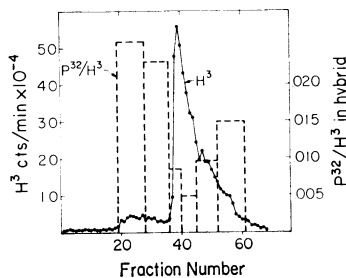
(f) *Isolation of closed circular mit-DNA* was carried out by centrifuging the SDS lysate of the 8100 × g membrane fraction in a CsCl-ethidium bromide density gradient.⁵ In some experiments, the band of closed circular mit-DNA was rebanded.

(g) *RNA-DNA hybridization* experiments were performed as described previously.⁶

(h) *Cytochrome oxidase assay* was carried out by a modification of the procedure of Smith:⁷ 0.1-ml aliquots of the sucrose gradient fractions were mixed in a cuvette with 2.9 ml 18 μM solution of reduced cytochrome C in 0.04 M PO₄ buffer, pH 6.2. The decrease of OD at 550 mμ at 25°C was measured at 10-sec intervals for 4–6 min.

Results.—(a) *Base sequence homology of membrane-associated RNA with mit-DNA:* Figure 1 shows the buoyant density profile in a CsCl-ethidium bromide

FIG. 1.—Comparative homology with membrane-associated heterogeneous RNA of different HeLa DNA components. The DNA from the 8100 × g membrane fraction of cells labeled with H³-thymidine was analyzed in a CsCl-ethidium bromide density gradient.⁵ Aliquots of pooled fractions were tested for hybridization capacity with membrane-associated RNA from 90-min P³²-labeled cells (1 μg DNA, 5 μg RNA). The results are expressed as P³²/H³ in the hybrids. The data are corrected for nonspecific background estimated with *E. coli* DNA.



gradient of DNA from the $8100 \times g$ membrane fraction from HeLa cells labeled with H^3 -thymidine. The heavy band (fractions 19–28) contains closed circular mit-DNA.^{5, 8, 9} The larger light band (fractions 36–60) consists of linear DNA molecules from nuclear contamination, but it would also contain any nicked or linear mit-DNA. The region between the light and dense bands corresponds to the position of the recently described middle band^{8, 9} (not resolved here): this contains catenated dimers and higher oligomers of mit-DNA. The capacity of fractions from different regions of the gradient to hybridize with P^{32} -labeled membrane-associated RNA is also shown in Figure 1. It is apparent that the DNA from both the heavy band and the intermediate region has a three to four times higher specific hybridizability with this RNA than the DNA from the light band. This result indicates that mit-DNA is involved in hybridization with this RNA fraction. The fractions on the light side of the main band have a somewhat higher hybridization capacity than the fractions of the center of the band, presumably due to the trapping of mit-DNA which occurs when the gradient is heavily loaded with DNA (here $60 \mu g$).

(b) *Rate of synthesis of mit-RNA:* Figure 2 shows the kinetics of labeling of the membrane-associated heterogeneous RNA and of the free polysome mRNA during prolonged exposure of HeLa cells to H^3 -uridine; correction was made for incomplete equilibration of the whole cell UTP pool with the exogenous H^3 -uridine at early times (up to 40 min).^{1, 10} The labeling of the RNA of the membrane fraction proceeds linearly from zero time, in agreement with an *in situ* synthesis of this RNA. The slight increase in slope at about 30 minutes, if significant, may indicate the arrival at the membrane fraction of an mRNA component of nuclear origin starting at that time. The decrease in the rate of labeling

