I. A STUDY BY ELECTRON MICROSCOPY OF THE RNA AND RNA-DNA HYBRIDS OF HeLa MITOCHONDRIA

II. REPLICATION OF CLOSED CIRCULAR MITOCHONDRIAL DNA IN MOUSE L CELLS

Thesis by

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To Barbara

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ABSTRACT

The thesis consists of two parts. In Part I, electron microscope analysis of HeLa mitochondrial RNA-DNA hybrids isolated from $CsSO_4$ gradients demonstrates that more than 85% of the heavy strand of HeLa mitochondrial DNA is transcribed *in vivo*.

A modified basic protein film method of spreading RNA in a strongly denaturing solvent for examination in the electron microscope has been developed and applied to determine the size of the HeLa mitochondrial specific ribosomal RNA (rRNA) components. Length measurements on purified 12S and 16S mitochondrial rRNA, on mixtures of the two, and on mixtures of 12S with 18S cytoplasmic rRNA have given molecular lengths of 0.27 μ, 0.42 μ, and 0.55 μ for the 12S, 16S, and 18S rRNAs, respectively. If these molecular lengths are proportional to molecular weight, and if the molecular weight of 18S cytoplasmic rRNA is taken as 0.71×10^6, as determined by sedimentation equilibrium, the molecular weights of the 12S and 16S components are 0.35×10^6 and 0.54×10^6, respectively. These molecular weight values are in good agreement with the relative values predicted from sedimentation velocity measurements, but not with the relative values based on gel electrophoresis.

Electron microscopy of hybrids between the heavy strand of HeLa mitochondrial DNA and HeLa mitochondrial rRNA demonstrates that the genes for 16S and 12S HeLa mitochondrial rRNAs are situated adjacent, or very close, to each other on mitochondrial DNA. The length of the DNA segment separating the two genes is estimated to correspond to less than 500 nucleotide pairs.

A coupling procedure has been developed for the efficient covalent attachment of periodate oxidized RNA to an insoluble matrix of hydrazide-derived sepharose, which is subsequently available for nucleic acid hybridization.

In Part II of the thesis, the properties of a new structure of mitochondrial DNA are discussed. In two strains of mouse L cells, it was found that approximately one half of the closed mitochondrial DNA molecules contain a short three-stranded DNA region in which a short single strand of progeny DNA containing about 350 nucleotides is inserted into the closed circular duplex with the displacement of a corresponding stretch of the still circular parental strand. The three-stranded region is called a D-loop because of its formal shape and because it appears to have been formed by displacement replication. Double-length mitochondrial DNA molecules often contain two D-loops which are always at diametrically opposed positions on the 20 megadalton circle. This latter result is taken to indicate that one of the forks in each D-loop marks a unique site for the initiation of replication.

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Part I

A STUDY BY ELECTRON MICROSCOPY OF THE RNA AND RNA-DNA HYBRIDS OF HeLa MITOCHONDRIA

A. General Introduction

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Transcription of Mitochondrial DNA in HeLa Cells

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In the last few years the investigation of the physical properties of mitochondrial DNA in eucaryotic cells has brought to light two remarkable facts. The first is that mitochondrial DNA has undergone an evolution, going from the simpler to the higher cellular organisms, in a direction opposite to that of the nuclear genome. Whereas the latter has increased in size and complexity, mitochondrial DNA has become smaller and simpler. Mitochondrial DNA molecules from lower eucaryotic cells (protozoa, fungi) and plant cells have, in fact, been found to be considerably larger (from 3 to 12 times) than those of animal cells (Suyama and Miura, 1968; Wolstenholme and Gross, 1968; Wood and Luck, 1969; Borst, 1970); moreover, as far as one can judge from the few available data concerning the sequence length of mitochondrial DNA, estimated from renaturation kinetics (Borst, 1970), the reduction in size of mitochondrial DNA during evolution appears to have been accompanied by reduction in its informational content, with the lost functions presumably having been taken over by the nucleus.

The second remarkable fact is that mitochondrial DNA from all animal species examined, from insects to echinoderms to the highest vertebrates, is in the form of circular molecules about five microns in length (Borst, Van Bruggen, and Ruttenberg, 1968). Thus, the amount of information that animal mitochondrial DNA contains appears to have been preserved over hundreds of millions of years. This suggests that the progressive reduction in informational content of mitochondrial DNA has reached in animal cells the limit compatible with the survival of the mitochondrial DNA itself. This in turn would imply that whatever the function is which has proven to be advantageous for animal cells to keep under the control of cytoplasmic genes, it cannot be taken over in any part by nuclear genes.

As concerns the possible nature of this function, strong constraints are obviously placed on it by the small size of animal cell mitochondrial DNA itself. One can think of the information required for the replication and transcription of mitochondrial DNA and their control, and for the synthesis of certain components of the intramitochondrial protein synthesizing machinery which cannot be substituted by their cytoplasmic counterparts for the translation of mitochondrial messages. It is likely, however, that these represent ancillary functions which would not by themselves confer evolutionary advantage upon the persistence of mitochondrial DNA in animal cells. One would like to think that there is a unique function of mitochondrial DNA related to the synthesis of some essential mitochondrial constituent. On the basis of various pieces of circumstantial evidence, a good candidate for this unique function is the synthesis of some protein component of mitochondrial membranes with a structural or organizational role (Attardi and Attardi, 1969). It is clear, at any rate, that mitochondria are very far from being closed autonomous systems within the cell. The primitive symbiotic bacteria, which were the probable precursors of mitochondria, adapted to growth in the microchemostat provided by the ancestral eucaryotic cell, have slowly become, in the course of evolution, domesticated cell organelles constructed according to a nuclear blueprint, though still with some mitochondrial controlling elements. As a physical corollary of the nuclear control of mitochondrial assembly, mitochondrial membranes, for a long time known to be a site of exchange and transport of small molecules, are thought today to be also an active site of flux inward, and possibly outward, of macromolecules: proteins, RNA, and lipids.

The mitochondrial genetic system, because of its relative simplicity and its compartmentalized character, offers unique advantages for the study of some of the basic problems concerning structure, replication and transcription of DNA, and processing, transport and translation of RNA in eucaryotic cells.

We shall describe in this paper the work that we have carried out in the last two years on the transcription of mitochondrial DNA in a human cell line grown in vitro, HeLa cells.

RAPIDLY LABELED HETEROGENEOUS MITOCHONDRIAL RNA

When exponentially growing HeLa cells are exposed to a labeled RNA precursor, radioactivity

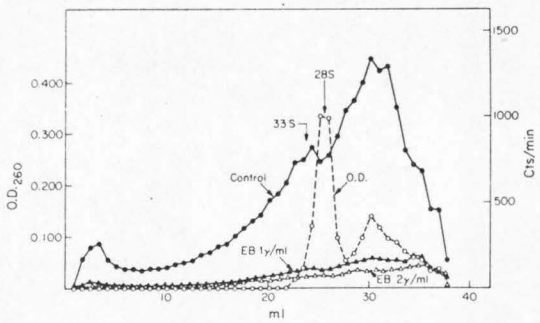

FIGURE 1. Effect of ethidium bromide on the labeling of heterogeneous mitochondria-associated RNA. 2×10^8 HeLa cells were exposed to 5-^{3}H-uridine (22.7 c/mM, 1.4 μc/ml) for 15 min in the absence (●——●) or in the presence of 1 μg/ml (▲——▲), or 2 μg/ml (△ - - - △) ethidium bromide. The crude mitochondrial fraction was isolated by differential centrifugation, as previously described (Attardi and Attardi, 1968), from the cell homogenate, treated for 10 min with 0.03 M EDTA, centrifuged at 11,000 × g for 10 min, washed by centrifugation with 0.25 M sucrose in 0.01 M Tris pH 6.7 at 25°C and 0.015 M EDTA, and then centrifuged to equilibrium in the Spinco SW 25.1 rotor at 25,000 rpm for 18 hr at 3°C in a 30 to 48% sucrose gradient in 0.01 M Tris buffer pH 6.7. RNA was extracted from the mitochondrial band (which was contaminated with rough endoplasmic reticulum elements) by phenol in the presence of 1.25% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.5% sodium cholate and 2.6 mg/ml bentonite, precipitated three times with ethanol, dissolved in 0.01 M acetate buffer pH 5.0 and 0.1 M NaCl, and run through a 5 to 20% (w/w) sucrose gradient in the same buffer (above a 4 ml cushion of 64% sucrose) in the SW 27 Spinco rotor (2.54 × 8.83 cm buckets) at 20,000 rpm for 10 hr at 3°C.

is incorporated linearly without any detectable lag (after correction for pool equilibration) into RNA associated with the mitochondrial fraction, which can be separated by differential centrifugation and buoyant density fractionation in sucrose gradient (Attardi and Attardi, 1967, 1968). Sedimentation analysis of RNA phenol-SDS extracted from this fraction after short 5-^{3}H-uridine pulses shows that the labeled RNA consists of heterogeneous components with sedimentation constants ranging from 4 S to 50 S and more (Attardi and Attardi, 1967, 1969). Figure 1 illustrates the effect of ethidium bromide, an intercalating phenanthridine dye, which has been shown to inhibit selectively the synthesis of mitochondria-associated RNA (Zylber, Vesco, and Penman, 1969), on the labeling during a 15 min 5-^{3}H-uridine pulse of the heterogeneous RNA. The mitochondrial fraction had been treated with 3×10^{-2} M EDTA to remove the bulk of ribosomes of the rough endoplasmic reticulum which contaminates the mitochondrial fraction. The OD_{260} profile shows a prominent 28 S peak (pertaining in its great majority, if not exclusively, to membrane-stuck 50 S subunits not removed by the EDTA-treatment from the contaminating rough endoplasmic reticulum elements), a broad band centered around 18 S (consisting of components which are resolved in long sedimentation runs [see below]) and slower sedimenting material. The heterogeneous pulse-labeled RNA consists of components sedimenting between 4 S and more than 50 S. A broad peak at about 33 S is reproducibly recognizable after a 15 min pulse. A concentration of ethidium bromide of 1 μg/ml, which does not appreciably affect nuclear RNA synthesis, inhibits uniformly by about 85% the labeling of different size components of the heterogeneous mitochondria-associated RNA; the inhibition is about 90% at a concentration of

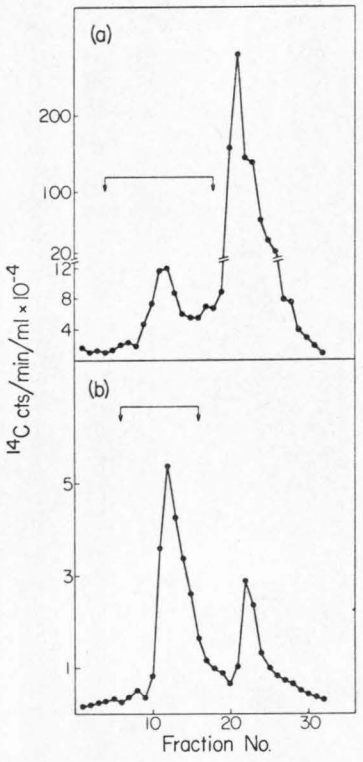

FIGURE 2. (*a*) Purification of ^{14}C-labeled HeLa closed circular mitochondrial DNA by CsCl-ethidium bromide density gradient centrifugation. The mitochondrial DNA was released by SDS, as previously described (Attardi and Attardi, 1969), from the crude mitochondrial fraction of HeLa cells labeled for 60 hr in the presence of 2-^{14}C-thymidine. After addition of CsCl to 1 M and centrifugation at 15,800 × g for 15 min, ethidium bromide was added to the supernatant to 200 μg/ml, and the solution was adjusted to 1.55 g/cm^3 with CsCl and centrifuged in the Spinco SW 41 rotor at 35,000 rpm for 48 hr at 20°C. Fractions were collected dropwise from the bottom of the tube. (*b*) After assay of radioactivity, the fractions corresponding to the smaller heavy band (designated by arrows in *a*) were pooled and recentrifuged under the same conditions in a CsCl-ethidium bromide density gradient. The fractions containing the heavier band, which consisted exclusively of closed circular mitochondrial DNA (Radloff et al., 1967), were pooled and freed of the dye by passage through a Dowex 50 W-X8 column (Radloff et al., 1967).

the drug of 2 μg/ml. The almost complete sensitivity to ethidium bromide, in short-term experiments, of the labeling of mitochondria-associated heterogeneous RNA presumably reflects a direct effect of this drug on the transcription of a closed circular DNA template: in fact, this intercalating dye is known to distort the structure of supercoiled circular DNA (Radloff, Bauer, and Vinograd, 1967; Crawford and Waring, 1967; Bauer and Vinograd, 1968). These experiments therefore support the idea that mitochondrial DNA is the template of the fast-labeled heterogeneous mitochondria-associated RNA. In agreement with this interpretation are the results of RNA-DNA hybridization experiments utilizing closed circular mitochondrial DNA purified by two cycles of cesium chloride density gradient centrifugation in the presence of ethidium bromide (Fig. 2) (Radloff et al., 1967; Attardi and Attardi, 1969). As shown in Table 1, after a 7 or 15 min 5-^{3}H-uridine pulse, both the RNA components sedimenting slower than 28 S and the heavier components hybridize with great efficiency with mitochondrial DNA and only to a very limited extent with total cell or nuclear DNA.

As shown in Table 2, the nucleotide composition of the mitochondria-associated heterogeneous RNA,

TABLE 1. BASE SEQUENCE HOMOLOGY TO MITOCHONDRIAL DNA OF PULSE-LABELED MITOCHONDRIA-ASSOCIATED HETEROGENEOUS RNA

Expt. no.	Pulse (min)	Fraction	DNA	count/ min in hybrid	% input count/ min in hybrid
1	7	10-26 S	Mitochondrial	194	13.3
		10-26 S	Total cell	5	0.3
		33-50 S	Mitochondrial	284	25.6
		33-50 S	Nuclear	10	0.9
2	15	8-35 S	Mitochondrial	30	11.4
		8-35 S	Total cell	<1	<0.4
		35-50 S	Mitochondrial	51	14.1
		35-50 S	Total cell	<1	<0.3

The mitochondrial fraction was isolated from HeLa cells exposed for 7 or 15 min to 5-^{3}H-uridine and the RNA was phenol-SDS extracted and run through sucrose gradients as in Fig. 1. RNA components from different portions of each gradient were precipitated with ethanol and dissolved in SSC/10. The incubation mixtures, containing 2 to 3.6 μg heat-denatured mitochondrial DNA and 10 to 22 μg RNA in a total vol of 2 ml 2 × SSC, were incubated at 70°C for 5 hr. After cooling, the mixtures were treated with 10 μg/ml heated pancreatic RNase for 60 min at 22°C and the RNA/DNA hybrids isolated by Sephadex chromatography as described in Jeanteur and Attardi (1969). (Washing of the nitrocellulose membranes with 0.5 M KCl, 0.01 M Tris buffer was at 56°C.) The data are corrected for nonspecific background estimated with *E. coli* DNA.

