A MEMBRANE-ASSOCIATED RNA OF CYTOPLASMIC ORIGIN IN HELA CELLS

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The genetic evidence of the existence in yeast[1] and molds[2] of cytoplasmic determinants controlling the structure and function of mitochondria and the demonstration that mitochondria from all organisms contain DNA[3-7] have indicated that transcription and translation processes dependent on cytoplasmic genes occur in eukaryotic cells. More recent observations in *Neurospora* have suggested that a cytoplasmic, presumably mitochondrial, genetic determinant controls the structural proteins of different membrane systems (mitochondria, microsomes, nuclear membrane)[8, 9]; these structural proteins, from their amino acid composition, fingerprinting pattern, and immunological behavior, appear to be identical or to have a common component.[8, 9] These findings suggest that cytoplasmic genes may have a wider role in the cell than hitherto suspected and may direct a considerable fraction of mRNA and protein synthesis.

These considerations prompted an investigation of cytoplasmic DNA-directed RNA synthesis in HeLa cells. The evidence obtained indicates that, in these cells, polysomes associated with cytoplasmic membranes contain an mRNA fraction which is distinct from mRNA of free polysomes, and appears to be of cytoplasmic origin.

Materials and Methods.—(a) *Cells:* Conditions of growth of HeLa cells in suspension have been described previously.[10] These cultures were free of any detectable PPLO contamination.

(b) *Buffers:* The buffers used are: (1) T: 0.01 M tris buffer (pH 7.1); (2) TM: 0.01 M tris buffer (pH 7.1), 0.00015 M $MgCl_2$; (3) TKM: 0.01 M tris buffer (pH 7.1), 0.01 M KCl, 0.00015 M $MgCl_2$; (4) SMET:[11] 0.07 M sucrose, 0.21 M d-mannitol, 0.0001 M EDTA, 0.001 M tris buffer (pH 7.2).

(c) *Labeling conditions:* Exponentially growing HeLa cells ($1-3 \times 10^5$ cells/ml) were exposed for various times to H^3-5-uridine (17.3–30.0 c/mM, 1.25–6.25 μc/ml). Long-term labeling of phosphatidyl choline was carried out by growing cells for 48 hr in the presence of C^{14}-choline chloride (25 μc/ml). For incorporation of P^{32}-orthophosphate, the cells were washed twice and resuspended in phosphate-free Eagle's medium (with dialyzed serum); carrier-free-orthophosphate was utilized at 50 μc/ml.

(d) *Preparation of subcellular fractions:* In order to minimize the possibility of aggregation of free polysomes with membranes, the Mg^{++} concentration in the homogenization medium was reduced to the minimum compatible with stability of polysomes, $1.5 \times 10^{-4} M$.[12] The labeled cells were washed three times with NKM (0.13 M NaCl, 0.005 M KCl, 0.001 M $MgCl_2$) and then resuspended in 6 vol TKM. After 3 min the suspension was homogenized with an A. H. Thomas homogenizer (10–15 strokes), sucrose was added to 0.25 M, and the homogenate centrifuged at $1160 \times g$ for 3 min to sediment nuclei, unbroken cells, and big cytoplasmic debris. The supernatant (*total cytoplasmic fraction*) was spun at $8100 \times g$ for 10 min; the pellet was resuspended in 0.25 M sucrose in T buffer and, after a spin at $1000 \times g$ for 2 min to sediment any possible residual nuclei and a small amount of aggregated debris, was recentrifuged at $8100 \times g$ for 10 min to yield the *first membrane fraction.* The first $8100 \times g$ supernatant was centrifuged at $15{,}800 \times g$ for 20 min to separate the *second membrane fraction* (pellet) from the *free polysome fraction.* Any deviations from this procedure are specified in the legends of the figures.

For sedimentation analysis, subcellular fractions were centrifuged for 70–80 min at 24 krpm at 3°C in the SW 25 rotor of the Spinco L ultracentrifuge through a 15–30% (w/w) sucrose gradient in TKM prepared above 3 ml of 64% (w/w) sucrose.