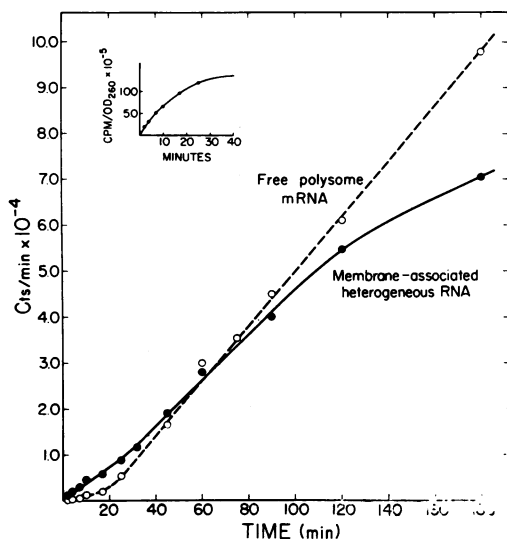


FIG. 2.—Flow of radioactivity into membrane-associated heterogeneous RNA and free polysome mRNA. HeLa cells ($3 \times 10^5/ml$) were exposed to H^3 -5-uridine ($3.3 \mu c/ml$) and 300-ml samples removed at different times; from each sample the $8100 \times g$ membrane fraction was isolated and banded in sucrose gradient; free polysomes were isolated by centrifugation of the $15,800 \times g$ supernatant of the cytoplasmic fraction in a $0.75 M$ – $2.0 M$ sucrose gradient in TKM for 5 hr.^{1, 3} the total H^3 cpm associated with these fractions was determined and corrected for small variations in the yield of total OD_{260} . RNA was released by SDS and analyzed in sucrose gradient in SDS buffer.¹⁵ The proportion of ribosomal and nonribosomal components was estimated as in ref. 15. Determination of the labeling of the total cell uridine nucleotide pools (*insert*) and correction of the RNA labeling data for incomplete UTP pool equilibration with external H^3 -uridine were performed as described previously.¹

after about 100 minutes (see also Fig. 6) suggests a saturation of radioactivity of some components of this RNA fraction. The labeling of the free polysome mRNA, after a 20–30-minute acceleration period, increases at a constant rate, which presumably represents the rate of its arrival from the nucleus. It appears from Figure 2 that the initial rate of labeling of the membrane-associated RNA is equivalent to about two thirds of the rate of arrival at the cytoplasm of free polysome mRNA (see *Discussion*).

(c) *Export of mit-RNA: (I) Kinetics of appearance of labeled RNA in mitochondria and extramitochondrial membrane structures:* An appreciable fraction (10–15%) of polysomes in HeLa cells are associated with tubules and vesicles of rough ER.^{3, 11, 12} the major part of these polysomes is recovered in the $8100 \times g$ membrane fraction and can be released from the membrane components by NaDOC treatment.^{1, 3} In both buoyant density fractionation and sedimentation velocity runs (Fig. 3), the distribution of membrane structures containing

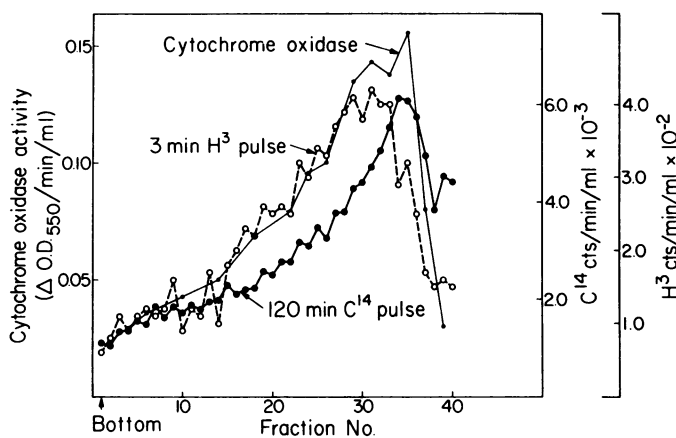


FIG. 3.—Sedimentation pattern of components of the $8100 \times g$ membrane fraction isolated from a mixture of HeLa cells labeled for 3 min with H^3 -uridine and for 120 min with C^{14} -uridine. 15–30% sucrose gradient in T buffer, SW 25.3 rotor, 7 krpm, 35 min.

RNA labeled during one hour or longer exposure to C^{14} -uridine was found to be significantly different from that of the cytochrome oxidase activity; on the contrary, the latter distribution coincided reasonably well with that of the structures containing RNA labeled for 3 minutes with H^3 -uridine. These results suggested that with increasing pulse length labeled RNA accumulates in extramitochondrial structures, presumably elements of rough ER. In order to obtain further evidence on this point, the sensitivity to pancreatic RNase of the RNA in the isolated membrane components was investigated: intramitochondrial RNA *in situ* is protected from the action of nucleases.^{13, 14} As an internal standard to monitor the extent of any possible mitochondrial damage, cells labeled for 3 minutes with H^3 -uridine (and mixed after harvesting with cells labeled for various times with C^{14} -uridine) were used: in the membrane fraction from 3-minute labeled cells, the majority of the RNA synthesized on mit-DNA, if not