TABLE 2. NUCLEOTIDE COMPOSITION OF RNA COMPONENTS FROM THE MITOCHONDRIAL FRACTION AND FROM THE FREE MONOMER-POLYSOME FRACTION OF HELA CELLS

Component	Moles % A	C	U + Ψ(T)	G	% GC	A/G	Estimated number of methyl groups per 100 nucleotides
Mitochondrial fraction							
30 min ^{32}P-pulse-labeled heterogeneous RNA* 9–25 S	33.9	24.5	22.6	18.9	43.4	1.79	
26–48 S	31.4	23.9	25.3	19.4	43.3	1.62	
16 S	32.0	25.7	23.4	18.9	44.6	1.69	0.9
12 S	32.7	26.3	22.6	18.3	44.6	1.79	1.0
4 S	27.5	22.2	28.9	21.4	43.6	1.29	4.6
Free monomer-polysome fraction							
28 S	16.4	31.3	17.1	35.2	66.5	0.47	1.4**
4 S	17.6	26.9	21.6	33.9	60.8	0.52	10.1
mRNA (10–38 S)*	24.8	21.4	27.9	25.8	47.2	0.96	
Mitochondrial DNA							
Heavy strand	22.2		31.8		46***		
Light strand	31.8		22.2		46***		

The 16 S and 12 S RNA components were isolated by two cycles of sucrose gradient centrifugation from the RNA of the mitochondrial fraction of HeLa cells labeled for 22 hr with ^{32}P-orthophosphate (1.75 μc/ml) and L-^{3}H-methyl methionine (5.4 mc/μmole, 2.5 μc/ml) in modified medium containing 10^{-3} M phosphate and 3 μg/ml methionine, in the presence of 0.04 μg/ml actinomycin D. To minimize the labeling of the purine rings, adenosine and guanosine were added at a concentration of 10^{-4} M to the growth medium for 24 hr prior to exposure to the isotopes and during the labeling period. The 4 S RNA from the mitochondrial fraction and the 28 S and 4 S RNA from the free monomer-polysome fraction were isolated from cells labeled with ^{32}P-orthophosphate and L-^{3}H-methyl methionine under the same conditions described above but in the absence of actinomycin D. The 4 S RNA from the mitochondrial fraction and from free ribosomes were isolated by polyacrylamide gel electrophoresis of the low mol wt components (up to about 7 S) of the sedimentation pattern (Fig. 6). ^{32}P-nucleotide composition after alkali digestion was carried out as previously described (Attardi, Parnas, Hwang, and Attardi, 1966). The % of methylated nucleotides was calculated from the ^{3}H to ^{32}P ratios and from the known content of methyl groups of cytoplasmic 28 S RNA. 5 S RNA isolated by polyacrylamide gel electrophoresis showed no detectable labeling with L-^{3}H-methyl-methionine (Fig. 6), thus excluding labeling of the purine rings. The A and T content of the heavy and light strands of mitochondrial DNA was determined from the ratio of 2-^{14}C-thymidine incorporated into each of the separated strands and from the GC content of human mitochondrial DNA. * Attardi and Attardi, 1967; ** Brown and Attardi, 1965; *** Clayton and Vinograd, 1967.

pulse-labeled for 30 min with ^{32}P, is strikingly different (especially for the high A and low G content) from that of 28 S rRNA and from that of free polysome mRNA. Furthermore, this base composition is complementary, as concerns the A and U content, to that of the heavy strand of mitochondrial DNA; this is in agreement with the observation that this strand is the transcriptionally active strand of mitochondrial DNA (see below).

Discrete RNA Components Coded by Mitochondrial DNA

After a 7 min pulse of 5-^{3}H-uridine, and more clearly after a 15 min pulse, discrete labeled components sedimenting at about 16 S, 12 S, and 4 S can be seen to emerge over the background of heterogeneous RNA (Attardi et al., 1969). These labeled components can be easily recognized in long sedimentation runs which fully display the components sedimenting slower than 28 S RNA. Figure 3*a* shows the labeling pattern of these components from the EDTA-treated mitochondrial fraction of cells exposed to a 90 min 5-^{3}H-uridine pulse. The OD_{260} profile shows near the bottom of the tube the prominent peak (partially pelleted) of 28 S RNA, near the center of the gradient an 18 S RNA peak with a shoulder at about 16 S, and two clear peaks at 12 S and 4 S. The labeling profile shows peaks at 12 S and 4 S; in contrast, the considerable labeling of the 18 S RNA species after a 90 min pulse masks in part the 16 S component, which is recognizable only as a shoulder. However, if the labeling is carried out in the presence of 0.04 μg/ml actinomycin D to block selectively rRNA synthesis (Dubin, 1967; Penman, Vesco, and Penman, 1968), the labeling of 18 S RNA is abolished and the 16 S component stands out clearly (Fig. 3*b*). Under the same conditions, the labeling of the 12 S and 4 S peaks is also not affected to any appreciable extent. Ethidium bromide, at a concentration of 1 μg/ml, besides strongly inhibiting the labeling of the heterogeneous RNA, abolishes that of the 16 S, 12 S, and to a great extent (about 80%) that of the 4 S components (Fig. 3*c*). After 4 hr labeling with 5-^{3}H-uridine in the presence of 0.04 μg/ml actinomycin D the 16 S, 12 S, and 4 S components appear as very prominent peaks (Fig. 4). The ethidium bromide sensitivity and actinomycin D resistance of the synthesis of these components suggests that they are coded by mitochondrial DNA. This interpretation has been fully corroborated by RNA-DNA hybridization tests in which the sedimentation distribution of long-term labeled RNA from an EDTA-treated mitochondrial fraction of HeLa cells was scanned for capacity to hybridize with mitochondrial DNA (Attardi and Attardi, 1969). In these experiments, in fact, the sedimentation profile of RNA components homologous to mitochondrial DNA has revealed a broad band between 9 and 15 to 16 S, with a peak at 12 S and a prominent 4 S peak; the shoulder of RNA homologous to mitochondrial DNA at 15 to 16 S presumably corresponds to the 16 S ethidium bromide-sensitive species which was not resolved in that analysis.

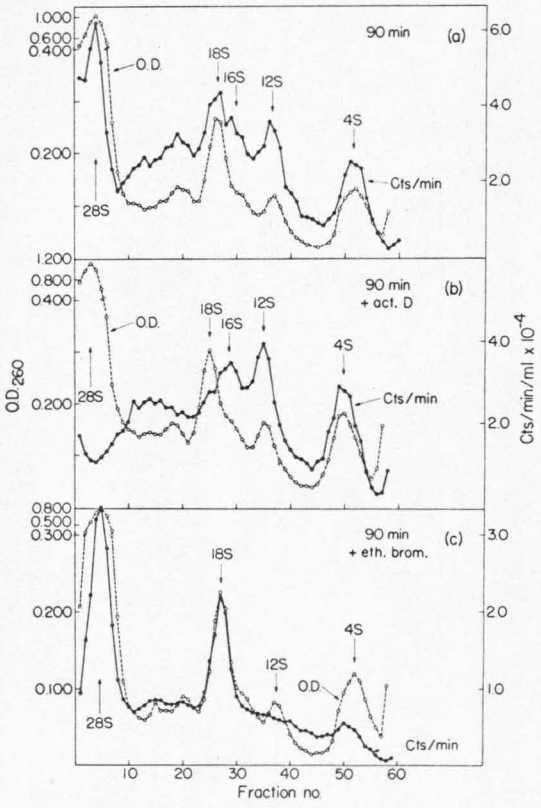

Figure 3. Sedimentation pattern of RNA components sedimenting slower than 28 S RNA from the mitochondrial fraction of HeLa cells exposed to 5-^{3}H-uridine for 90 min in the absence or in the presence of actinomycin D or ethidium bromide.

2.4×10^8 HeLa cells were labeled for 90 min with 5-^{3}H-uridine (22.8 c/mM, 1.4 μc/ml) in the absence (*a*) or in the presence of 0.04 μg/ml actinomycin D (*b*) or 1 μg/ml ethidium bromide (*c*). RNA was extracted from the EDTA-treated and isopycnically separated mitochondrial fraction, and run through a 15 to 30% (w/w) sucrose gradient in SDS buffer (0.5% dodecyl SO_4, 0.1 M NaCl, 0.01 M Tris buffer pH 7.0, 0.001 M EDTA) in the SW 25.3 Spinco rotor at 25,000 rpm for 28 hr at 20°C.

An analysis by polyacrylamide gel electrophoresis of individual components isolated by sucrose gradient centrifugation (Fig. 5) shows that the RNA species sedimenting at 12 S moves through the gel, with respect to the 18 S and 28 S rRNA markers, as would be expected from its

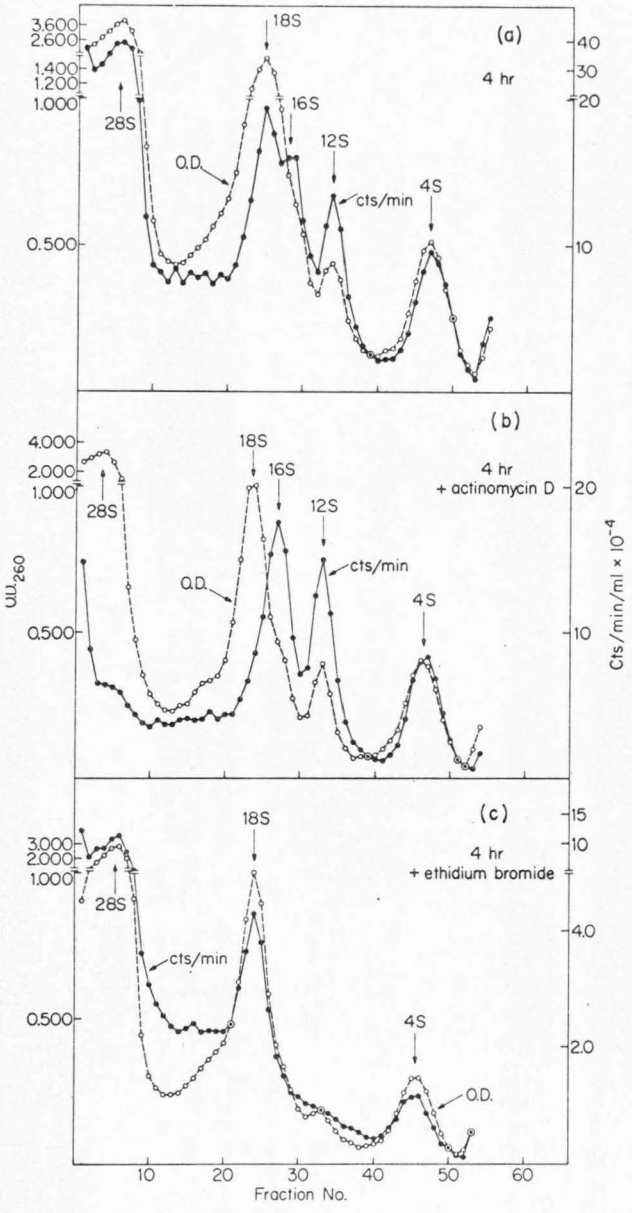

FIGURE 4. Sedimentation pattern of RNA components sedimenting slower than 28 S RNA from the mitochondrial fraction of HeLa cells exposed to 5-^{3}H-uridine for 4 hr in the absence or in the presence of actinomycin D or ethidium bromide.

3.1 × 10^8 HeLa cells were labeled for 4 hr with 5-^{3}H-uridine (31.0 c/mM, 1.4 μc/ml) in the absence (*a*) or in the presence of 0.04 μg/ml actinomycin D (*b*) or 1 μg/ml ethidium bromide (*c*). The mitochondrial fraction was isolated by differential centrifugation, treated with 0.03 M EDTA and, after pelleting, utilized immediately for RNA extraction. The RNA was analyzed in a 15 to 30% sucrose gradient in SDS buffer (SW 27 Spinco rotor with 1.59 × 10.16 cm buckets, 25,000 rpm, 25 hr at 20°C)

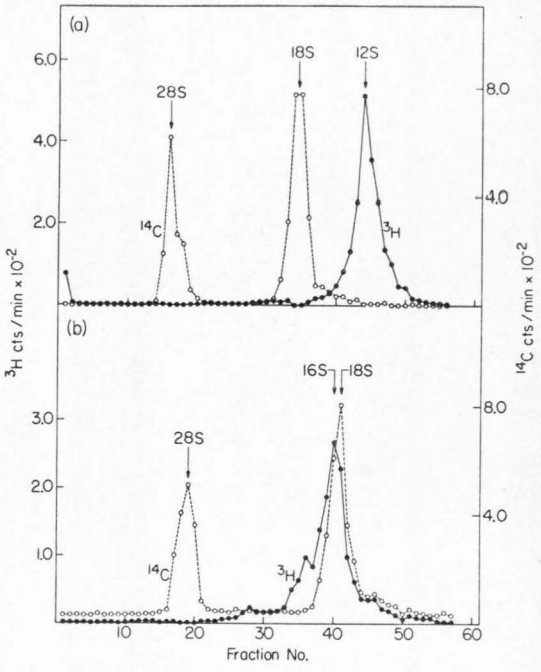

FIGURE 5. Electrophoretic analysis of 16 S and 12 S RNA. The peak fractions of the 16 S and 12 S components in Fig. 4*b* were analyzed by polyacrylamide gel electrophoresis (2.7%) at 5 ma per 8 cm gel for 6 hr (16 S RNA) or for 4.5 hr (12 S RNA), in the presence of ^{14}C-labeled 28 S and 18 S RNA extracted from isolated subunits of free ribosomes.

sedimentation properties in the absence of conformational differences. On the contrary, the 16 S RNA component of the sedimentation pattern moves through the gel slightly behind the 18 S RNA marker, that is, slower than expected from its sedimentation behavior. A discrepancy between sedimentation and electrophoretic properties has also been reported by others for mitochondrial rRNA species of lower eucaryotic cells (Halvorson, Horimoto, Scragg, and Nikhorocheff, 1970; Edelman, Verma, and Littauer, 1970; Stegeman, Cooper, and Avers, 1970), and is presumably due to the less compact conformation of these species as compared to the rRNA markers. On the basis of their electrophoretic mobilities the mol wt of the 16 S and 12 S species were estimated to be 0.7 × 10^6 and 0.4 × 10^6, respectively. Analysis of the sedimentation behavior of these species in formaldehyde gradients after denaturation with formaldehyde has shown that they sediment as sharp peaks (with the 16 S RNA moving behind the 18 S rRNA) without any appreciable amount of trailing material, indicating that they are represented by continuous polynucleotide chains.

RNA components similar to the 16 S and 12 S

RNA described here have been detected in the mitochondrial fraction from hamster cells treated for a long time with actinomycin D (Dubin and Montenecourt, 1970). Likewise, the 16 S and 12 S RNA appear to correspond to the "21 S" and "12 S–13 S" electrophoretic components previously described in the mitochondrial fraction of HeLa cells (Vesco and Penman, 1969) and *Xenopus* cells (Dawid, 1969).

An analysis by polyacrylamide gel electrophoresis of low molecular weight components from the mitochondrial fraction of cells labeled with ^{32}P-orthophosphate and ^{3}H-methyl-methionine has shown a prominent methylated 4 S peak and a small non-methylated 5 S component, which migrated in the gel similarly to the 4 S and 5 S components isolated from free ribosomes (Fig. 6). As shown in Table 2, the 16 S and 12 S components have a base composition which is very similar to that of the heterogeneous RNA. The mitochondria-associated 4 S RNA has a nucleotide composition characterized by relatively high A and U content; it therefore resembles that of the 16 S, 12 S and ^{32}P-pulse-labeled heterogeneous RNA in its high A content, but differs from it significantly in its high U content, and is strikingly different from 4 S RNA associated with cytoplasmic ribosomes which is of high GC content. The 16 S and 12 S components are methylated, though to a lesser extent than the 28 S rRNA. The mitochondria-associated 4 S RNA is also methylated and appears to have less than one-half as many methyl groups as cytoplasmic 4 S RNA; this represents probably an overestimate of the methylation level of mitochondria-associated 4 S RNA since it is likely that this is contaminated by about 20% cytoplasmic 4 S RNA from endoplasmic reticulum bound ribosomes, as estimated from the incomplete ethidium bromide sensitivity of its labeling. The ethidium bromide-sensitive 4 S RNA present in the mitochondrial fraction presumably contains at least some of the mitochondria-specific transfer RNA (tRNA) species which have been described in animal cells, including HeLa cells (Buck and Nass, 1968, 1969; Nass and Buck, 1969; Galpor and Darnell, 1969; Smith and Marcker, 1969).

The labeling of the 16 S, 12 S and ethidium bromide-sensitive 4 S RNA proceeds in time in a proportional fashion and extrapolates back to the same initial time (5 to 8 min); this is in keeping with the idea of a coordinated synthesis and accumulation of these components in the mitochondrial fraction and, furthermore, argues against any precursor to product relationship between them.