Separation of cytoplasmic fractions on the basis of density was carried out by centrifuging them for 16 hr at 24 krpm at 3°C in the SW 25 rotor through a 1.0 to 2.0 *M* sucrose gradient in T buffer.

(e) *Extraction and analysis of RNA:* Conditions for RNA extraction and sedimentation analysis, isotope determinations, and base composition analysis have been described in detail elsewhere.[13]

(f) *Determination of the labeling of the pools of uridine nucleotides:* A cell suspension was exposed to H^3-5-uridine (25.4 c/mM, 3.3 μc/ml). At various intervals, aliquots were removed and quickly cooled; the cells were washed three times with NKM, then precipitated with 0.5 N $HClO_4$. The $O.D._{260}$ and the H^3 cpm of the acid-soluble fraction were measured. The samples were then neutralized with KOH, the insoluble $KClO_4$ was separated by centrifugation, and the labeled components of the acid-soluble pool fractionated by Dowex 1-X8 (formate form) chromatography.[14] For all times of labeling analyzed (4, 7, 10, 17 min), more than 90% of the cpm were found to be associated with UMP, UDP and UDP derivatives, and UTP.

(g) *Extraction of total and cytoplasmic DNA:* Total HeLa and *E. coli* DNA were extracted by the Marmur procedure.[15] For the isolation of cytoplasmic DNA, 20 ml packed HeLa cells were gently homogenized in SMET buffer; the nuclei were separated, and the total cytoplasmic fraction was treated according to the Marmur procedure, followed by CsCl density gradient centrifugation. About 1% of total cell DNA was recovered. As this figure is higher than that reported for the proportion of mitochondrial DNA in cultured mammalian cells (0.2%),[5] it is likely that this "cytoplasmic" DNA preparation is contaminated by nuclear DNA.

(h) *RNA-DNA hybridization* experiments were performed as described previously.[16]

Results.—(a) *Distribution of rapidly labeled RNA among cytoplasmic fractions:* Figure 1*A* shows the sedimentation pattern of the total cytoplasmic fraction from HeLa cells. A considerable amount of fast-sedimenting material has been prevented from pelleting by the dense sucrose layer at the bottom of the tube. These fast-sedimenting components contain the bulk of cytoplasmic phospholipids (about 85% of phosphatidyl choline) (Fig. 2), and 10–15% of total cell rRNA; they are known to include mitochondria, vesicles and tubules of the smooth and rough E.R., and other membranaceous structures that electron-microscopic examination has revealed in HeLa cells.[17, 18] One recognizes in the middle portion of the gradient, in Figures 1*A* and 2, a band of polysomes, which presumably correspond to the free

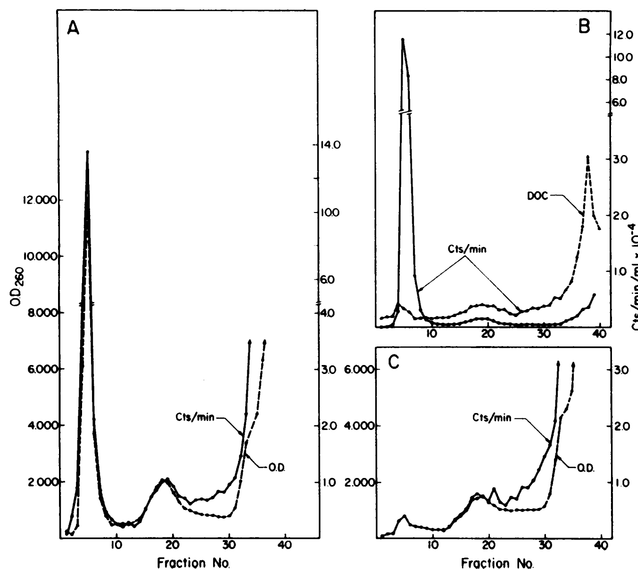


FIG. 1.—Sedimentation pattern of cytoplasmic fractions from HeLa cells labeled for 30 min with H^3-5-uridine. (*A*) Total cytoplasmic fraction. (*B*) Membrane fraction separated by centrifugation at 15,800 × *g* for 20 min and analyzed as such (●) or after treatment with 1% NaDOC (▲). (*C*) 15,800 × *g* supernatant. 15–30% sucrose in TKM (over 3 ml 64% sucrose), 80 min, 24 krpm.