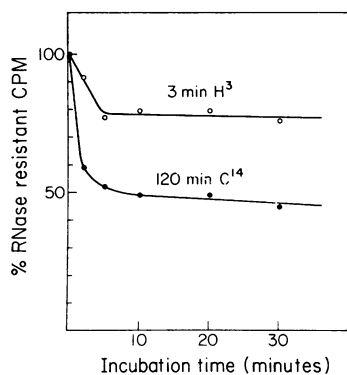


FIG. 4.—Kinetics of RNase digestion *in situ* of membrane-associated heterogeneous RNA. A portion of the $8100 \times g$ membrane fraction utilized in the experiment of Fig. 3 was banded in a sucrose gradient: aliquots from the peak fraction of the main band were treated with $50 \mu\text{g/ml}$ pancreatic RNase at 2°C in $0.1 M$ NaCl, $0.01 M$ Na citrate.

all, would presumably be intramitochondrial. Conditions of RNase digestion were found which clearly distinguished in the membrane-associated RNA a fraction which was quickly digested, from the remainder which was inaccessible to the enzyme or only slowly attacked by it (Fig. 4).

Figure 5 shows the buoyant density distribution in sucrose gradient of the structures containing RNase-sensitive or RNase-resistant 3-minute H^3 -labeled RNA and 30- or 120-minute C^{14} -labeled RNA. While the proportion of H^3 label sensitive to RNase is very similar in the two experiments, the proportion of C^{14} sensitive to RNase increases with pulse length. The significance of the 3-minute H^3 -RNA fraction sensitive to RNase is not clear: it may result from the damage of a portion of the mitochondria, or it may reflect the rapidity with which one fraction of mit-RNA is exported (see below), or both. The distribution of

RNase-sensitive C^{14} -containing structures is significantly displaced toward higher densities with respect to that of the RNase-resistant C^{14} -labeled structures (Fig. 5). An increase in RNase sensitivity of the C^{14} -labeled membrane-associated RNA was also observed after a cold chase or actinomycin treatment. It should be pointed out that for C^{14} -uridine pulses longer than 30 minutes, an increasing fraction of label is in rRNA. As described elsewhere,³ the great majority (>97%) of the ribosomes of the $8100 \times g$ membrane fraction are associated with the rough ER and ~60 per cent of the rRNA they contain is made acid-soluble under the conditions of RNase treatment used here. In the present experiments, the radioactivity associated with RNase-sensitive or RNase-resistant rRNA (estimated by assuming 60% sensitivity of the fraction of label in rRNA¹⁵) was subtracted from the total RNase-sensitive or RNase-resistant radioactivity: the difference would represent mainly label in heterogeneous RNA. The data thus obtained in different experiments (Fig. 6) indicate that with increasing pulse length or after a pulse chase an increasing proportion of labeled heterogeneous RNA is associated with extramitochondrial structures. The interpretation that the RNase-resistant fraction of the membrane-associated heterogeneous RNA corresponds to intramitochondrial RNA, and the RNase-sensitive fraction to mRNA of polysomes of the rough ER, is supported by a different type of observation. After labeling times longer than half an hour, a considerably greater proportion of label is found in heterogeneous RNA components in the membrane fraction than in free polysomes (Table 1): this "excess," estimated under the assumption that the polysomes of the rough ER receive newly synthesized rRNA and mRNA in the same relative proportion as free polysomes, is very close to the RNase-resistant fraction found in the experiments

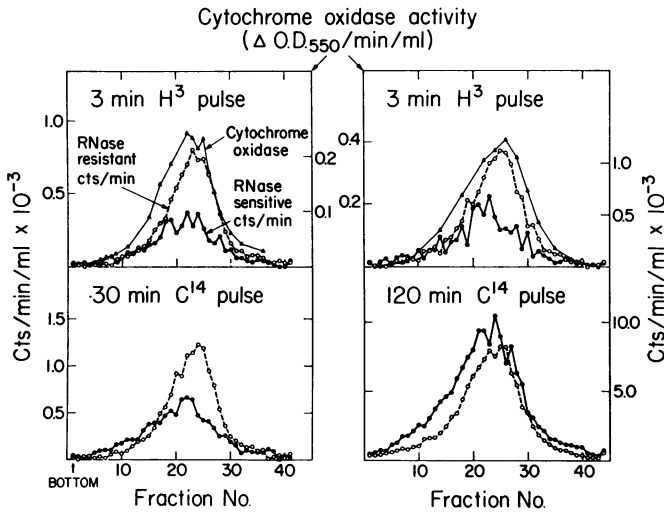


Fig. 5.—Buoyant density distribution in sucrose gradient of membrane structures containing RNase-sensitive or RNase-resistant RNA from a mixture of cells labeled for 3 min with H^3 -uridine and for 30 or 120 min with C^{14} -uridine. The $8100 \times g$ membrane fraction was banded in sucrose gradient: an aliquot of each gradient fraction was treated with $50 \mu g/ml$ pancreatic RNase for 10 min at $2^\circ C$ as in Fig. 4. Correction for a low level of DNA labeling by C^{14} -uridine was made by subtracting the TCA-precipitable radioactivity resistant to hydrolysis by $0.5 M NaOH$ for 20 hr at $30^\circ C$.