The 16 S and 12 S components appear to be unstable, with an estimated half-life of less than 4 hr, if the synthesis of mitochondrial DNA-coded RNA is blocked by ethidium bromide (Fig. 4c). That this instability is in part a consequence of the abnormality induced by the drug treatment is suggested by the observation that the specific activity of the 12 S RNA after 22 hr labeling with ^{32}P-orthophosphate, in the presence of sufficient unlabeled phosphate to allow normal growth, is only slightly (10 to 15%) higher than that of the stable 28 S and 18 S rRNA components. However, it is likely that the 16 S and 12 S RNA

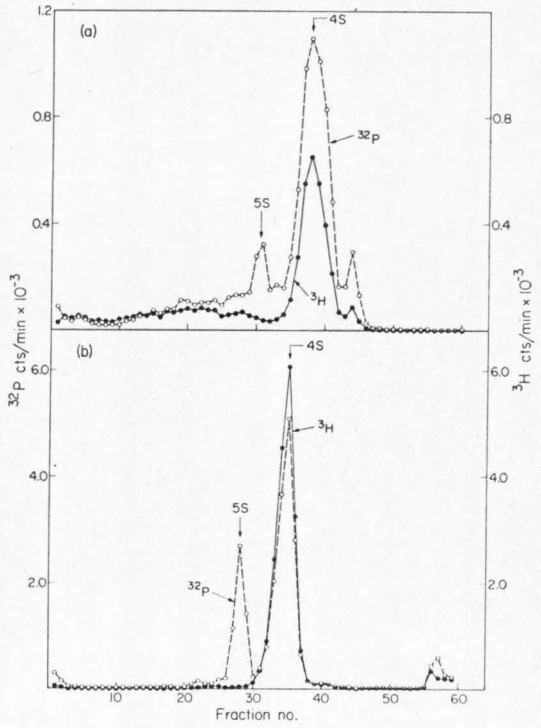

FIGURE 6. Electrophoretic analysis of low mol wt RNA components from the mitochondrial fraction of HeLa cells labeled for 22 hr with ^{3}H-methyl-methionine and ^{32}P-orthophosphate.

(*a*) HeLa cells (which had been grown for 24 hr in the presence of 10^{-4} M adenosine and 10^{-4} M guanosine) were exposed for 22 hr to ^{32}P-orthophosphate (1.75 μc/ml) and L-^{3}H-methyl methionine (5.4 mc/μmole, 2.5 μc/ml) in modified medium containing 10^{-3} M phosphate and 3 μg/ml methionine, in the presence of 10^{-4} M adenosine and 10^{-4} M guanosine. The RNA was extracted from the EDTA-treated and isopycnically separated mitochondrial fraction and analyzed in sucrose gradient as explained in Fig. 3. The components sedimenting in the 1 to 7 S region were collected by ethanol precipitation and centrifugation and analyzed by polyacrylamide gel electrophoresis (10% acrylamide gel at 5 ma) for 3 hr.

(*b*) RNA was extracted from the free monomer-polysome fraction from the same experiment and analyzed in sucrose gradient as in Fig. 3. The components sedimenting in the 1 to 7 S region of the gradient were collected by ethanol precipitation and centrifugation and analyzed by polyacrylamide gel electrophoresis as in (*a*).

have a certain physiological turnover; supporting this is the fact that these components become labeled with 5-^{3}H-uridine significantly faster than the mitochondria-associated 4 S RNA. The latter component has been reported to be metabolically stable (Zylber and Penman, 1969; Knight, 1969), and, in agreement with this, its specific activity after 22 hr labeling with ^{32}P-orthophosphate is essentially equal to that of the stable rRNA components.

Evidence for Complete Transcription of Mitochondrial DNA

In order to obtain information as to the fraction of mitochondrial DNA being transcribed in exponentially growing HeLa cells, RNA-DNA hybridization experiments have been carried out between mitochondrial DNA and mitochondria-associated RNA from cells uniformly labeled with 5-^{3}H-uridine. Since the available evidence indicates that there are no major internal repetitions in mammalian mitochondrial DNA (Borst, 1970), it was expected that measurements of the fraction of mitochondrial DNA hybridizable at saturation with mitochondria-associated RNA would provide an estimate of the in vivo transcription. Three approaches have been followed. The first has been to determine the maximum amount of mitochondria-associated RNA which can be hybridized to a known amount of mitochondrial DNA in hybridization saturation experiments; the second approach has utilized centrifugation in Cs_2SO_4 density gradients to analyze the density of RNA-DNA hybrids formed at saturation; the third approach has involved analysis of RNA-DNA hybrids by electron microscopy. While the results of the first approach depended heavily on the accuracy of determination of the specific activity of the RNA and DNA involved in hybrid formation, the second and third approach were completely independent of these parameters.

Since the great tendency of mitochondrial DNA to renature would have made the quantitation of RNA-DNA hybridization experiments difficult, advantage was taken of the fact that the two complementary strands of human mitochondrial DNA can be separated in alkaline CsCl gradients (Corneo, Zardi, and Polli, 1968) due to their different GT/CA ratio (Vinograd, Morris, Davidson, and Dove, 1963). Figure 7 shows the result of such a separation. The ratio of radioactivity associated with the two bands in this experiment was 1.4. Since in an experiment utilizing unlabeled mitochondrial DNA the ratio of OD_{260} associated with the two bands was close to unity, suggesting that no preferential loss of the light strands occurred during the fractionation, the difference in ^{14}C-thymidine radioactivity associated with the two

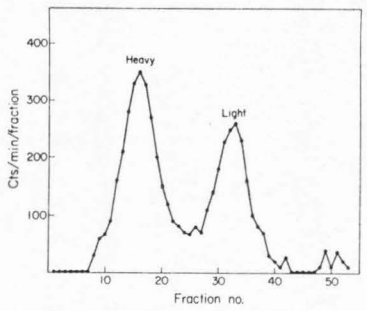

Figure 7. Separation of the complementary strands of ^{14}C-labeled HeLa mitochondrial DNA in alkaline CsCl density gradient. A solution containing 13 μg closed circular mitochondrial DNA in 4.0 ml 0.055 M K_3PO_4 and 0.01% SDS was brought to a refractive index of about 1.405 with solid CsCl and to pH 12.4 with KOH. The mixture was centrifuged in a polyallomer tube in the Spinco 65 angle rotor at 42,000 rpm for 42 hr at 20°C.

strands presumably reflects solely the difference in thymine content. From a value of 1.4 for the ratio of thymine content in the two strands and from the GC content of human mitochondrial DNA (46%; Clayton and Vinograd, 1967) the proportion of A and T in the two strands can be calculated (Table 2).

An essential requirement for experiments aimed at a quantitative analysis of RNA-DNA hybrids was to label both the metabolically unstable and stable RNA species to a similar specific activity. For this purpose, the level of 5-^{3}H-uridine in the medium was adjusted at various intervals over a period of 46 hr, so that even fast turning-over RNA would have a specific activity similar to the average specific activity of stable RNA species. Fractionation in sucrose gradient of the labeled RNA extracted from the EDTA-treated mitochondrial fraction confirmed that the non-rRNA components sedimenting faster than 30 S, which is presumably fast turning over (Attardi et al., 1969), had about the same specific activity as the rRNA species.

As shown in Fig. 8, HeLa mitochondrial RNA of different sedimentation values hybridizes almost exclusively with the heavy strand. This is in agreement with the observation that the base composition of the pulse-labeled mitochondrial RNA is complementary, as concerns the A and U content, to that of the heavy strand of mitochondrial DNA. The low level of hybridization observed with the light strand (about 5% of that obtained with the heavy strand) is presumably due in part to a small amount of contaminating heavy strand; in fact, this level was considerably reduced (to about 2%) by using light strand run twice through an alkaline CsCl density gradient. A more sensitive type of analysis will be required to establish whether a small section of the light strand (estimated to correspond

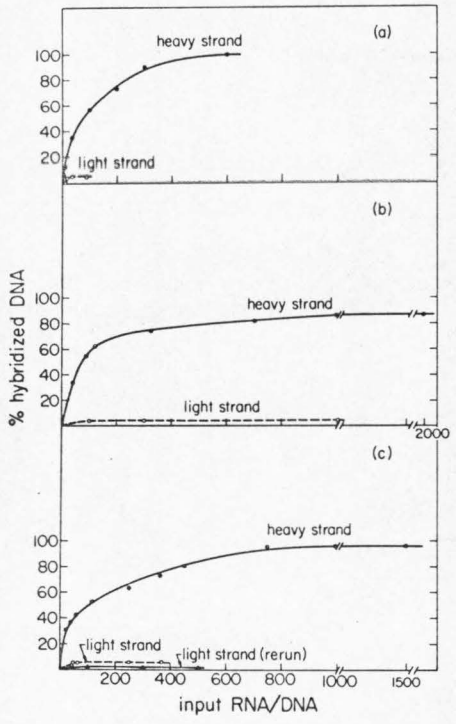

FIGURE 8. Hybridization of separated strands of ^{14}C-labeled HeLa mitochondrial DNA with increasing amounts of RNA from the EDTA-treated and isopycnically separated mitochondrial fraction of HeLa cells labeled for 46 hr with 5-^{3}H-uridine.

HeLa cells (initial concentration 10^5 cells/ml) were labeled for 46 hr with 3.4 μc/ml 5-^{3}H-uridine (22.8 c/mmole) added in various portions in such a way that the average radioactivity incorporated per cell during the total incubation period was about equal to that incorporated during the last 10.5 hr incubation.

The RNA was analyzed in sucrose gradient as in Fig. 1, and the sedimentation profile was divided into three cuts corresponding to S values >40, between 22 and 40, and <22. The material present in each cut was collected by ethanol precipitation and centrifugation, and the >40 S components were rerun twice through sucrose gradient under the same conditions. The material sedimenting between 30 and 70 S after the second rerun, and the 22 to 40 S and <22 S components of the first run were digested with 100 μg/ml electrophoretically purified DNase for 60 min at room temperature, extracted with SDS-phenol, precipitated with 2 vol ethanol, redissolved in 2 ml 2 × SSC, and run through a Sephadex G-100 column equilibrated with 2 × SSC at room temperature.

The fractions of the eluate containing the peak of radioactivity from each column were pooled and utilized for hybridization experiments by the Gillespie and Spiegelman procedure (1965). The final specific activity was 31,000 count/min/μg for the <22 S and 22 to 40 S components, and 28,000 count/min/μg for the 30 to 70 S components. Aliquots of 0.0125 μg (*a*), or 0.025 μg (*b* and *c*) of ^{14}C-thymidine-labeled heavy or light strand of mitochondrial DNA were immobilized on nitrocellulose membranes and incubated with the RNA samples (*a*: 30 to 70 S; *b*: 22 to 40 S; *c*: <22 S) in 2 ml of 4 × SSC containing 0.01 M Tris buffer pH 7.8 for 24 hr at 68°C. After incubation, the filters were washed with 2 × SSC, incubated in 5 ml 2 × SSC containing 20 μg/ml heated pancreatic RNase for 1 hr at room temperature and again washed with 2 × SSC. The data are corrected for non-specific background (less than 10% of the count/min hybridized with the heavy mitochondrial DNA strand), as determined without DNA or with purified SV 40 DNA (component I) (Aloni, Winocour, Sachs, and Torten, 1969).

to a mol wt $\leq$ 100,000) is indeed transcribed. The exclusive transcription of the heavy strand of mitochondrial DNA had been previously reported for rat liver cells (Borst and Aaij, 1969). The present experiments confirm these observations for exponentially growing cells.

It appears from Fig. 8 that the saturation levels for the heavy strand of mitochondrial DNA obtained with the fast sedimenting components (30 to 70 S) and with the slow sedimenting components (<22 S) approached 100%, whereas only about 85% of the heavy strand appeared to be saturated with the RNA components in the intermediate range of sedimentation constants (22 to 40 S).

Evidence supporting the validity of the saturation values given above has been derived from an analysis of the density in Cs_2SO_4 gradients of RNA-DNA hybrids formed at saturation. On the basis of the available data (Sinsheimer and Lawrence, 1964) on complete RNA-DNA hybrids involving ϕX174 DNA (which has a similar base composition [Sinsheimer, 1959] to that of the heavy strand of HeLa mitochondrial DNA, as judged from the A to T ratio and the GC content of the latter, and the base composition of the pulse-labeled heterogeneous RNA), fully hybridized DNA segments were expected to band in Cs_2SO_4 density gradients at a density of about 1.491 g/cm^3, whereas non-hybridized segments were expected to band at a density of about 1.452 g/cm^3, with the partially hybridized segments occupying the intermediate density region.

In the present experiments, all the ^{14}C-labeled heavy strand of mitochondrial DNA which was annealed in liquid medium with saturating amounts of mitochondria-associated RNA components with S values <22 was recovered in the form of a sharp band at a density of 1.491 g/cm^3 in correspondence with a ^{3}H-RNA peak (Fig. 9*a*). The RNA to DNA ratio in the peak was estimated from the specific activities of RNA and DNA to be about 1.0. No mitochondrial DNA was found in the density position of non-hybridized DNA or at intermediate densities. The unhybridized heavy strand was found to band at the expected density of 1.452 g/cm^3 (Fig. 9*b*), like the marker ϕX174 DNA (Fig. 9*f*), while the input RNA banded at about 1.638 g/cm^3 (Fig. 9*c*). The ^{3}H-RNA present in the hybrids formed with the fast sedimenting components (30 to 70 S) banded also, in the majority, at a density of 1.491 g/cm^3 (Fig. 9*d*). The banding at the position expected for 1:1 RNA-DNA hybrids of the

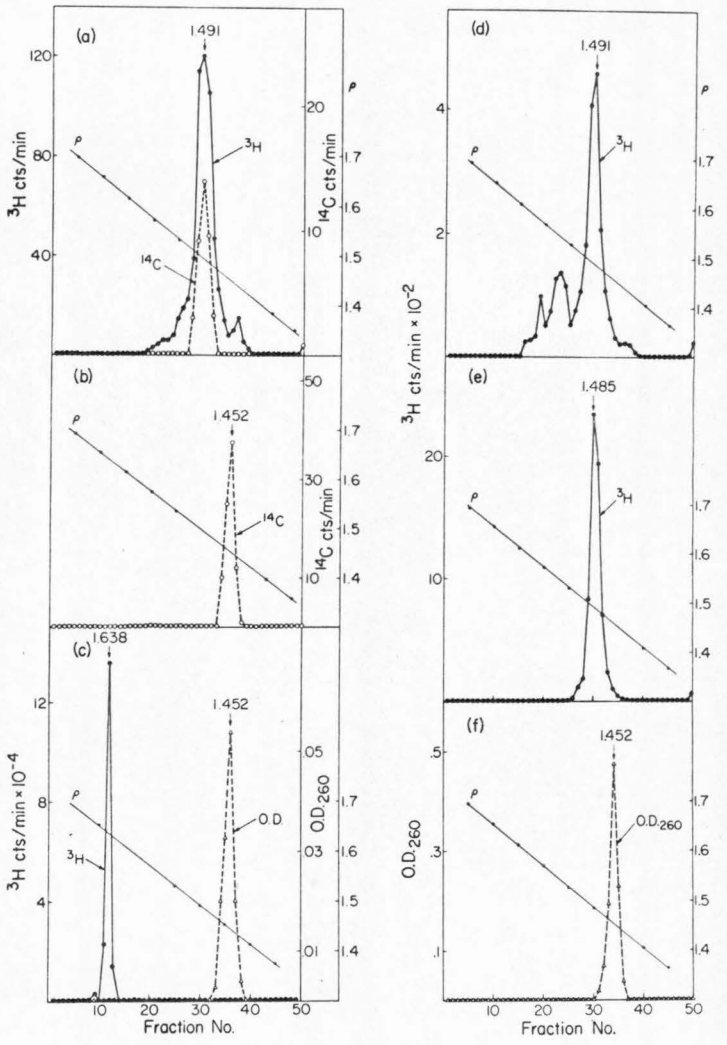

FIGURE 9. Analysis of RNA-DNA hybrids in Cs_2SO_4 density gradient. In the experiment shown in (*a*), 0.20 μg of ^{14}C-labeled heavy strand of mitochondrial DNA was incubated at 70°C for 5 hr in 1.5 ml 4 × SSC containing 0.1 M Tris buffer pH 8.0, with 200 μg of the ^{3}H-RNA components lighter than 22 S (see legend for Fig. 8); in the experiment shown in (*d*) 0.08 μg of unlabeled heavy strand of mitochondrial DNA was incubated under the same conditions with 72 μg of ^{3}H-RNA of the 30 to 70 S sedimentation range; in the experiment shown in (*e*) 0.20 μg of unlabeled heavy strand was incubated with 360 μg of ^{3}H-RNA from the 22 to 40 S sedimentation region. After incubation, the mixtures were diluted with an equal vol of H_2O, brought to pH 7.2, and digested with pancreatic RNase (200 μg/ml) and T_1 RNase (100 units/ml) for 1 hr at room temperature. The RNA-DNA hybrids were then isolated by Sephadex G-100 chromatography (Jeanteur and Attardi, 1969), redigested with 5 μg/ml pancreatic RNase and 5 units/ml T_1 RNase, extracted with SDS-phenol, and after dialysis vs. 2 × SSC, adjusted to 1.530 g/cm^3 with Cs_2SO_4 in a total vol of 5.0 ml, and centrifuged at 32,000 rpm for 5 days at 20°C in a Spinco SW39 rotor. In (*b*) 0.025 μg or ^{14}C-labeled mitochondrial DNA heavy strand, in (*c*) 5 μg of ^{3}H-RNA (22 to 40 S), and in (*f*) 40 μg ϕX174 DNA were run in Cs_2SO_4 density gradients under the same conditions specified above. After collection of fractions from the bottom of the tube and refractive index measurement on selected fractions, the whole fractions (*d*, *e*) or 50 μl aliquots (*a*) were diluted with 1 ml of 6 × SSC and filtered through nitrocellulose membrane filters. In (*b*) and (*c*) the fractions were precipitated with 10% TCA.

heavy strand of mitochondrial DNA annealed with the 30 to 70 S RNA components or with <22 S components confirmed the results of the DNA saturation experiments suggesting full length base sequence complementarity of the heavy strand with mitochondria-associated RNA. The hybrids formed with the 22 to 40 S components banded at a slightly lower density (~1.485 g/cm^3) (Fig. 9*e*), again in agreement with the results of the DNA saturation experiments.