polysomes which are seen in the cytoplasmic matrix in electron-microscopic pictures. Centrifugation for 5 hr in a 0.75 M–2.0 M sucrose gradient of this polysome fraction prepared (as described in *Materials and Methods*) from C^{14}-choline labeled cells reveals essentially no lipoprotein material in correspondence with the polysome peak.

After a 30-min pulse with H^3-uridine, about twice as much of the newly synthesized RNA appears in the region of the fast-sedimenting material as is associated with free polysomes (Fig. 1*A*).

Differential centrifugation of the total cytoplasmic extract separates the membrane fraction, contaminated by a small amount of free polysomes (Fig. 1*B*), from the bulk of these (Fig. 1*C*). Treatment of the membrane fraction with 1% NaDOC releases almost all H^3, which now sediments in the upper two thirds of the gradient in the region corresponding to polysomes, monomers, and lighter components (Fig. 1*B*). The H^3 sedimenting in the polysome region shows the sensitivity to RNase characteristic for free polysomes,[19] suggesting that it is associated with polysomal structures.

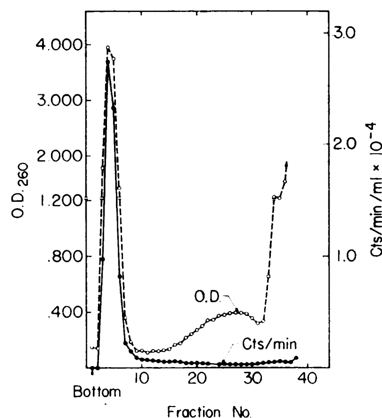


FIG. 2.—Sedimentation pattern of total cytoplasmic fraction from HeLa cells uniformly labeled with C^{14}-choline. Same gradient as in Fig. 1, 70 min, 24 krpm.

In the experiments described below, the cytoplasmic membranaceous material was separated into two subfractions, as explained in *Materials and Methods*. The first membrane fraction contains the bulk of mitochondria,[11] contaminated by other membranaceous structures, some of which are rich in RNA. The second membrane fraction consists of slower-sedimenting mitochondria and other membrane elements, and a relatively small amount of free polysomes.

As the bulk of membrane-associated RNA was found to be in the first membrane fraction, all the investigations described below were carried out with this fraction.

(b) *Buoyant density distribution in sucrose gradient of the membrane components:* As shown in Figure 3, these components band in a region of the sucrose gradient corresponding to ρ = 1.17–1.195. Mitochondria, as revealed by the OD_{415} due to cytochrome *c*, band at $\rho \sim 1.19$, in agreement with the known density of these structures in sucrose gradient.[11] The buoyant density of the structures carrying the pulse-labeled RNA depends on the medium utilized for homogenization and sucrose gradient, in particular, on the Mg^{++} concentration. After complete removal of Mg^{++} by EDTA in the homogenization medium, the structures containing newly synthesized RNA band at $\rho \sim 1.18$ and are fairly well separated from the mitochondrial band (Fig. 3*A*). If the homogenization medium and sucrose gradient contain neither EDTA nor Mg^{++}, the material carrying labeled RNA is displaced towards higher densities (Fig. 3*B*); if Mg^{++} (1.5×10^{-4} M) is added to the homogenization medium, this material almost completely overlaps the mitochondrial band (Fig. 3*C*). It is known that divalent cations cause shrinkage (with resulting increased density) and aggregation of vesicles deriving from E. R., presumably as a result of the reduction of the surface potential of these structures.[20] From their