of Figure 6, and very likely corresponds to the intramitochondrial RNA (Table 1). It appears from Figure 6 that the radioactivity of the intramitochondrial RNA becomes saturated after about 75 minutes: if this saturation reflects the turnover of a labile fraction (see *Discussion*), it may account for the decrease in the over-all rate of labeling of the membrane-associated heterogeneous RNA (Fig. 2).

(II) *Comparative homology with mit-DNA of membrane-associated RNA labeled during short and long pulses:* In order to test whether the accumulation of newly synthesized RNA in extramitochondrial structures results from export of mit-RNA or from arrival of RNA of nuclear origin, the capacity of membrane-associated RNA labeled during a very short H^3 -uridine pulse to hybridize with mit-DNA was compared with that of membrane-associated RNA labeled during a two-hour C^{14} -uridine pulse (when a considerable fraction of it is extramitochondrial (Fig. 6)). In these experiments, because of the nuclear origin of the rRNA in the membrane-bound ribosomes,^{1, 3, 16} labeled rRNA would not contribute to hybrid formation. If all the extramitochondrial nonribosomal RNA labeled in a long pulse were of nuclear origin, on the basis of the data of Figure 6 and Table 1 one would expect the ratio of H^3 to C^{14} in the hybrid to be about twice that in the input heterogeneous RNA. On the contrary, as is shown in Table 2, the heterogeneous membrane-associated RNA labeled in a long pulse has a degree of homology with mit-DNA which is similar to that of the RNA labeled in a short pulse. The slightly higher (from 0 to 25% in different experiments) hybridiza-

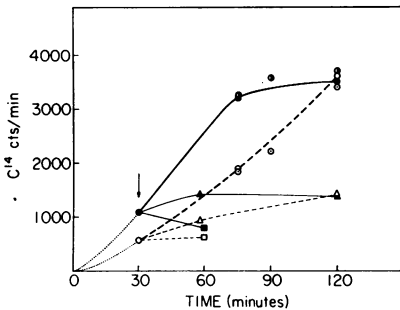


FIG. 6.—Distribution of membrane-associated heterogeneous RNA between RNase-sensitive and -resistant structures in continuous incorporation and pulse-chase experiments. The $8100 \times g$ membrane fraction was isolated from mixtures of 1.2×10^8 cells labeled for 3 min with H^3 -uridine ($10 \mu C/ml$) and 1.2×10^8 cells labeled for different times with C^{14} -uridine ($0.06 \mu C/ml$); the total RNase-sensitive and RNase-resistant fractions of membrane-associated RNA were determined as in Fig. 5, and corrected for the contribution of rRNA as indicated in the text. The C^{14} data are normalized for small variations in the yield of membrane components on the basis

of total H^3 -uridine incorporation (= C^{14} cpm per 1000 H^3 cpm), and for possible contribution to RNase sensitivity by mitochondrial damage on the basis of a constant 25% RNase sensitivity of H^3 -RNA (see text).

●—● RNase-resistant, ○—○ RNase-sensitive fraction, after labeling with C^{14} -uridine for different times; ▲—▲ RNase-resistant, △—△ RNase-sensitive fraction, after 30 min C^{14} -uridine pulse, then 0.01 *M* cold uridine chase; ■—■ RNase-resistant, □—□ RNase-sensitive fraction, after 30 min C^{14} -uridine pulse, then actinomycin ($10 \mu g/ml$) treatment; ⊙ polysome associated, and ⊙ nonpolysome-associated heterogeneous RNA of the $8100 \times g$ membrane fraction, after different exposure times to C^{14} -uridine, was estimated as explained in the legend of Table 1.

tion capacity of the seven-minute H^3 -RNA compared to the two-hour C^{14} -RNA may be due to the fact that the sequences of mit-RNA are not represented in the same proportion in preparations labeled during a short and a long pulse, or to the late arrival at the membrane fraction of an mRNA component of nuclear origin: the latter possibility is suggested by the hybridization results obtained with total HeLa cell DNA (Table 2), and possibly by the change in slope at about 30 minutes in the labeling of the membrane-associated RNA (Fig. 2).