As a third approach to the problem of the fraction of mitochondrial DNA hybridizable at saturation with mitochondrial RNA, hybrids between <22 S RNA components and mitochondrial DNA banded in a Cs_2SO_4 gradient were examined for their content of duplex structure by electron microscopy utilizing the basic protein film technique (Kleinschmidt and Zahn, 1959), staining with uranyl acetate (Wetmur, Davidson, and Scaletti, 1966). Several of the hybrid molecules mounted by the above technique and photographed in dark field are shown in Fig. 10 (center). For comparison, the heavy strand of mitochondrial DNA and double-stranded ϕX RF DNA mounted under the same conditions are presented in Fig. 10 (left and right, respectively); single-stranded DNA appears collapsed in comparison with the uniform extended appearance of duplex DNA.

An examination of a population of 480 molecules isolated from the band at $\rho = 1.491$ g/cm^3 in the Cs_2SO_4 density gradient, and therefore presumed to be RNA-DNA hybrids, revealed that the great majority had a uniform duplex appearance. Only very few regions appearing as bushes, and possibly

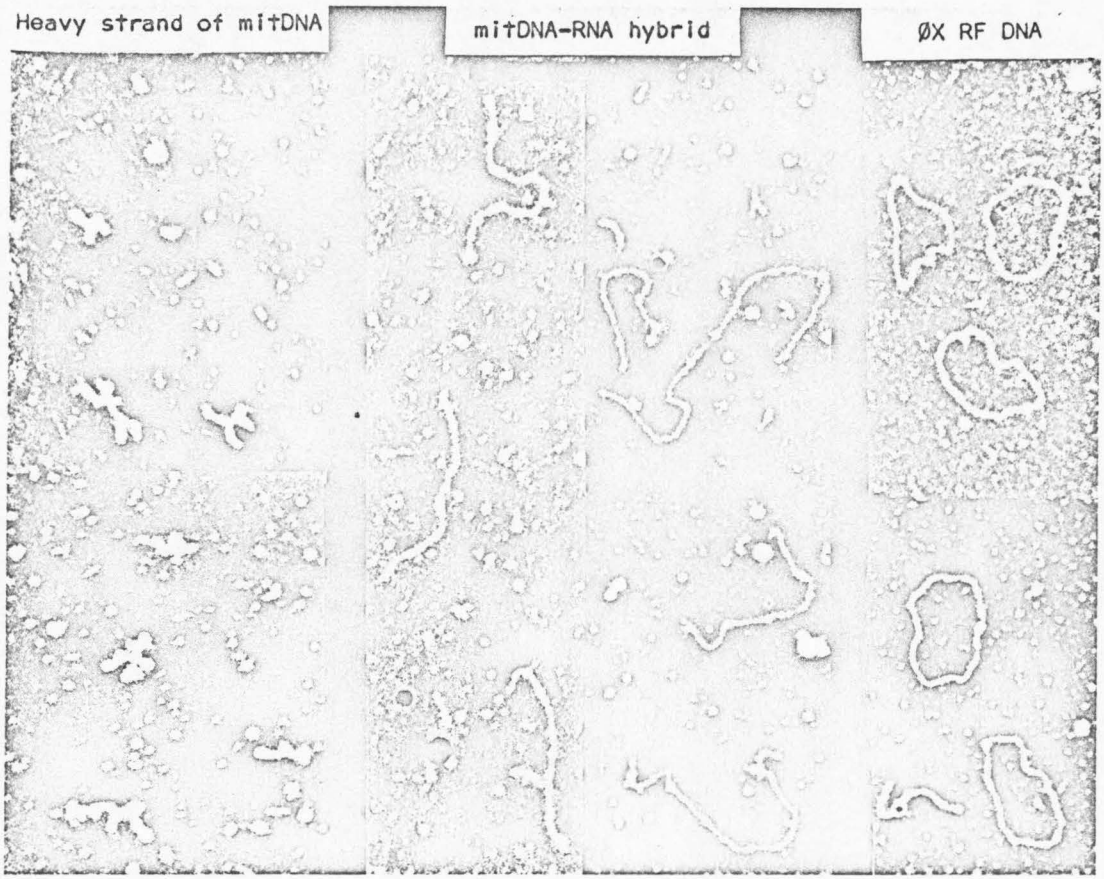

FIGURE 10. Electron micrographs of heavy strand of mitochondrial DNA, mitochondrial RNA-DNA hybrids, and ϕX RF DNA. The basic protein film technique (Kleinschmidt and Zahn, 1959) was used, with a spreading solution of 0.5 M ammonium acetate, 0.1 M Tris, 0.005 M EDTA at pH 8, 0.5 μg/ml nucleic acid, 0.2 mg/ml cytochrome c, and a hypophase of 0.2 M ammonium acetate pH 8. Films were picked up on 3.5% parlodion-coated 200 mesh screens and stained with uranyl acetate (Wetmur, Davidson, and Scaletti, 1966). The grids were examined by dark-field electron microscopy on a Philips EM300 electron microscope utilizing the electronic beam tilt and a 40 μ objective aperture.

corresponding to single-stranded DNA stretches of 500 to 700 bases, as judged by their dimensions, were seen in these hybrid molecules. From other studies it has been estimated that a bush corresponding to a length of 500 to 700 bases would be the smallest stretch that could be recognized (Davis and Davidson, 1968; Davis, Simon, and Davidson, 1970). An examination of 500 hybrid molecules with length greater than 1.0 μ for the presence of single-stranded DNA bushes of 500 to 700 bases showed an average number of 0.63 regions per micron of hybrid which could represent such bushes. On this basis the fraction of DNA in the hybrid that is base-paired to RNA can be conservatively estimated to be about 0.85. This value could be, however, greatly overestimated if a large number of short, unrecognizable (less than 500 nucleotides) non-base-paired regions are interspersed between double-stranded regions in the hybrids. The weight average length of the hybrids (1.02 μ) was found to be much smaller than the weight average length of duplex mitochondrial DNA (4.8 μ): this discrepancy could conceivably be due in part to such short unrecognizable non-base-paired regions resulting in an apparent reduction in the contour length of DNA. However, an analysis by the formamide technique (Davis et al., 1970) of the size distribution of a sample of heavy strand of mitochondrial DNA isolated by alkaline CsCl gradient centrifugation and carried through all the steps followed in the hybridization, including incubation in Cs_2SO_4, revealed a general agreement with the size distribution of the RNA-DNA hybrids (Fig. 11). It is clear, therefore, that the

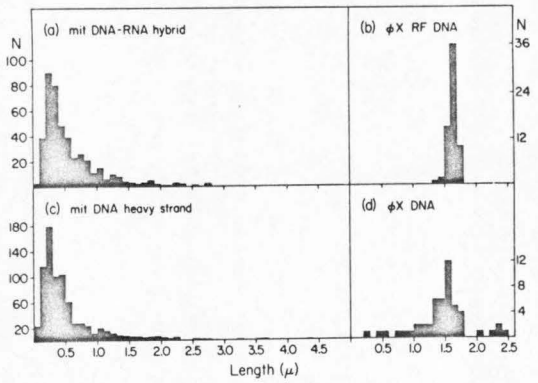

FIGURE 11. Length distributions of mitochondrial RNA-DNA hybrids, heavy strand of mitochondrial DNA, ϕX RF DNA, and ϕX DNA. Mitochondrial RNA-DNA hybrids and ϕX RF DNA were mounted as described in Fig. 10. The heavy strand of mitochondrial DNA was mounted by a formamide technique (Davis, Simon, and Davidson, 1970) with single-stranded ϕX174 DNA incorporated as an internal length standard. The weight average length of this internal ϕX DNA was normalized to the weight average length of the ϕX RF DNA; single-stranded mitochondrial DNA lengths measured relative to the internal ϕX marker are therefore reported as lengths that would exist in duplex DNA.

average size of the hybrids does not result from the presence of a large number of short single-stranded regions interspersed between double-stranded regions but, on the contrary, reflects mainly the small size of the DNA used in hybridizations (due to chemical cleavage or mechanical shearing during the alkaline CsCl banding [Borst, Van Bruggen, Ruttenberg, and Kroon, 1967] and to thermal breakage during hybridization). It can thus be concluded that 0.85 is a reasonable lower limit for the fraction of DNA that is base-paired to RNA in the hybrids isolated by buoyant density centrifugation.

The results of the three experimental approaches described above have clearly indicated that all or almost all the sequences of the heavy strand of mitochondrial DNA in HeLa cells are represented in mitochondria-associated RNA. Since the evidence derived from measurements of DNA renaturation kinetics argues against the presence of major internal repetitions in mammalian mitochondrial DNA (Borst, 1970), the present results can be interpreted to indicate that the mitochondrial genome in HeLa cells is completely or almost completely transcribed. The occurrence of fast sedimenting RNA molecules, ethidium bromide-sensitive and homologous to mitochondrial DNA in the sedimentation profile of pulse-labeled mitochondrial RNA (Attardi et al., 1969), had suggested the possibility that reading of the mitochondrial DNA template takes place in the form of a continuous long RNA chain. The conclusion that mitochondrial DNA is completely or almost completely transcribed and the observation that this transcription occurs almost exclusively from the heavy strand is in agreement with such a possibility. Implied in this idea is that the slower sedimenting discrete RNA species coded by mitochondrial DNA described in the previous section derive from processing of the large precursors. The observed kinetics of labeling of different size molecules in pulse experiments is indeed consistent with a precursor to product relationship between the faster and the slower sedimenting molecules (Attardi et al., 1969). The 33 S peak skewed to the heavy side observed in pulse-labeled preparations may represent one of the first intermediates in the processing of mitochondrial RNA.

RNA-DNA hybridization experiments (Attardi and Attardi, 1969) have shown that the bulk RNA homologous to mitochondrial DNA in HeLa cells is represented by discrete species with sedimentation coefficients of 4 S, 12 S, and 16 S; the heterogeneous RNA components spread in the 4 S to 70 S region of the gradient and the discrete 33 S component represent a relatively minor part of the total mitochondrial DNA-coded RNA. However, these heterogeneous RNA components and the 33 S discrete species are presumably those involved in hybrid formation in the DNA saturation experiments carried out with the RNA heavier than 22 S. Although the 4 S, 12 S, and 16 S RNA components certainly participated in hybrid formation in the experiments carried out with the RNA sedimenting slower than 22 S, the high RNA to DNA input ratio needed to reach DNA saturation with this RNA fraction suggests the contribution to hybrid formation of more rare RNA sequences not represented in the above mentioned discrete species. If the model discussed above of continuous transcription of the heavy strand of mitochondrial DNA is correct, the presence of *all* transcribed sequences of mitochondrial DNA in the <22 S region of the gradient could result either from physiological processing of the large precursors, from artificial degradation during extraction, from the presence of incomplete nascent RNA chains starting on the template from multiple initiation points, or from a combination of these processes. The possibility that saturation of the heavy strand of mitochondrial DNA with the <22 S components is due to the fact that the genes corresponding to the discrete species 16 S, 12 S, and 4 S account for all its length is excluded by the results of RNA-DNA hybridization with these individual components discussed below. The saturation level, appreciably lower than 100%, observed with the RNA components of intermediate sedimentation constant (22 to 40 S in Fig. 3*a*), both in the saturation experiments and in the Cs_2SO_4 density gradient analysis,

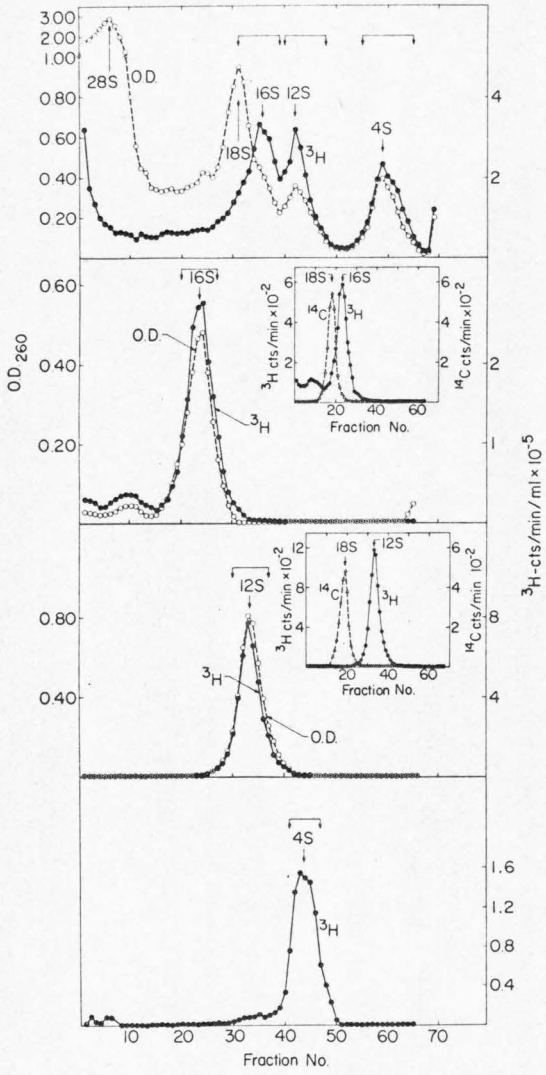

FIGURE 12. Purification of the 16 S, 12 S, and 4 S RNA components from the mitochondrial fraction of HeLa cells labeled for 4 hr with 5-^{3}H-uridine in the presence of actinomycin D.

RNA was extracted, as explained in the legend for Fig. 1, from the EDTA-treated crude mitochondrial fraction isolated by differential centrifugation from the homogenate of 8×10^8 HeLa cells labeled with 5-^{3}H-uridine for 4 hr in the presence of 0.04 μg/ml actinomycin D. The RNA was run through a 15 to 30% (w/w) sucrose gradient in SDS buffer in the SW27 Spinco rotor (1.59 × 10.16 cm buckets) for 29 hr at 25,000 rpm at 20°C. (*a*) The portions of the gradient corresponding to the 16 S and 12 S components (indicated by arrows) were pooled, collected by ethanol precipitation and centrifugation, and run through two cycles (*c*), or three cycles (*b*) of sucrose gradient centrifugation under the same conditions described above. In the inserts the sedimentation profiles of the final preparations run in the presence of ^{14}C-labeled 18 S rRNA marker are shown.

The components sedimenting in the 2 to 7 S region of the gradient in the pattern shown in (*a*) were collected by ethanol precipitation and centrifugation and run through a polyacrylamide gel (10% acrylamide gel at 5 ma) for 3.5 hr.

The portions of the patterns shown in (*b*, *c*, and *d*) indicated by arrows were collected by ethanol precipitation and centrifugation, subjected to DNase digestion—Sephadex chromatography, and utilized for RNA-DNA hybridization experiments (Fig. 13). ○ – – – ○, OD$_{260}$; ●——●, ^{3}H count/min; △ . . . . △, ^{14}C count/min.

may indicate that some sequences are absent in these components: a possible candidate for these missing sequences is 4 S RNA.