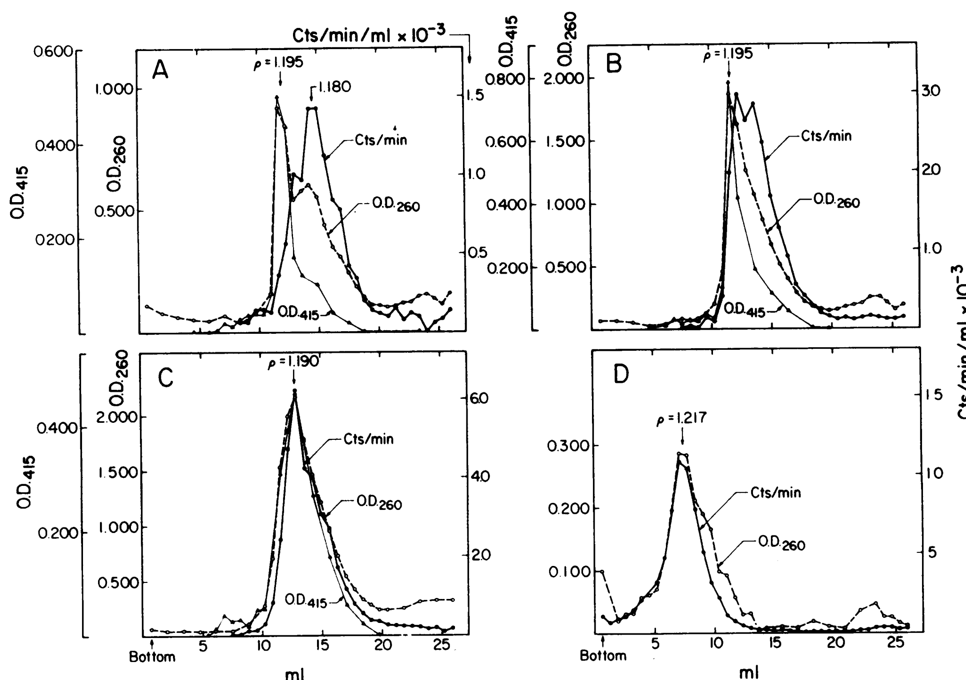
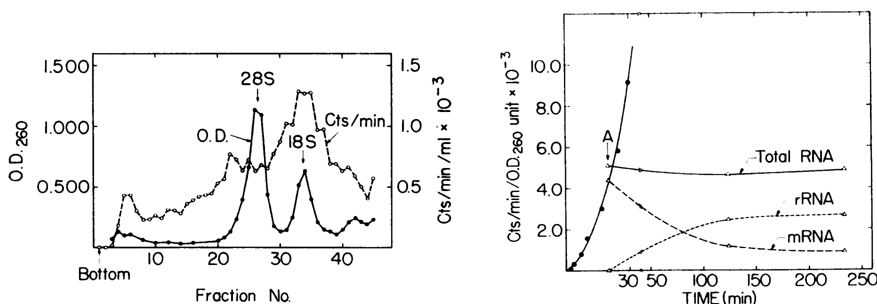


FIG. 3.—Equilibrium centrifugation in sucrose density gradients of membrane fractions from HeLa cells labeled for 15 min with H^3-5-uridine. The first membrane fraction was isolated from 3 batches of cells, homogenized, respectively, in SMET (*A*) or in T (*B*) or in TM buffer (*C*). (*D*) Nuclei obtained from the same cell suspension were extensively broken by homogenization, centrifuged at 1160 × *g* for 3 min, and the supernatant analyzed. 1.0–2.0 *M* sucrose in T buffer, 16 hr, 24 krpm.



(*Left*) FIG. 4.—Sedimentation pattern of membrane-associated RNA extracted from HeLa cells exposed for 7 min to H^3-5-uridine. 5–20% sucrose in acetate-NaCl buffer[13] (over 3 ml 64% sucrose), 8 hr, 25 krpm.