TABLE 1. *Polysome-associated and nonpolysome-associated heterogeneous RNA of the membrane fraction.*

Incubation time (min)	Fraction	Ribosomal RNA (% of total)	Heterogeneous RNA* (% of total)	Polysome-associated fraction of heterogeneous RNA† (%)	Non-polysome-associated fraction of heterogeneous RNA (%)	RNase-resistant fraction of heterogeneous RNA‡ (%)
75	Free polysome RNA	42	58			
	Membrane-associated RNA	21	79	36	64	63
90	Free polysome RNA	51	49			
	Membrane-associated RNA	28	72	38	62	58
120	Free polysome RNA	62	38			
	Membrane-associated RNA	44	56	48	52	49

* Includes small amount of tRNA and 5S RNA (5–10% of heterogeneous RNA).

† Calculated according to the formula $[(hRNA_F/rRNA_F) \times rRNA_M]/hRNA_M$, where hRNA and rRNA indicate heterogeneous and ribosomal RNA, respectively, and subscripts F and M refer to free polysomes and membrane fraction, respectively.

‡ Data derived from Fig. 6.

TABLE 2. Comparative homology with mit-DNA of membrane-associated heterogeneous RNA labeled during short and long pulses.

Membrane-associated RNA fraction	DNA	Cpm in Hybrid per μg DNA		Per Cent Input Cpm in Hybrid		H^3/C^{14} in RNA	
		H^3	C^{14}	H^3	C^{14}	Input	Hybrid
<i>Expt. 1:</i> (7 min H^3 -RNA + 120 min C^{14} -RNA)							
8-35S	Mitochondrial	8.3	9.5	11.4	9.1	0.72	0.88
35-50S	Mitochondrial	14.2	6.7	14.1	13.5	2.0	2.12
<i>Expt. 2:</i> (15 min H^3 -RNA + 120 min C^{14} -RNA)							
8-50S	Total	7.5	5.2	8.6	9.6	1.6	1.43
	"cytoplasmic"						(1.57*)
	Total cell	0.6	0.8	1.2	2.8	1.6	0.73
<i>Expt. 3:</i> (7 min H^3 -RNA + 120 min C^{14} -RNA)							
10-26S	Mitochondrial 1	77	55	10.4	8.1	1.1	1.39
	Mitochondrial 2	97	69	13.3	10.3	1.1	1.40
	Total cell	2.5	4	0.3	0.6	1.1	0.62

Expt. 1: 3.6 μg HeLa mit-DNA and 10 μg RNA (8-35S) or 15 μg RNA (35-50S); *expt. 2:* 22 μg "cytoplasmic DNA"¹ or total HeLa DNA and 20 μg RNA; *expt. 3:* 2 μg mit-DNA (two preparations) or total HeLa DNA and 22 μg RNA (all in 2 ml). The data are corrected for nonspecific background estimated with *E. coli* DNA. The total cpm and the H^3/C^{14} in input RNA refer to heterogeneous RNA only, estimated as in ref. 15.

* Corrected for contribution of hybridization with contaminating nuclear DNA,¹ as estimated from the data obtained with total cell DNA.

Discussion.—The main conclusion of this paper is that mit-DNA is the template of the membrane-associated heterogeneous RNA of cytoplasmic origin previously described in HeLa cells.¹ Base sequence homology of this RNA with mit-DNA and association of the newly synthesized RNA with mitochondria (recognized by buoyant density properties and cytochrome oxidase activity) have led to this conclusion. Mit-RNA is synthesized at a high rate: if the intramitochondrial UTP pool equilibrates with exogenous H^3 -uridine in parallel with the total cell UTP pool, this rate can be estimated to correspond to two thirds of the rate of entry into the cytoplasm of the free polysome mRNA. In *Neurospora* the pool of precursors for mit-DNA synthesis has been shown to turn over more slowly than the pool utilized for nuclear DNA synthesis, possibly because it is fed by an effectively large ribonucleotide pool deriving from turnover of RNA.¹⁷ A similar situation in HeLa cells might lead to an underestimate of the relative rate of mit-RNA synthesis. Assuming mit-DNA to be of the order of 0.1 per cent of total HeLa cell DNA,⁸ the rate of mit-RNA synthesis per unit DNA mass appears to be very high indeed. This RNA, however, does not accumulate in the cytoplasm in proportion to its synthesis, due to its metabolic instability.¹ It is possible that the saturation of radioactivity of intramitochondrial RNA after about 75 minutes of labeling reflects the turnover kinetics of a labile fraction. To what extent the high rate of transcription of mit-DNA is related to its informational role for protein synthesis is not known. Biochem-