TITRATION OF MITOCHONDRIAL GENES FOR 16 S, 12 S, AND 4 S RNA

In order to obtain information concerning the fraction of the heavy strand of mitochondrial DNA which is homologous to each of the three mitochondrial DNA-coded discrete RNA components described above, hybridization saturation experiments were carried out utilizing ^{14}C-labeled heavy strand and ^{3}H-labeled RNA species isolated from cells labeled for 4 hr with 5-^{3}H-uridine in the presence of 0.04 μg/ml actinomycin D. Purified 16 S and 12 S RNA were obtained by successive cycles of sucrose gradient centrifugation, while 4 S RNA was isolated by sucrose gradient centrifugation followed by polyacrylamide gel electrophoresis (Fig. 12). The final specific activity in count/min/μg was 20,000 for 12 S RNA, 18,000 for 16 S RNA, and 9,000 for 4 S RNA. While the small difference in specific activity between 12 S and 16 S RNA may reflect a slight contamination of the 16 S component by 18 S RNA, the lower specific activity of the 4 S RNA is probably the reflection of its metabolic stability as contrasted with a certain instability of the 16 S and 12 S RNA, as discussed above. In the present experiments, it was assumed that the approximate 20% of the mitochondria-associated RNA which pertains to extramitochondrial ribosomes (as mentioned above) has the same specific activity as the intramitochondrial components.

As shown in Fig. 13, the 12 S and 16 S RNA appear to saturate the heavy strand of mitochondrial DNA at levels of about 10% and 15%, respectively. These levels correspond fairly closely to those expected if there were one cistron for each of these RNA species per mitochondrial DNA molecule, assuming a mol wt of 4×10^5 for the 12 S RNA and 7×10^5 for the 16 S RNA, as estimated from their electrophoretic mobilities. The slight upward slope of the final portion of the saturation curves for these two species presumably reflects the presence of heterogeneous RNA contaminants. The saturation level obtained with combined 12 S and 16 S RNA, though not corresponding to that expected if the hybridization with these two species were strictly additive, does indicate that the 16 S

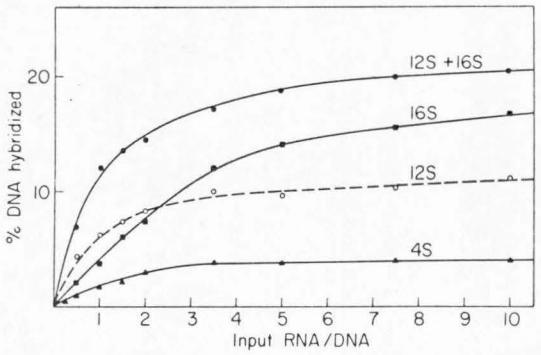

FIGURE 13. Hybridization of the heavy strand of ^{14}C-labeled HeLa mitochondrial DNA with increasing amounts of purified ^{3}H-labeled 16 S, 12 S, and 4 S components. The 16 S, 12 S, and 4 S components, purified as explained in the legend of Fig. 12, were subjected to DNase digestion (25 μg/ml) and Sephadex chromatography under the conditions described in Fig. 8. Aliquots of 0.06 μg (in the experiment with 4 S RNA) or 0.025 μg (in the other experiments) of the heavy strand of ^{14}C-labeled mitochondrial DNA were immobilized on nitrocellulose membranes and incubated with increasing amounts of RNA in 2 ml of 4 × SSC containing 0.01 M Tris buffer pH 7.8 for 24 hr at 68°C. The nitrocellulose membranes were washed and digested with pancreatic ribonuclease as in Fig. 8. The data are corrected for non-specific background (less than 15% of the hybrid values) as determined with SV40 DNA.

RNA cistron contains sequences different from those of 12 S RNA. This result, together with the kinetic evidence of lack of precursor to product relationship between the two species, indicates that the 16 S and 12 S cistrons are distinct. Further experiments are needed to answer the question whether the lack of additivity is the result of some sequences being in common between the two RNA species or, as seems more likely, of the presence in the 12 S preparation of degradation products of 16 S RNA.

Mitochondria-associated 4 S RNA gave with the heavy strand a saturation level of about 4%, corresponding to about eight genes for molecules of 25,000 average mol wt. It remains to be established whether the small stretch of the light strand hybridizable with mitochondrial RNA contains any 4 S RNA genes. It is interesting to notice that the relative rates of labeling of the 12 S and 4 S RNA components suggest that about ten molecules of 4 S RNA are synthesized per each 12 S molecule: this would indeed imply the existence of about ten cistrons for 4 S RNA per each 12 S cistron if the mitochondrial genome is expressed in a coordinate fashion.

ARE THE 16 S AND 12 S RNA CONSTITUENTS OF MITOCHONDRIAL MINIRIBOSOMES?

Mitochondria from *Neurospora*, yeast, and *Aspergillus* have been shown to contain specific ribosomes (Küntzel and Noll, 1967; Stegeman, Cooper, and Avers, 1970; Edelman et al., 1970). These ribosomes have RNA components similar in sedimentation constant to the bacterial RNA species (Küntzel and Noll, 1967; Wintersberger, 1967; Edelman et al., 1970), though possibly the major species (23 S), as judged from its electrophoretic mobility, is larger than its bacterial counterpart and approaches the size of the 25 S cytoplasmic RNA (Edelman et al., 1970). The RNA components of mitochondrial ribosomes in fungi appear to be coded by mitochondrial DNA (Wintersberger and Viehhauser, 1968; Wood and Luck, 1969; Halvorson et al., 1970). In animal cells, the search for specific mitochondrial ribosomes has so far been elusive, though the literature contains both electron microscope (André and Marinozzi, 1965) and biochemical observations (O'Brien and Kalf, 1967a,b; Ashwell and Work, 1970) of the occurrence of particles smaller than bacterial ribosomes, which have been tentatively interpreted as mitochondrial ribosomes. On the other hand, there is good evidence (Roodyn, Reis, and Work, 1961; Truman and Korner, 1962; Kroon, 1964; Wheeldon and Lehninger, 1966) of the existence in animal cells, including HeLa cells, (Attardi et al., 1969) as well as in lower eucaryotic cells (Linnane, 1968), of an intrinsic mitochondrial protein synthesizing system, which can be supported by oxidative phosphorylation processes, and which has a pattern of sensitivity towards various inhibitors of protein synthesis which is different from that of the cytoplasmic protein synthesizing machinery and strikingly resembles that of bacterial systems (Linnane, 1968). Therefore, barring the unlikely event that an unusual mechanism of protein synthesis operates in animal cell mitochondria, we have to assume that these organelles do contain ribosomes.

We have started investigating the possibility that the 16 S and 12 S RNA represent miniribosomal RNA components, equivalent to the specific RNA species of fungal mitochondria. We have obtained evidence that these RNA species are indeed components of ribonucleoprotein particles. In particular, three types of labeled ribonucleoprotein particles have been identified in lysates of mitochondria from cells labeled with 5-^{3}H-uridine in the presence of 0.1 μg/ml actinomycin D (Fig. 14). In the sedimentation pattern shown in Fig. 14, the OD_{260} profile reveals a peak near the bottom corresponding to 74 S ribosomes, and three smaller peaks sedimenting at about 60 S, 45 S, and 35 S (as estimated with reference to the 74 S position). Whereas the 74 S peak is not appreciably labeled, three labeled peaks can be seen in the 60 S, 45 S, and 35 S regions; the 60 S labeled peak appears to be

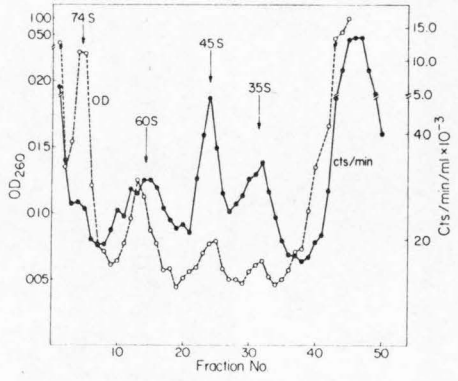

FIGURE 14. Sedimentation pattern of components released by Triton X-100 from the mitochondrial fraction of HeLa cells labeled for 2 hr with 5-^{3}H-uridine in the presence of actinomycin D. 2×10^8 HeLa cells in 700 ml modified Eagle's medium with 5% dialyzed calf serum were labeled for 2 hr with 5-^{3}H-uridine (31 mc/μmole, 1.4 μc/ml) in the presence of 0.1 μg/ml actinomycin D (added 30 min prior to the isotope). A mitochondrial fraction was prepared by differential centrifugation from the cell homogenate, and finally resuspended in 2.0 ml 0.05 M Tris buffer pH 6.7, 0.1 M KCl, 0.01 M $MgCl_2$, and lysed with 2% Triton X-100. The lysate was layered on a 15 to 30% sucrose gradient in the buffer specified above and run for 13 hr at 27,000 rpm in the SW27 Spinco rotor (2.54 × 8.83 cm buckets) at 3°C. ○ - - - - ○, OD_{260}; ●——●, count/min.

somewhat displaced towards the light side with respect to the OD_{260} peak, whereas the 45 S peak is very slightly displaced to the heavier side with respect to the 45 S OD peak (see also insert in Fig. 16). A rerun in sucrose gradient of the central fractions of the ^{3}H-labeled 60 S, 45 S, and 35 S peaks (Fig. 15) shows that the 60 S material gives a main discrete peak sedimenting very slightly slower than 60 S free ribosomal subunits, in addition to some labeled material spread throughout the gradient and a labeled peak in the 6–8 S region; the 45 S material gives a main discrete peak sedimenting slightly ahead of 45 S free ribosomal subunits, and the 35 S material a main discrete peak sedimenting at about 35 S relative to the latter subunits. The RNA extracted from the peak fraction of the 60 S labeled peak shows both a 16 S and a 12 S component, with some slower and faster sedimenting heterogeneous material (Fig. 16*a*). The RNA from the peak fraction of the 45 S peak shows mainly a 16 S component with a small amount of 12 S and slower sedimenting material (Fig. 16*b*), whereas the RNA from the peak fraction of the 35 S peak shows mainly a 12 S component (Fig. 16*c*).

A reasonable interpretation of the results described above is that the discrete components present in mitochondrial lysates, which are labeled with 5-^{3}H-uridine in the presence of actinomycin D and sediment at about 60 S, 45 S, and 35 S, represent mitochondrial-specific ribonucleoprotein particles containing, respectively, 16 S and 12 S RNA, 16 S RNA, and 12 S RNA in addition possibly to other RNA species. The apparently unequal molar ratio of the 12 S and 16 S RNA in the 60 S particles may be due to a certain degradation of the 16 S RNA to molecules sedimenting as 12 S during the long manipulations required for the isolation of these particles. This possibility

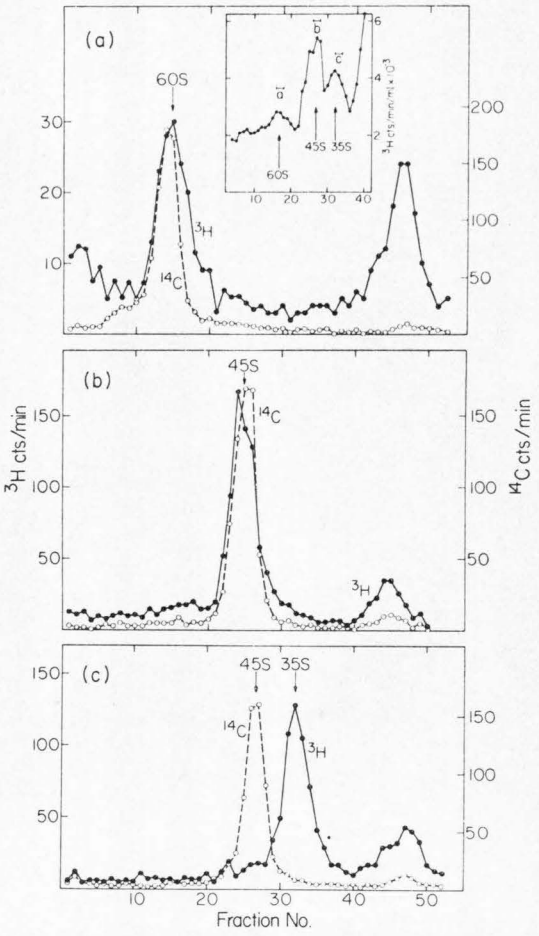

FIGURE 15. Rerun in sucrose gradient of discrete components released by Triton X-100 from the mitochondrial fraction of HeLa cells labeled for 2 hr with 5-^{3}H-uridine in the presence of actinomycin D. The Triton X-100 lysate of the mitochondrial fraction from 2×10^8 HeLa cells labeled with 5-^{3}H-uridine, under the conditions described in Fig. 14, was run on a 15 to 30% sucrose gradient in 0.05 M Tris buffer pH 6.7, 0.1 M KCl, and 0.01 M $MgCl_2$, as detailed in Fig. 14. The portions of the gradient indicated by arrows (insert) were pooled, and 0.7 ml (*a*) or 1.0 ml (*b* and *c*) of the pooled samples were rerun in sucrose gradient under the same conditions, in the presence of ^{14}C-labeled 60 S or 45 S free ribosomal subunits (isolated by centrifuging, under the conditions specified above, 2 ml of the 15,800 × *g* supernatant of the cytoplasmic extract of HeLa cells labeled for 6 hr with 25 μc 2-^{14}C-uridine). ●——●, ^{3}H count/min; ○ - - - - ○, ^{14}C count/min.

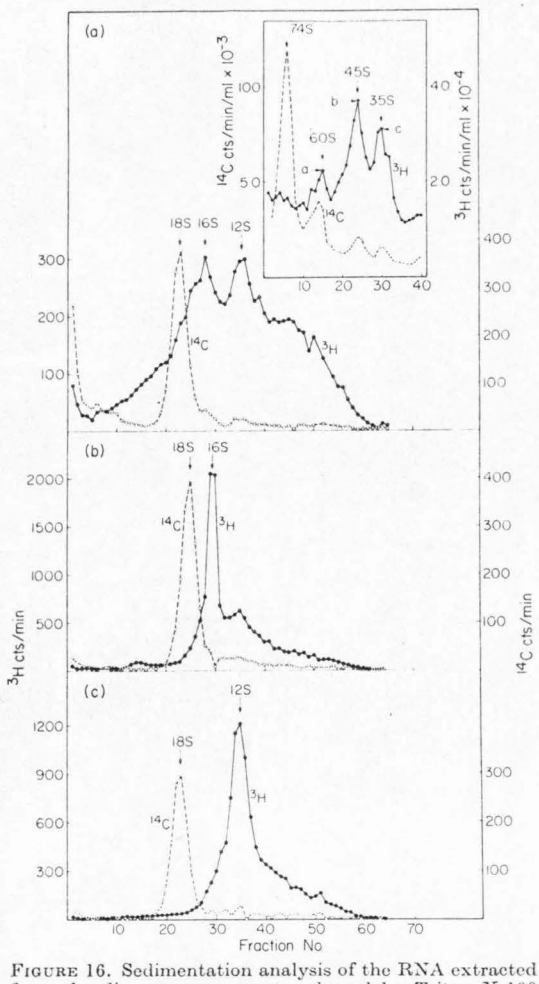

FIGURE 16. Sedimentation analysis of the RNA extracted from the discrete components released by Triton X-100 from the mitochondrial fraction of HeLa cells labeled with 5-^{3}H-uridine in the presence of actinomycin D. 1.3×10^{8} HeLa cells in 700 ml medium were labeled for 19 hr with 10 μc 2-^{14}C-uridine (50 μc/μmole), then centrifuged down, resuspended in 700 ml fresh medium and labeled for 2 hr with 4 mc 5-^{3}H-uridine in the presence of 0.1 μg/ml actinomycin D (added 30 min prior to the isotope). The mitochondrial fraction was isolated by differential centrifugation, lysed with Triton X-100, and run on a sucrose gradient as explained in the legend for Fig. 14. The fractions of the gradient indicated by arrows (insert) were precipitated with 2 vol ethanol after addition of 0.05 M NaCl, 2% SDS, and 10μg carrier unlabeled 18 S RNA. After centrifugation, each pellet was dissolved in SDS buffer and, after addition of a small amount of ^{14}C-labeled 18 S rRNA, run on a 15 to 30% sucrose gradient in SDS buffer in the SW27 rotor (1.59 × 10.16 cm buckets) for 28 hr at 20°C.

seems the more plausible as the 45 S particles, even rerun in sucrose gradient, have always been found to contain a small amount of 12 S material. The appreciable amount of heterogeneous RNA found in the 60 S peak fraction may also in part derive from degradation of the discrete RNA species. Some of it, however, probably pertains to the underlying heterogeneously sedimenting structures seen in the rerun experiments (Fig. 15*a*).