(*Right*) FIG. 5.—Effect of actinomycin D on the labeling of membrane-associated RNA. HeLa cells were exposed to H^3-5-uridine, and samples removed at different times; to one aliquot of the suspension actinomycin D (7.5 μg/ml) was added at 20 min, and samples removed from it at 104 and 230 min. From each sample the first membrane fraction was isolated as in Fig. 3*C*, and the H^3 associated with it measured. From the membrane fractions isolated at 20, 104, and 230 min, the RNA was extracted, and the proportion of mRNA (>6*S*) and rRNA estimated as described previously.[13]

density in sucrose gradients and their sensitivity to divalent cations, it seems likely that the structures containing newly synthesized RNA are vesicles of the rough E. R. As shown in Figure 3*D*, the material released from HeLa cell nuclei by extensive breakage bands at a higher density than the membrane components derived from the total cytoplasmic fraction; this observation confirms the cytoplasmic derivation of these components.

(c) *Sedimentation pattern of membrane-associated RNA:* Figure 4 shows the sedimentation profile of membrane-associated RNA extracted from cells exposed to H^3-uridine for 7 min. The OD_{260} reveals the two rRNA components in the ratio expected for ribosomes ($\sim$2.5).[21] Appropriate experiments have failed to show any difference in sedimentation properties between rRNA species from the membrane fraction and from free polysomes. For their sedimentation behavior the labeled RNA components are presumably of the messenger type; they are distributed over the whole gradient from the 6*S* to the 50*S* region, with a broad peak centered around 18*S*. The proportion of components sedimenting faster than 28*S* appears to be appreciably greater than in the mRNA extracted from free polysomes. After about 25 min labeling, radioactivity starts appearing in the 18*S* rRNA, and approximately 30 min later in the 28*S* rRNA; at the same time labeled rRNA components appear also in the free polysomes.

(d) *Base composition of cytoplasmic mRNA fractions:* Table 1 shows the

TABLE 1

NUCLEOTIDE COMPOSITION OF FRACTIONS OF HELA CYTOPLASMIC MESSENGER RNA*

Fraction	Moles (%) A	C	U(T)	G	GC%	A/U	A/G
Membrane-associated mRNA:†							
9–25*S*	33.9	24.5	22.6	18.9	43.4	1.50	1.79
26–48*S*	31.4	23.9	25.3	19.4	43.3	1.24	1.62
Free polysome mRNA:							
10–38*S*	24.8	21.4	27.9	25.8	47.2	0.89	0.96
"Toal" cytoplasmic mRNA:[10]							
9–40*S*	25.7	25.4	27.6	21.1	46.5	0.93	1.22
Total HeLa DNA[10]	29.8	20.0	30.1	20.1	40.1	0.99	1.48
Human mitochondrial DNA‡					45		

* Isolated from 30 min P^{32} pulse-labeled cells.
† The data represent averages of two analyses.
‡ From leucocytes of leukemic donor; GC content estimated from buoyant density in CsCl (J. Vinograd, personal communication).

"apparent" base composition of the mRNA extracted from the membrane fraction and from free polysomes after a 30-min P^{32} pulse. One can see that both the heavier (>25*S*) and lighter (9*S*–25*S*) components of the membrane-associated mRNA have a base composition strikingly different (especially for the high A content) from that of free polysomal mRNA. The base composition previously found[10] for "total" cytoplasmic mRNA (that is, the RNA extracted, after a 30-min pulse, from free polysomes and that portion of the membrane-associated polysomes which is released by 0.5% NaDOC) is also shown: the pattern of base ratios appears to be roughly that of a mixture of the two classes of mRNA.