ical and genetic evidence in *Neurospora*^{17, 18} and yeast^{16, 19} supports the idea that mit-DNA is genetically active. Several types of observations strongly suggest that mit-RNA does not remain confined inside mitochondria, but is transported in part to extramitochondrial structures. Biochemical and EM evidence has indicated that the great majority (>97%) of the ribosomes of the 8100 × g membrane fraction are associated with ER elements:³ this would put an upper limit of 0.3–0.4 per cent of the total cell ribosomal content for the amount of intramitochondrial ribosomes. If a substantial part of mit-RNA functions as messenger for protein synthesis, as is suggested by its association with polysomal structures,^{1, 3} it seems unlikely that it could be served by very few intramitochondrial ribosomes. Furthermore, RNA-DNA hybridization experiments have shown that the labeled heterogeneous RNA which accumulates in extramitochondrial membrane structures in a two-hour pulse has a degree of homology with purified mit-DNA which is similar to that of the intramitochondrial RNA. On the basis of the available evidence, it seems thus justifiable to conclude that mit-RNA in HeLa cells is exported in part for its utilization to elements of the rough ER, where it becomes associated with ribosomes of nuclear origin.^{1, 3} The possibility that a fraction of this RNA functions inside the mitochondria is suggested by the published reports concerning mitochondrial protein synthesis.²⁰

Abbreviations used: mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; mit-DNA, mitochondrial DNA; mit-RNA, mitochondrial RNA; ER, endoplasmic reticulum; EM, electron-microscopic; NaDOC, sodium deoxycholate; SDS, sodium dodecyl-sulfate; TCA, trichloroacetic acid; RNase, ribonuclease; Tris, tris(hydroxymethyl)amino-methane; UTP, uridine triphosphate.

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¹ Attardi, B., and G. Attardi, these PROCEEDINGS, **58**, 1051 (1967).

² Houssais, J. F., and G. Attardi, these PROCEEDINGS, **56**, 616 (1966).

³ Attardi, B., B. Cravioto, and G. Attardi, in preparation.

⁴ Attardi, G., H. Parnas, M.-I. H. Hwang, and B. Attardi, *J. Mol. Biol.*, **20**, 145 (1966).

⁵ Radloff, R., W. Bauer, and J. Vinograd, these PROCEEDINGS, **57**, 1514 (1967).

⁶ Attardi, G., P. C. Huang, and S. Kabat, these PROCEEDINGS, **53**, 1490 (1965).

⁷ Smith, L., *Arch. Biochem. Biophys.*, **50**, 285 (1954).

⁸ Hudson, B., and J. Vinograd, *Nature*, **216**, 647 (1967).

⁹ Clayton, D. A., and J. Vinograd, *Nature*, **216**, 652 (1967).

¹⁰ Latham, H., and J. E. Darnell, *J. Mol. Biol.*, **14**, 1 (1965).

¹¹ Epstein, M. A., *J. Biophys. Biochem. Cytol.*, **10**, 153 (1961).

¹² Journey, L. J., and M. N. Goldstein, *Cancer Res.*, **21**, 929 (1961).

¹³ Barnett, W. E., and D. H. Brown, these PROCEEDINGS, **57**, 452 (1967).

¹⁴ Rifkin, M. R., D. D. Wood, and D. J. L. Luck, these PROCEEDINGS, **58**, 1025 (1967).

¹⁵ Girard, M., H. Latham, S. Penman, and J. E. Darnell, *J. Mol. Biol.*, **11**, 187 (1965).

¹⁶ Fukuhara, H., these PROCEEDINGS, **58**, 1065 (1967).

¹⁷ Reich, E., and D. J. L. Luck, these PROCEEDINGS, **55**, 1600 (1966).

¹⁸ Woodward, D. O., and K. D. Munkres, in *Symposium on Organizational Biosynthesis*, ed. H. J. Vogel, J. O. Lampen, and V. Bryson (New York: Academic Press, 1967), p. 489.

¹⁹ Mounolou, J. C., H. Jakob, and P. Slonimski, *Biochem. Biophys. Res. Commun.*, **24**, 218 (1966).

²⁰ Wheeldon, L. W., and A. L. Lehninger, *Biochemistry*, **5**, 3533 (1966) (see also for citations).