The known tendency of free RNA to form heavier complexes with proteins present in cell extracts (Girard and Baltimore, 1966; Baltimore and Huang, 1970) raised the possibility that the particles isolated here from mitochondrial lysates arise from such a type of artifact. In order to test this possibility, reconstruction experiments were done in which ^{3}H-labeled purified 12 S and 16 S RNA were added to unlabeled mitochondrial suspensions immediately before lysis. Analysis in sucrose gradients of these lysates revealed that the 12 S and 16 S RNA did sediment faster than expected for free RNA, with broad peaks centered around 25 S and 35 S, respectively, and with some labeled material spreading down to the bottom of the gradient (Fig. 17). However, no discrete 35 S or 60 S peaks were seen in these reconstruction experiments with the 12 S RNA, nor any discrete 45 S

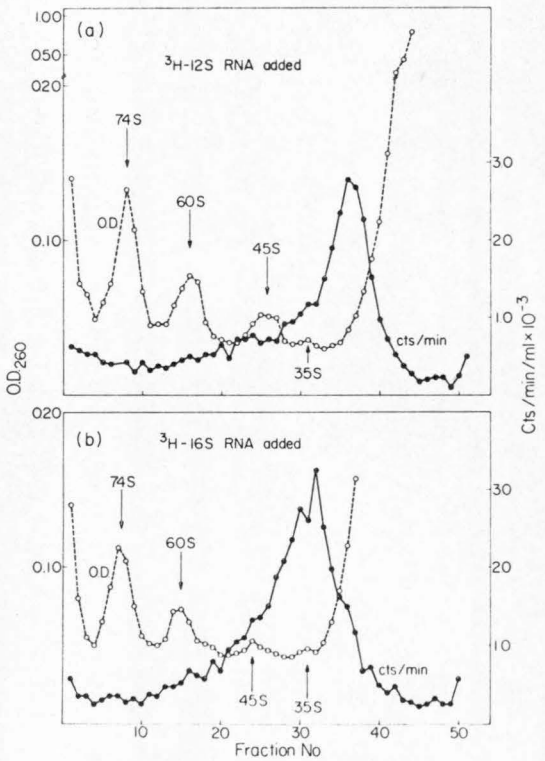

FIGURE 17. Sedimentation patterns of mixtures of purified ^{3}H-labeled 12 S or 16 S RNA and the Triton X-100 lysate of the mitochondrial fraction from unlabeled cells. A sample of ^{3}H-labeled 12 S (*a*), or 16 S RNA (*b*), purified as described in Fig. 12, (about 5000 count/min) was added to the suspension of the mitochondrial fraction from about 2.6×10^{8} unlabeled HeLa cells immediately before lysis with Triton X-100; the lysate was then run in sucrose gradient under the conditions detailed in the legend for Fig. 14. ○ - - - - ○, OD$_{260}$; ●——●, count/min.

or 60 S peaks with 16 S RNA. Therefore, it is reasonable to conclude that the particles observed by us do exist in vivo. Preliminary experiments of banding in CsCl density gradients after glutaraldehyde fixation (Baltimore and Huang, 1968) of the 60 S and 45 S particles, dialyzed vs. 0.2 M NaCl, 0.01 M EDTA, 0.01 M Tris buffer pH 7.4 and rerun in sucrose gradients in the same buffer, have given a density of about 1.41 g/cm^3 for both types of particles. This density would correspond to an RNA content of about 45%.

As concerns the nature of these mitochondria-specific ribonucleoprotein particles, a plausible, though certainly not exclusive, interpretation is that the 45 S and 35 S particles represent the subunits of mitochondrial 60 S ribosomes. The 55 S particles described in rat liver mitochondria (O'Brien and Kalf, 1967a,b; Ashwell and Work, 1970) and reportedly capable of incorporating labeled leucine in a form chasable by puromycin, sensitive to chloramphenicol, and insensitive to cycloheximide may correspond to the 60 S particles described here. We are currently investigating this possibility. If the 16 S and 12 S RNA turn out to be RNA components of mitochondria-specific miniribosomes, this will imply that the reduction in informational content of mitochondrial DNA in animal cells to the extreme limit compatible with its survival has involved a decrease of the rRNA cistrons to an unprecedented small size.

Relationship of Mitochondrial DNA Transcription to the Cell Cycle

Information useful for an understanding of the genetic role of mitochondrial DNA and the control of its expression can be derived from an analysis of the transcription of this DNA in relationship, on one hand, to the cell cycle, and on the other, to the growth and division of mitochondria. An investigation has been carried out on the synthesis of RNA on a mitochondrial DNA template in different stages of the cell cycle. Physiologically synchronized populations of HeLa cells were obtained by a technique based on the selective detachment of mitotic cells from their solid substrate (Terasima and Tolmach, 1963; Robbins and Marcus, 1964). Cell populations at various stages of the cell cycle were exposed to 5-^{3}H-uridine pulses, and the labeling of mitochondria-associated RNA was analyzed. As shown in Fig. 18 (*a*, *b*, *c*), the labeling during a 20 min 5-^{3}H-uridine pulse of the different size components of heterogeneous mitochondria-associated RNA in cells in the G_2 phase is five times higher than that of cells in the G_1 phase and twice as high as that of cells in the S phase. The relatively low level of labeling of mitochondria-associated RNA in G_1 is accounted for in part by the 15 to 20% of unsynchronized cells present in the cell population. A similar behavior is observed for the labeling, during a 60 min 5-^{3}H-uridine pulse (carried out in the presence of 0.04 μg/ml actinomycin D), of the discrete 16 S, 12 S, and 4 S components (Fig. 18 *d*, *e*, and *f*). Differences in the specific activity of the precursor pools in different phases of the cell cycle could conceivably account for these results. However, an analysis of the labeling during a 20 min 5-^{3}H-uridine pulse of the intramitochondrial UTP pool revealed that the specific activity of this precursor was about equal in the S and G_2 phases and only about 30% lower in the G_1 phase. It is clear, therefore, that the observed differences in the labeling of RNA coded by mitochondrial DNA in different phases of the cell cycle cannot be accounted for by pool effects and must, on the contrary, reflect real differences in the rate of synthesis of this RNA. The present results indicate that transcription of mitochondrial DNA is considerably accelerated in the S phase and reaches a maximum in the G_2 phase.

It is interesting to note that an analysis of the replication of mitochondrial DNA in different stages of the cell cycle has likewise revealed that this replication occurs in the S and G_2 phases (Pica-Mattoccia and Attardi, in prep). The time relationship with respect to the cell cycle between mitochondrial DNA replication and transcription may be a reflection of the fact that the two processes are somehow linked. A possibility worth investigating is that the conversion from the superhelical to open circular form which is required for mitochondrial DNA replication opens the way for a subsequent wave of transcription. The very high rate of mitochondrial RNA synthesis (Attardi et al., 1969) demands that each mitochondrial DNA molecule be transcribed many times during each cell cycle. On the basis of a mitochondrial DNA content of about 1000 molecules per HeLa cell as in L cells (Nass, 1969), and an average rate of synthesis of mitochondrial RNA in unsynchronized cell populations corresponding to at least 4×10^6 nucleotides/min/cell (estimate derived from the relatives rates of labeling with 5-^{3}H-uridine of free polysome 28 S RNA and mitochondria-associated RNA [Attardi et al., 1969], and from the finding that the labeling of the extramitochondrial UTP pool is faster than that of the intramitochondrial pool), it can be estimated that each mitochondrial DNA molecule must on the average be transcribed at least 500 times per cell cycle. This high number of transcriptive events may occur in a short time interval for each mitochondrial DNA molecule. If one assumes in fact a time of about 2.3 min for a complete transcription of the heavy strand of

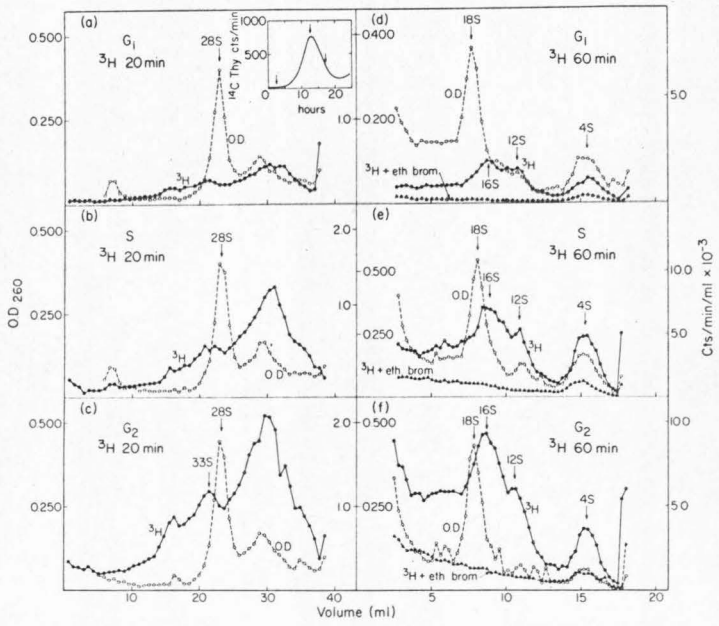

FIGURE 18. Labeling of mitochondria-associated RNA from HeLa cells subjected to 5-^{3}H-uridine pulses at different stages of the cell cycle. Physiologically synchronized HeLa cells were obtained by the Terasima and Tolmach method (1963). Samples ($\sim 6 \times 10^6$ cells in 100 ml medium) at different stages of the cell cycle (G_1 at about 3 hr, S at about 12 hr, and G_2 at about 16.5 hr from the time of resuspension into fresh medium of the mitotic cells [see insert]) were exposed to 5-^{3}H-uridine (28.4 mc/μmole; 20 μc/ml) for 20 min (*a*, *b* and *c*), or for 60 min in the presence of 0.04 μg/ml actinomycin D and in the presence or absence of 1 μg/ml ethidium bromide (*d*, *e* and *f*), and mixed with a constant excess of unlabeled cells (2×10^8 cells). A crude mitochondrial fraction was isolated from the cell homogenate by differential centrifugation and treated with EDTA as described in the legend of Fig. 1, and utilized for RNA extraction with SDS-phenol either directly (*d*, *e* and *f*), or after buoyant density fractionation in sucrose gradient. The RNA samples were centrifuged through 15 to 30% sucrose gradients in SDS buffer in the SW27 Spinco rotor with 2.54×8.83 cm buckets for 11.5 hr at 20,000 rpm at 20°C (*a*, *b* and *c*) or in the SW 27 rotor with 1.59×10.16 cm buckets for 25 hr at 25,000 rpm at 20°C.

In the insert, the rate of ^{14}C-methylthymidine incorporation into total cell DNA in the synchronized HeLa cell population at various stages of the cell cycle is shown. Arrows point to times at which samples were removed from the synchronized population for mitochondrial RNA labeling. ○ – – – ○, OD_{260}; ●——●, ^{3}H count/min; ▲– – – –▲, ^{3}H count/min of ethidium bromide treated samples.

mitochondrial DNA as for the transcription of a 45 S gene (Greenberg and Penman, 1966), and considers that at least 100 RNA polymerase molecules could operate simultaneously on each mitochondrial DNA molecule as on the active rRNA genes of amphibian oocytes (Miller and Beatty, 1969), it can be calculated that 500 cycles of transcription could take place in less than 15 min. Therefore, it is conceivable that during the transcriptive portion of the cell cycle each mitochondrial DNA molecule supports a relatively short burst of RNA synthesis, and that mitochondrial RNA synthesis occurs asynchronously in different mitochondria of the same cell.

SEPARATION OF MITOCHONDRIA ACTIVE IN RNA SYNTHESIS

The evidence discussed above indicating that transcription of mitochondrial DNA in HeLa cells occurs predominantly in the S and G_2 phases implies that in any unsynchronized cell population only a fraction of mitochondria are at any moment active in RNA synthesis. An independent experimental approach has shown that this is indeed the case. A fractionation of the components of a crude mitochondrial fraction in dextran-sucrose gradients has allowed, in fact, a partial separation of a minority of transcriptionally active mitochondria from the bulk of inactive ones. As shown in Fig. 19, the distribution in dextran-sucrose gradients of the structures which are labeled during short 5-^{3}H-uridine pulses in an ethidium bromide-sensitive form is strikingly different from the distribution of the structures having cytochrome oxidase activity. The latter shows a broad band near the center of the gradient; a small amount (5 to 10%) of cytochrome oxidase-positive structures have sedimented to or near the bottom of the gradient (being in part prevented from pelleting by the dense sucrose cushion). The structures containing the pulse-labeled ethidium bromide-sensitive RNA form near the center of the gradient a band which is consistently displaced toward the heavy side with respect to the main band of cytochrome oxidase activity; furthermore, a substantial portion of the pulse-labeled RNA (30 to 40%) is found in correspondence with the fast sedimenting structures.

After a 5 min ^{3}H-uridine pulse the central band of structures containing the ^{3}H-labeled RNA shows a shoulder in correspondence with the main peak of cytochrome oxidase activity. From the ratio of enzyme activity to ^{3}H count/min on the light side of the cytochrome oxidase band one would estimate roughly that at most 15% of the ^{3}H radioactivity in the central band is associated with the bulk of structures having cytochrome oxidase activity. With increasing pulse length there

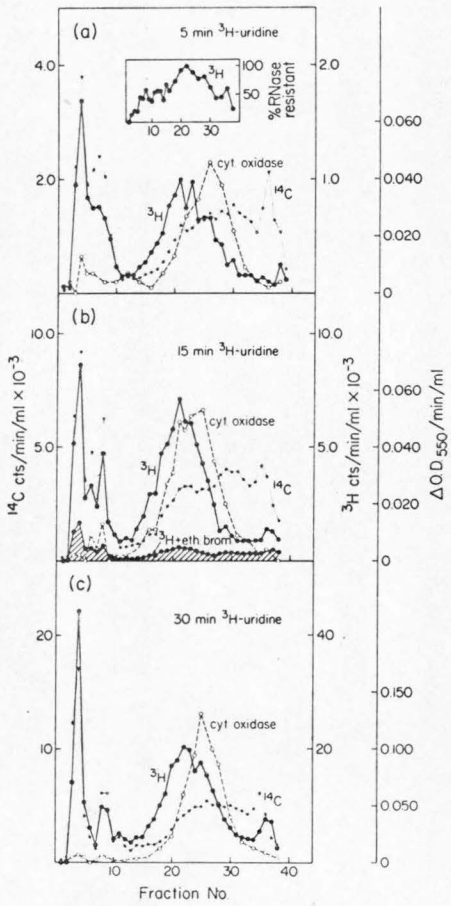

FIGURE 19. Fractionation in dextran-sucrose gradients of the components of the crude mitochondrial fraction from mixtures of HeLa cells subjected to short 5-³H-uridine pulses and cells labeled for 24 hr with 2-¹⁴C-uridine.