(e) *Metabolic behavior of cytoplasmic mRNA fractions:* As shown in Figure 5, addition of actinomycin D (7.5 μg/ml) immediately stops the labeling of membrane-associated RNA; in contrast, the flow of radioactivity into free polysomal RNA (in particular, into rRNA) continues for several hours, although at a decreasing

rate, up to a final fourfold increase (not shown in figure). Sedimentation analysis of the RNA extracted from the membrane fraction after different times of exposure to the drug reveals a fairly rapid decline (estimated half-life less than 1 hr) of the mRNA fraction, which is compensated for by the appearance of labeled rRNA; this becomes the predominant labeled component (more than 65%) after about 1-$^1/_2$ hr actinomycin action. In contrast, the free polysome-associated mRNA is relatively stable: in fact, only about 20% of free polysomes break down during the 4-hr exposure to actinomycin D, and it is uncertain whether this is due to dissociation of polysomes or destruction of mRNA[22, 23]; furthermore, the absolute amount of labeled mRNA in the intact polysomes does not decrease appreciably during this time. These results suggest that membrane-associated mRNA has a considerably faster turnover than the free polysome-associated mRNA.

(f) *Kinetics of labeling of cytoplasmic mRNA fractions:* The observed differences in base composition, sedimentation properties, and metabolic behavior indicated that the membrane-associated mRNA and the free polysome mRNA represent two distinct mRNA populations. In order to obtain some information concerning the site of synthesis of these two mRNA classes, an analysis of the kinetics of appearance of label in the two fractions was carried out. Figure 6 shows that the membrane-associated mRNA becomes labeled much faster than the free polysome-associated mRNA. In order to correct these kinetics for incomplete equilibration of the UTP pool with H^3-uridine, the labeling of this pool after different times of exposure of the cells to the H^3 precursor was analyzed. After correction for differences in pool equilibration, it appears that the increase of label in the membrane-associated messenger is linear from zero time, whereas the appearance of label in the free polysome mRNA still shows an appreciable delay (Fig. 6). A reasonable interpretation of these results is that the membrane-associated mRNA is cytoplasmic in origin (in particular, synthesized in the membranaceous structures themselves); on the other hand, the slower appearance of labeled mRNA in free polysomes could reflect the time required for the equilibration of the pool of precursor mRNA molecules and/or for the processing of these mRNA molecules in the nucleus and their transport into the cytoplasm.

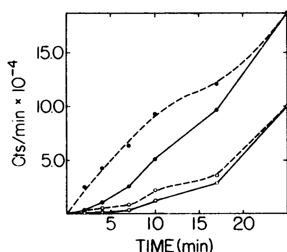


FIG. 6.—Flow of radioactivity into the membrane-associated RNA and free polysome RNA. HeLa cells were exposed to H^3-5-uridine, and samples removed at different times; from each sample the first membrane fraction and the free polysomes were separated as in Figs. 1 and 3, and the total H^3 associated with them measured and corrected for small variations in the yield of total OD_{260}. Correction for differences in UTP pool equilibration with external H^3-uridine was made by assuming that the amount of label in the RNA synthesized after a given incubation time is proportional to the integral of the curve of the UTP pool labeling. ●, Membrane-associated RNA. ○, Free polysome-associated RNA. Dashed lines are used for the corrected kinetics curves.

(g) *RNA-DNA hybridization experiments:* As a more direct approach to the problem of the site of synthesis of the membrane-associated mRNA, hybridization experiments were carried out between this mRNA and the free polysome mRNA, on the one hand, and total HeLa DNA and "cytoplasmic" DNA, on the other. It appears in Table 2 that the membrane-associated mRNA has a markedly greater sequence homology with "cytoplasmic" DNA than with total DNA. The amount

TABLE 2

COMPARATIVE HOMOLOGY OF MESSENGER RNA FRACTIONS WITH "CYTOPLASMIC" AND TOTAL HELA DNA

	Membrane-Associated mRNA		Free Polysome mRNA	
	Cpm in hybrid per μg DNA*	Input cpm in hybrid (%)	Cpm in hybrid per μg DNA*	Input cpm in hybrid (%)
Experiment 1:				
"Cytoplasmic" DNA	206	32.4	0.7	0.8
Total DNA	21	5.7	0.4	0.8
Experiment 2:				
"Cytoplasmic" DNA	97	26.0	0.8	1.3
Total DNA	15	5.2	0.4	0.8

Each annealing mixture contained 10 μg/ml DNA and 10 μg/ml total RNA, except in expt. 2 where 20 μg/ml DNA and 20 μg/ml free polysome RNA were used. Incubation at 70°C for 4 hr; RNase digestion with 10 μg/ml, 60 min at 22°C; washing of hybrid on S&S membranes at 55°C.[16] Expts. 1 and 2 utilized two different samples of membrane-associated mRNA (30 min and 20 min H^3 pulse, respectively).