A crude mitochondrial fraction was isolated by differential centrifugation, as previously described (Attardi and Attardi, 1968), from the homogenate of a mixture of 2.1×10^7 cells labeled for 24 hr with 2-¹⁴C-uridine [56 mc/mmole; 0.013 (*a*) or 0.044 μc/ml] and 1.9×10^8 cells labeled for a different time with 5-³H-uridine (28.5 mc/μmole; 4.4 μc/ml) in the absence or presence of 1 μg/ml ethidium bromide. Each crude mitochondrial fraction, resuspended in 1.0 ml 0.25 M sucrose, 0.0001 M Tris buffer pH 6.7, was layered onto a 0 to 20% (w/w) linear gradient of dialyzed dextran T40 (Pharmacia, Uppsala) in 15.5% (w/w) sucrose, 0.0001 M Tris buffer pH 6.7 prepared over a 2.5 ml cushion of 1.7 M sucrose, 0.01 M Tris buffer pH 6.7 in a 2.54×8.83 cm tube for the SW27 Spinco rotor. Centrifugation was for 4 hr at 7000 rpm at 3°C. One ml fractions were collected, diluted with 1 vol of 0.020 M Tris buffer pH 6.7 and assayed for total acid-precipitable radioactivity and cytochrome oxidase activity (Attardi et al., 1969). For the latter purpose, 0.4 ml aliquots of the diluted fractions were kept frozen overnight at −20°C, thawed, refrozen at −70°C and assayed the next day. In (*a*) the aliquots for cytochrome oxidase assay from the lowest 30 fractions of the dextran gradient were diluted with 0.25 M sucrose, 0.10 M Tris buffer pH 6.7 to a constant dextran concentration. For the test of RNase sensitivity *in situ*, 0.2 ml aliquots of diluted gradient fractions were further diluted with 1.0 ml 0.1 M NaCl, 0.01 M sodium citrate containing 50 μg/ml pancreatic RNase and incubated for 10 min at 2°C. ●——●, ³H count/min; ○---○ OD_{550}/min/ml; ● . . . ● ¹⁴C count/min.

is a tendency for the ³H band to overlap progressively more with the cytochrome oxidase band, so that after a 30 min pulse approximately 25% of the count/min of the ³H band appears to be associated with the bulk of cytochrome oxidase-positive structures. An analysis of the RNase sensitivity *in situ* of the structures containing the pulse-labeled RNA shows a striking difference between those sedimenting at or near the bottom of the gradient and those of the central band: whereas the latter show 80 to 100% RNase resistance of the ³H label, the fast sedimenting structures show a 20 to 40% resistance, with a gradient of RNase resistance going from the bottom of the gradient toward the peak tubes of the central ³H band. A reasonable interpretation of these results is that the central band of ³H pulse-labeled structures represents the transcriptionally active mitochondria. Differences in size or density of these mitochondria from the majority would account for their displacement in dextran-sucrose gradients from the main distribution of cytochrome oxidase activity. The observation that, after long labeling, the structures corresponding to the main band of cytochrome oxidase activity contain the bulk of the 16 S and 12 S RNA components would tend to exclude the possibility that the present results are due to the majority of mitochondria having somehow lost their pulse-labeled RNA during the isolation and centrifugation of the mitochondrial fraction. The fact that the transcriptionally active mitochondria have a very small amount of cytochrome oxidase activity associated with them may be due to the fact that the organelles involved in RNA synthesis have little or no cytochrome oxidase, or more likely, that only a small fraction of mitochondria at any given time is active in RNA synthesis. Since this fraction appears to be considerably smaller than expected if transcription should occur in all mitochondria throughout the S and G_2 phases, the observations reported above suggest that the transcriptive activity may occur in different mitochondria asynchronously in burst-like form, as discussed in the previous section. It will be interesting to see whether this minor fraction of mitochondria which is transcriptionally active is also active in mitochondrial DNA replication.

As concerns the fast sedimenting structures containing 30 to 40% of the ethidium bromide-sensitive pulse-labeled RNA, they may also represent transcriptionally active mitochondria and they may be responsible for the 5 to 10% cytochrome oxidase activity which is found in their correspondence; however, if these pulse-labeled

structures are mitochondria, one has to assume that they are particularly fragile in order to account for the RNase sensitivity *in situ* of their ^{3}H-labeled RNA. Alternatively, the accessibility of the label to RNase attack may indicate that these fast sedimenting structures are in great part extramitochondrial structures containing newly synthesized RNA exported from the mitochondria. This possibility is currently under investigation. It should be mentioned that previously reported indirect evidence suggested the occurrence of such an export of mitochondrial RNA (Attardi et al., 1969).

In the experiments illustrated in Fig. 19, the cells subjected to short 5-^{3}H-uridine pulses had been mixed with cells labeled for 24 hr with ^{14}C-uridine; this long-term label is known to be mainly associated with ribosomes of rough endoplasmic reticulum contaminating the mitochondrial fraction (Attardi, Cravioto, and Attardi, 1969), and therefore provides a convenient marker to follow the distribution of these structures in dextran-sucrose gradients. It appears from Fig. 19 that the ^{14}C profile is markedly different from that of mitochondria, with about 60% of the ^{14}C label being distributed in the upper half of the gradient and the rest being associated with the fast sedimenting structures. It should be noticed that the ^{14}C radioactivity shows a sensitivity *in situ* to RNase which is rather uniform throughout the gradient (from 55 to 62%), and similar to that previously reported for endoplasmic reticulum bound ribosomes (Attardi et al., 1969).

CONCLUSIONS AND PERSPECTIVES

The evidence discussed in this paper suggests that there is in mitochondrial DNA of HeLa cells one cistron for 16 S RNA, one cistron for 12 S RNA and about eight cistrons for 4 S RNA. The three discrete RNA components coded by mitochondrial DNA so far identified appear thus to account for about 25% of the length of the heavy strand. On the other hand, the RNA-DNA hybridization data presented above indicate clearly that the entire or almost entire heavy strand is transcribed in HeLa cells. Therefore, about three-quarters of the information contained in this strand and expressed in HeLa cells remains as yet unidentified in terms of primary gene products. If the 16 S and 12 S RNA are constituents of mitochondrial ribosomes and 4 S RNA represents mitochondria-specific tRNA, i.e., if these RNA species are components of the mitochondrial protein synthesizing apparatus, it is legitimate to assume that in the as yet unexplored three-quarters of the heavy strand there must be information for specific messengers to be translated by the mitochondrial machinery. Whether any part of the information encoded in mitochondrial DNA is destined to be utilized outside mitochondria, possibly for the synthesis of extramitochondrial constituents, as suggested by indirect evidence, is an important question which needs to be answered.

As concerns the nature of the heterogeneous RNA components which are fast-labeled in pulse experiments, they probably include nascent mitochondrial RNA chains. If the heavy strand is transcribed in the form of a gigantic RNA molecule destined to be processed to smaller size functional molecules, then intermediates or waste products of this processing could contribute to the heterogeneous RNA. The latter would also include mitochondrial DNA coded messenger RNA species destined to be utilized inside the mitochondria or exported. The understanding of the significance of the heterogeneous fast-labeled RNA and of its relationship to the discrete RNA species will have to wait for the elucidation of the mode of transcription of mitochondrial DNA and of the processing of mitochondrial RNA.

If transcription of mitochondrial DNA occurs in the form of a continuous, long RNA chain, regulation at this level of the expression of the mitochondrial genome would have to be an all-or-none phenomenon. Possibilities of differential regulation of mitochondrial genes would thus have to be restricted to the level of translation of specific messengers or to the level of stability of the primary gene products. That the last mentioned mechanism of regulation does indeed operate is suggested by the different metabolic stabilities of the 16 S and 12 S RNA, on one side, and 4 S RNA, on the other. The evidence indicating that only a minor fraction of mitochondria are active in RNA synthesis at any given moment in each cell points to the existence of intramitochondrial signals, possibly related to the DNA replication cycle, which control the wave of transcription in each mitochondrial DNA molecule. On the other hand, the fact that the transcriptive activity occurs predominantly in the S and G_2 phases indicates that mitochondrial DNA transcription is also under the control of extramitochondrial factors.

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B. Determination of the Fraction of the Heavy Strand of HeLa Mitochondrial DNA that is Transcribed

APPENDIX

Electron Microscopic Visualization of Mitochondrial RNA–DNA Hybrids

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Hybrids between mitochondrial RNA (<22 s components) and mit-DNA banded in a Cs_2SO_4 gradient, as described in Results section (c)(ii), were examined by electron microscopy for their content of duplex structure. The basic protein film technique (Kleinschmidt & Zahn, 1959) was used, with a spreading solution of 0·5 M-ammonium acetate, 0·1 M-Tris, 0·005 M-EDTA at pH 8, 0·5 μg nucleic acid/ml., 0·2 mg cytochrome *c*/ml., and a hypophase of 0·2 M-ammonium acetate, pH 8. Films were picked up on 3·5% Parlodion-coated 200-mesh screens and stained with uranyl acetate (Wetmur, Davidson & Scaletti, 1966). Staining was used in order to allow detection of small regions of non-hybridized single-stranded DNA. The grids were examined by dark-field electron microscopy on a Philips EM300 electron microscope utilizing the electronic beam tilt and a 40 μ objective aperture. The dark-field technique enhanced the contrast of the stained structures.

Several of the hybrid molecules mounted by the above technique and photographed in dark-field are shown in Plate AI(b). For comparison, the heavy strand of mit-DNA and double-stranded ϕX RF DNA mounted under the same conditions are presented in Plate AI(a) and (c), respectively. Single-stranded DNA appears collapsed in

TABLE A1

Length measurements of mitochondrial RNA–DNA hybrids, mit-DNA heavy strand, φX RF DNA and φX DNA

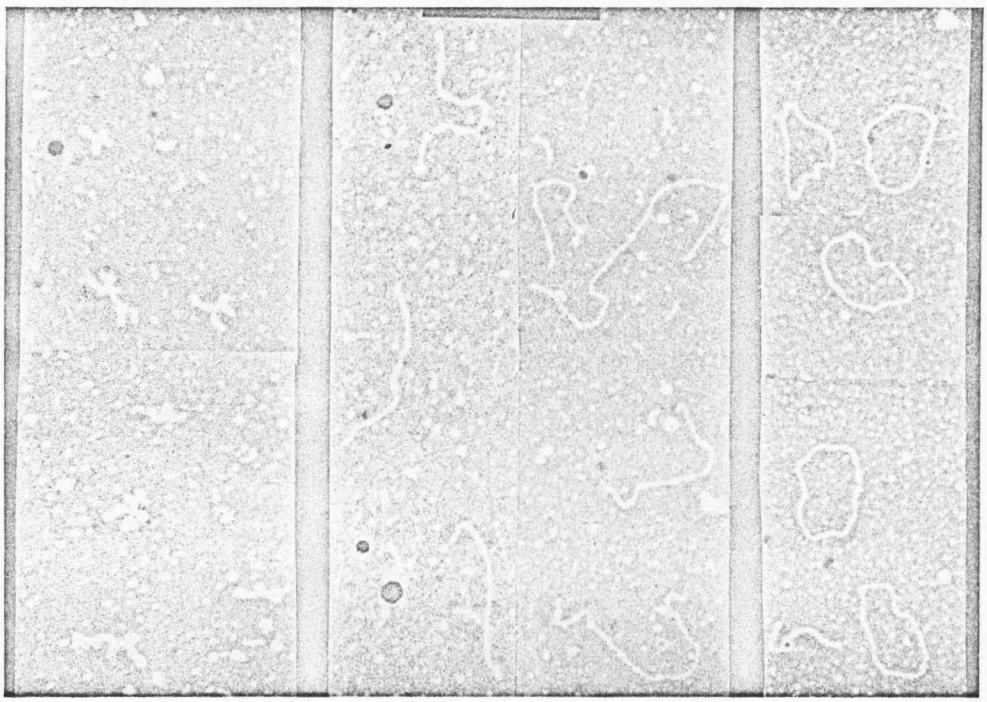
	Number of molecules observed	Number of molecules measured	$<L>_n$ (μ)	$<L>_w$ (μ)	T_n (μ)	T_w (μ)
Mit-RNA–DNA hybrid	480	451	0·62	1·02	0·50	0·70
φX RF DNA	62	62	1·67	1·70	0·21	0·29
Mit-DNA heavy strand	864	851	0·51	1·10	0·55	1·01
φX DNA	51	51	1·35	1·54	0·52	0·44

$<L>_n$ = number average length = $\Sigma_i N_i L_i / \Sigma_i N_i$, where N_i is the number of molecules of length L_i (Tanford, 1963).
$<L>_w$ = weight average length = $\Sigma_i N_i L_i^2 / \Sigma_i N_i L_i$.
T_n = standard deviation based on $<L>_n$.
T_w = standard deviation based on $<L>_w$.

comparison with the uniform extended appearance of duplex DNA. An examination of a population of 480 molecules isolated from the band at $\rho = 1{\cdot}491$ g/ml. in the Cs_2SO_4 density-gradient, and therefore presumed to be RNA–DNA hybrids, revealed that the great majority had a uniform duplex appearance. Only very few regions appearing as bushes, and possibly corresponding to single-stranded DNA stretches of 500 to 700 bases, as judged by their dimensions, were seen in these hybrid molecules (see below). From other studies it has been estimated that a bush corresponding to a length of 500 to 700 bases would be the smallest stretch that could be recognized (Davis & Davidson, 1968: Davis, Simon & Davidson, 1970). In the population investigated here, 451 of the 480 molecules were measurable. This proportion of measurable molecules is typical for the spreading procedure for double-stranded DNA utilized in this work. Photographs of the molecules taken on 35-mm film were traced at a final magnification of 100,000. The magnification was determined with a germanium replica of a 54,864 line per inch diffraction grating. The observed length distribution of RNA–DNA hybrids is shown in Figure A1(a). The length distribution of duplex φX RF mounted under identical conditions is presented in Figure A1(b). The averages and standard deviations for the distributions are presented in Table A1. In the interpretations below, we assume the same length per base pair for an RNA–DNA hybrid as for a DNA duplex.

In order to estimate the fraction of DNA that is base-paired to RNA in the hybrid, hybrids with lengths greater than 1·0 μ were carefully examined for the presence of single-stranded DNA bushes of 500 to 700 bases. A measurement of 500 hybrid molecules with a weight average length of 1·4 μ yielded per micron of hybrid an average of 1·29 regions which could represent bushes of 500 to 700 bases, as judged from their dimensions. A measurement of 50 φX RF molecules yielded an average of 0·66 such a region per micron of duplex DNA length (that is, per 3000 base pairs). These regions on φX RF DNA probably represent large aggregates of cytochrome c or precipitates of uranyl oxide. Assuming a similar proportion of spurious bushes in the RNA–DNA hybrids, the difference, that is an average number of 0·63 region per micron of hybrid may indeed represent single-strand DNA bushes of 500 to 700 bases.

Heavy strand of mit-DNA mit-DNA–RNA hybrids ϕX RF DNA



(a) (b) (c)

PLATE A1.

[facing p. 268

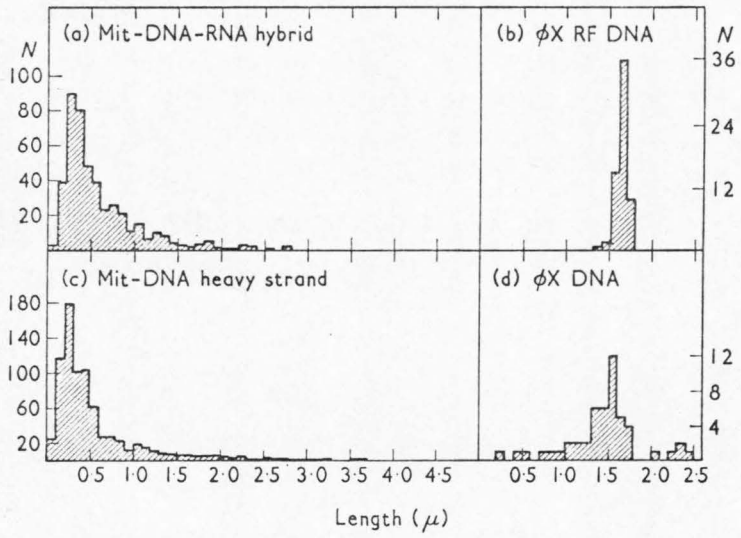


FIG. A1. Length distributions of mit-RNA–DNA hybrids, mit-DNA heavy strand, φX RF DNA and φX DNA.

On this basis, the fraction of DNA in the hybrid that is base-paired to RNA can be conservatively estimated to be about 0·85.

The value of 0·85 could be greatly overestimated if a large number of short, unrecognizable (less than 500 nucleotides) non-base-paired regions are interspersed between double-stranded regions in the hybrids. Such regions could arise from the presence in DNA of short sequences not represented in the RNA population, or by depurination of the DNA sample resulting in localized regions of mismatching, or by partial degradation of the hybrid by RNase. The much smaller average size of the hybrids ($<L>_w = 1{\cdot}02\ \mu$) in comparison with duplex mit-DNA ($<L>_w = 4{\cdot}8\ \mu$, calculated from the data in Radloff, Bauer & Vinograd, 1967) (Table A1) could conceivably be due in part to such bushes resulting in an apparent reduction in the contour length of DNA. The small size might also arise from shear breakage of the hybrids. As a third and rather probable possibility, the small size of hybrids may be principally a reflection of the average size of the DNA used in the hybridizations.