* Recovered after Sephadex chromatography; the data are corrected for nonspecific background estimated with *E. coli* DNA (amounting to 10–15% of the highest hybrid value in each experiment).

of specific mRNA present in the hybridization mixture (estimated to be 2–5% of total RNA[24]) is presumably nonsaturating for the quantity of "cytoplasmic" DNA utilized, but closer to saturating levels for the relatively small fraction of cytoplasmic DNA present in the total DNA preparation. Therefore, the genuine cytoplasmic DNA present in total DNA may give per μg DNA a higher level of hybrid than that contained in the "cytoplasmic" DNA preparation. The observed difference in degree of homology of membrane-associated mRNA with "cytoplasmic" and total DNA is thus a minimum value. The free polysome-associated mRNA appears to hybridize with the two DNA preparations to a much closer extent than the membrane-associated mRNA. This hybridization with "cytoplasmic" DNA presumably occurs with contaminating nuclear DNA. The difference in hybridization of free polysome mRNA with "cytoplasmic" and total DNA is probably insignificant: it may derive from a small contamination of free polysomes by membrane-associated polysomes which would have a large effect on the hybridizing capacity of free polysomal mRNA, since the membrane-associated mRNA has tenfold specific activity and much greater hybridization efficiency. The high proportion of membrane-associated mRNA that hybridizes with "cytoplasmic" DNA (25–30% under the conditions utilized here), as compared to that observed for free polysome mRNA, shows that the cytoplasmic DNA contains a high concentration of genes homologous to the membrane-associated mRNA. This result strongly suggests that the membrane-associated mRNA is, in part at least, the product of cytoplasmic (presumably mitochondrial) genes.

Discussion.—The results presented in this paper have indicated that in HeLa cells a fraction of polysomes (amounting to about 10–15% of the total) are associated with membranes and contain a messenger fraction which, for sedimentation properties, base composition, and metabolic behavior, is distinct from mRNA of free polysomes; on the basis of the kinetics of its appearance in the cytoplasm, and especially of its sequence homology with cytoplasmic DNA, this mRNA appears to be of cytoplasmic origin, presumably synthesized on a mitochondrial DNA template. As to the membrane structures with which this RNA is associated, several lines of evidence suggest that they are distinct from mitochondria. In the absence of divalent cations, these structures band in sucrose density gradients at a lower density than the bulk of mitochondria; furthermore, they show the sensitivity to divalent cations described for elements of E. R.[20] Their relatively high content in

RNA (present in structures with the properties of typical polysomes) also speaks against their mitochondrial nature, in view of the low RNA content reported for mammalian mitochondria.[25, 26] The probable nuclear origin of the ribosomes serving the membrane-bound mRNA, which is suggested by the similar kinetics of appearance of labeled rRNA in bound and free polysomes, further supports the idea that the membrane-bound polysomes are extramitochondrial. On the basis of the present evidence it is thus tentatively concluded that the polysome-carrying membrane structures observed here correspond to the tubules and vesicles of the rough E. R. that electron microscopy has revealed in HeLa cells.[17, 18] The membrane-associated mRNA studied here would thus represent mitochondrial mRNA which is exported to the rough E. R. A fraction of this RNA, however, presumably remains inside the mitochondria since these organelles are able to support protein synthesis.[27, 28] Work is in progress to establish conclusively the nature of the cytoplasmic DNA template of the membrane-bound mRNA and to test the hypothesis of the possible involvement of this RNA in directing the synthesis of structural components of mitochondria and other cytoplasmic membranes.

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Abbreviations used: mRNA, messenger RNA; rRNA, ribosomal RNA; E. R., endoplasmic reticulum; NaDOC, sodium deoxycholate.

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