In order to estimate a limit for the amount of non-base-paired DNA present as short segments in these hybrids, the size distribution of a sample of heavy strand of mit-DNA isolated by alkaline CsCl gradient centrifugation and carried through all the steps followed in the hybridization, including incubation in Cs_2SO_4 (as detailed in Materials and Methods section (f)(ii)), were analyzed. For this purpose, the DNA was mounted by a formamide technique (Davis *et al.*, 1970), in which both double-stranded and single-stranded DNA appear as extended filaments. Single-stranded φX174 DNA was incorporated as an internal length standard. The length distribution of the circular φX DNA observed in the mit-DNA sample is plotted in Figure A1(d). The weight average length of this internal φX DNA has been normalized to the weight average length of the φX RF DNA. Single-stranded mit-DNA lengths measured relative to the internal marker of φX are therefore reported as lengths that would exist in duplex DNA. The distribution thus obtained is presented in Figure A1(c). A larger number of molecules has been measured here to compensate for the larger standard deviation

in the distribution of lengths measured by this method in comparison with the duplex DNA method. It is clear that preparation of the DNA has resulted in a considerable degradation. This degradation could have occurred as a result of chemical cleavage or mechanical shearing during the alkaline CsCl banding (Borst, Van Bruggen, Ruttenberg & Kroon, 1967), and of heating the DNA sample during hybridization. Chemical cleavage of the chain at depurination sites could also have occurred. The distributions of single-stranded DNA and of RNA–DNA hybrids, although they appear very similar in form, are not identical. By a χ^2 test there is less than one chance in 100 that the two distributions are identical. The major difference in the two distributions lies in the relative numbers of molecules in the two distributions with lengths less than 0·5 μ. This is evidenced by the larger number average length for the hybrid molecules (0·62 μ) in comparison with the number average length of heavy strands (0·51 μ). This may be due to the hybridization of two short DNA strands to one longer RNA.

Nevertheless, the general agreement of the two distributions (within about 15% relative error) indicates that the distribution of DNA chain lengths is mainly responsible for the distribution of hybrid size, and that the hybrids do not contain a large number of short, unrecognizable ($<$500 nucleotides) single-stranded regions interspersed between double-stranded regions. Therefore, the estimate of 0·85 is a reasonable lower limit for the fraction of DNA that is base-paired to RNA in the hybrids isolated by buoyant density centrifugation.

We wish to thank Professor Norman Davidson for his valuable advice and help in the course of this work.

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C. Size Determination by Electron Microscopy of HeLa Mitochondrial Ribosomal RNAs

This section is the preprint of

Expression of the Mitochondrial Genome in HeLa Cells
VI. Size Determination of Mitochondrial Ribosomal RNA by Electron Microscopy

by Donald Robberson, Yosef Aloni, Giuseppe Attardi and Norman Davidson

This paper has been accepted for publication by the Journal of Molecular Biology.

EXPRESSION OF THE MITOCHONDRIAL GENOME IN HeLa CELLS VI. *SIZE DETERMINATION OF MITOCHONDRIAL RIBOSOMAL RNA BY ELECTRON MICROSCOPY

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*Paper V in this series is Attardi and Ojala (1971)

Abstract

A modified basic protein film method of spreading RNA in a strongly denaturing solvent for examination in the electron microscope has been developed and applied to determine the size of the HeLa mitochondrial specific rRNA components.† Length measurements on purified 12 S and 16 S mitochondrial rRNA, on mixtures of the two, and on mixtures of 12 S with 18 S cytoplasmic rRNA have given molecular lengths of 0.27 μ, 0.42 μ, and 0.55 μ for the 12 S, 16 S, and 18 S rRNAs. If these molecular lengths are proportional to molecular weight, and if the molecular weight of 18 S cytoplasmic rRNA is taken as 0.71×10^6, as determined by sedimentation equilibrium, the molecular weights of the 12 S and 16 S components are 0.35×10^6 and 0.54×10^6, respectively. These molecular weight values are in good agreement with the relative values predicted from sedimentation velocity measurements, but not with the relative values based on gel electrophoresis.

1. Introduction

Recent work from several laboratories (Vesco & Penman, 1969 a, b; Dawid, 1969; Attardi et al., 1969; Dubin & Montenecourt, 1970) has shown the occurrence in mitochondria of animal cells of two high mol. wt. RNA species, which are the structural components of mitochondria-specific 60 S ribosomes (Swanson & Dawid, 1970; Attardi & Ojala, 1971). The size of the two RNA species is at present uncertain. Thus, in polyacrylamide gel electrophoresis runs, the larger RNA species from HeLa cells has been found to have a mobility which would correspond, in the absence of conformational influences, to a sedimentation constant (S_E value‡) of 18 to 21 S (Vesco & Penman, 1969a; Attardi et al., 1970), while the smaller RNA species has shown an electrophoretic behavior corresponding to a 14-15 S component (Vesco & Penman, 1969a; Attardi et al., 1970). On the other hand, sedimentation analysis in sucrose gradients in 0.1 M salt has given sedimentation constants of about 16 S and 12 S for the two species as estimated relative to 18 S rRNA (Attardi et al., 1969). Also after heat-formaldehyde denaturation, the larger mitochondrial rRNA component sediments more slowly than does 18 S rRNA in a sucrose gradient in the presence of formaldehyde (Attardi & Attardi, 1971). A similar discrepancy between sedimentation and electrophoretic properties of mitochondrial RNA species has been reported for other systems

(Dawid, 1969; Dubin & Montenecourt, 1970; Edelman, Verma & Littauer, 1970). In order to obtain information by a different approach on the size of the two mitochondrial rRNA species from HeLa cells and to try to resolve the discrepancy between their sedimentation and electrophoretic behavior, length measurements of molecules of the two species have been carried out by electron microscopy. The RNA molecules were mounted by a modified basic protein film method under strongly denaturing conditions in the belief that any secondary or tertiary structure effects that might influence the sedimentation or electrophoresis results would then not influence the molecular lengths in the electron microscopy.

2. Materials and Methods

(a) Isolation and purification of mitochondrial RNA

The two HeLa mitochondrial RNA species, 16 S and 12 S, were purified as previously described (Aloni & Attardi, 1971), from cells labeled with [^{3}H-5] uridine in the presence of 0.04 γ/ml actinomycin D (to suppress the labeling of cytoplasmic rRNA) by three sequential centrifugations through 15% to 30% (w/w) sucrose gradients in sodium dodecyl SO_4 buffer: 0.5% dodecyl SO_4, 0.1 M NaCl, 0.01 M Tris buffer, pH 7.0, 0.001 M EDTA). In order to eliminate DNA contaminants, one preparation of 16 S and 12 S RNA was further purified by polyacrylamide gel electrophoresis, as detailed in the legend of Figure 4.

The preparations were checked for the presence of single-strand breaks by denaturation with formaldehyde and sedimentation in sucrose gradients in the presence of formaldehyde (Attardi & Attardi, 1971). The sedimentation profiles presented in Figure 1 demonstrate the size homogeneity of the RNA components used in this study. The 18 S cytoplasmic rRNA, isolated from purified subunits as previously described (Amaldi & Attardi, 1968), was also shown to be homogeneous in size by sedimentation in sucrose gradient in the presence of formaldehyde (Fig. 1).

Samples of purified RNA were precipitated with ethanol and dissolved in 1×10^{-3} M Tris buffer, 2.5×10^{-4} M disodium EDTA, pH 8.0 (25°C) at a final concentration of 40-50 γ/ml.

(b) Purification of formamide

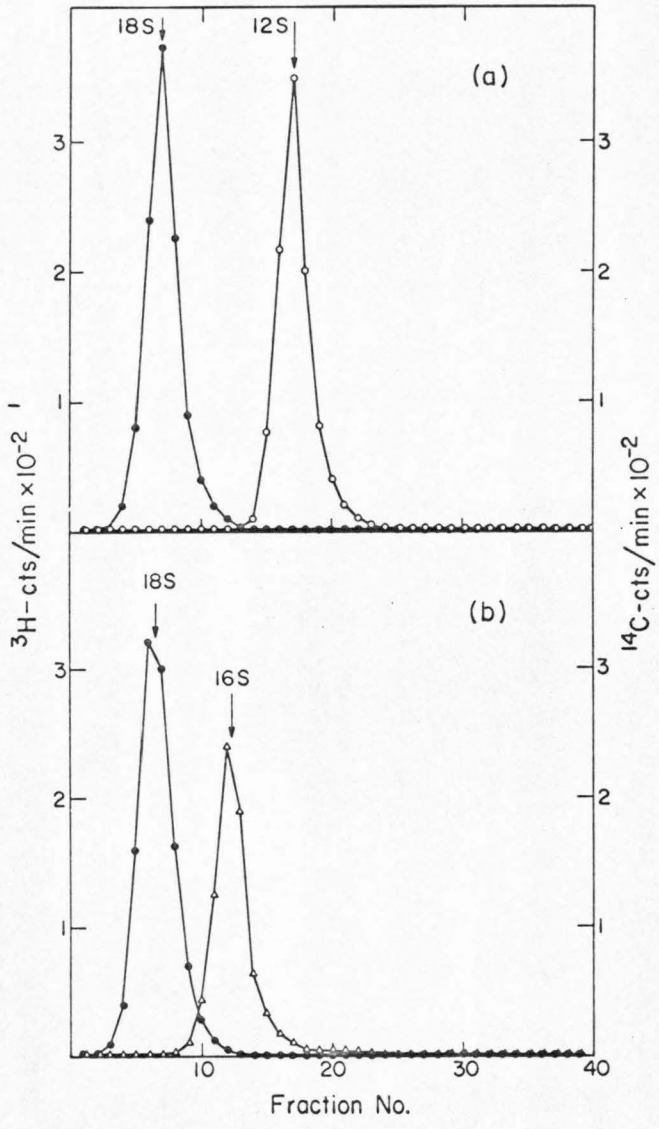
Commercially available 99% formamide (Matheson, Coleman & Bell) was purified from ionic and u.v. absorbing impurities by crystallization at 0-2°C. Approximately 350 ml. quantities in covered 400 ml. beakers were immersed in an ice bath. The liquid was stirred with a magnetic stirrer in a 4°C constant temperature room. When 40-50% of the formamide had crystallized, the bulk of the supernatant was decanted, and crystals plus remaining supernatant placed in the top portion of a sintered glass funnel especially adapted to fit the 250 ml. capacity buckets of an International centrifuge. Crystals were

Fig. 1. Sedimentation analysis in sucrose gradients in the presence of formaldehyde of RNA samples used for electron microscopy.

Samples of the purified 12 S and 16 S RNA components, mixed with ^{14}C-labeled 18 S marker were heated in 0.2 ml. of 18% neutralized formaldehyde, 0.001 M EDTA at 70°C for 5 min. and quickly cooled to 0°C; the samples were diluted 2.5 times with 0.001 M EDTA and run through a 5 to 20% (w/w) sucrose gradient in 0.02 M phosphate buffer (pH 7.4), 0.1 M NaCl 1% formaldehyde in the SW 41 rotor at 40,000 rev./min for 18 hr at 4°C.

(a) 12 S mitochondrial RNA plus 18 S cytoplasmic RNA;

(b) 16 S mitochondrial RNA plus 18 S cytoplasmic RNA.


18S
12S
(a)
18S
16S
(b)
^{3}H-cts/min x10^{-2}
^{14}C-cts/min x10^{-2}
Fraction No.

freed of the remaining liquid by centrifuging 3 min at 1500 rev./min, and subsequently stored at -70° C.

(c) Preparation of RNA samples for electron microscopy

A sample to be spread for electron microscopy was thawed in ice and 0.5 µl was diluted into 100 µl of a 4 M solution of solid urea (Mann ultra pure) in purified formamide. The diluted RNA sample was heated at 53°C for 30 sec. After cooling to 25°C, ~2 µl of a solution containing 2.5 mg/ml. of cytochrome C (Sigma, 2X crystallized) and 2 M Tris, 0.05 M disodium EDTA was added. The latter solution was prepared by mixing equal volumes of a solution containing 5 mg/ml. cytochrome C and of 4 M Tris OH, 0.1 M disodium EDTA, adjusted to pH 8 (25°C) by the addition of concentrated HCl. Thus, the concentrations in the final solution were: RNA, 0.20-0.25 γ/ml.; cytochrome C, ~50 γ/ml.; urea, 4 M; Tris buffer, ~0.04 M; EDTA, 0.001 M; and formamide, ~80 percent (w/v).

Samples of RNA at initial concentrations as low as 10 µg/ml. have been successfully spread by diluting 2.5 µl of the sample into 100 µl of the formamide-urea, followed by addition of cytochrome C and Tris EDTA as described above.

Thirty-five microliters of the final solution were spread onto ~ 140 ml. of a hypophase of 1×10^{-6} M Tris, 2.5×10^{-7} M disodium EDTA, pH ~ 7.8, containing 2 µl of diethylpyrocarbonate. The hypophase had been poured into a rectangular plastic

tray (10 × 10 × 1 cm) onto which two teflon bars were symmetrically placed to confine the spread film to an area of approximately 30 cm^2. The spreading solution was layered onto a glass microscope slide (cleaned in 1:1 conc. nitric acid:H_2O) just above the interface with the hypophase. Films spread in this manner were picked up within 15 sec on parlodion-coated 200-mesh copper grids at approximately 3 mm from the point of application. Grids were stained with uranyl acetate at a concentration of 1×10^{-4} M essentially as described before (Davis & Davidson, 1968), except that an additional rinse in 90% ethanol before and after staining was used, the isopentane rinse being eliminated. Finally, the grids were rotary-shadowed at an angle of 8° with Pt-Pd. The uranyl acetate staining contributes little to the contrast in this case.

In the various basic protein film procedures for electron microscopy of nucleic acids, whether this be double- or single-stranded DNA or RNA, not all grids prepared are satisfactory. Grids are rejected by somewhat subjective criteria based on apparent excessive stretching and alignment of the molecules, or excessive kinkiness and nonuniformity of width. By these criteria, the spreading procedure described here is more difficult to apply than the procedures described for duplex DNA (Kleinschmidt & Zahn, 1959), or for mixtures of single- and double-strand DNA (Davis, Simon & Davidson, 1971). In some

experiments, the RNA specimens were mounted by using a spreading solution of 47% formamide, 0.2 M Tris, 0.02 M disodium EDTA (pH 8.5), 0.05 mg/ml. cytochrome C, and a hypophase of 17% formamide, 0.01 M Tris, 0.001 M disodium EDTA (pH 8.5). The cytochrome C solution used in the latter experiments was filtered through 0.01 μ millipore membrane to remove most of a contaminant polymeric material. The grids were stained with uranyl acetate as before.

(d) Electron microscopy

Specimen grids prepared by the above procedure were examined in a Philips EM 300 electron microscope at 60 KV with a 40 μ objective aperture. RNA samples prepared by the formamide-urea procedure, stained and shadowed, were examined by light-field microscopy. RNA samples prepared by the formamide procedure, and stained but not shadowed, were examined by dark-field microscopy utilizing the electronic beam tilt available on the Philips 300electron microscope. Dark-field microscopy was used to increase the contrast of the stained specimens. Photographs were taken on 35-mm film at an instrument magnification of 2,970X. Tracings of the molecules were made on a Nikon comparator at a final magnification of 48,500X. The magnification was calibrated with a diffraction grating replica (Ladd).

(e) Estimation of DNA contamination

In order to avoid nicking by traces of RNase possibly existing in DNase preparations, the RNA samples used in this study were not digested with DNase. The DNA contamination was estimated by examining the samples under conditions which would not fully denature the RNA.

3. Results

Typical fields of each of the RNA species are presented in Plates Ia, b, and c. Typical fields observed for the mixture of 12 S or 16 S mitochondrial RNA with 18 S cytoplasmic rRNA are shown in Plates Id and e. It is apparent that the 12 S RNA is considerably shorter than the 18 S RNA and that the 16 S RNA is somewhat shorter than the 18 S RNA. Fields such as these, which were rich in molecules (~100 per frame) possessing a more or less uniform appearance and not obviously stretched and aligned, were used for length measurements. At least two frames were used to construct histograms and all molecules observed were scored.

The frequency distributions of lengths for each of the purified RNA species are presented in Figure 2a, b, and c. Each distribution exhibits a single major peak with an approximately Gaussian form; the mean length of the 18 S RNA appears to be greater than the mean length of the 16 S RNA, which in turn is greater than the mean length of the 12 S RNA. The

Plate I: Formamide-urea spreading of RNA.

(a) 12 S mitochondrial RNA; (b) 16 S mitochondrial RNA; (c) 18 S cytoplasmic RNA; (d) mixture of 12 S and 16 S mitochondrial RNA components; (e) mixture of 12 S mitochondrial RNA and 18 S cytoplasmic RNA.

Bar length equals 1 μ.

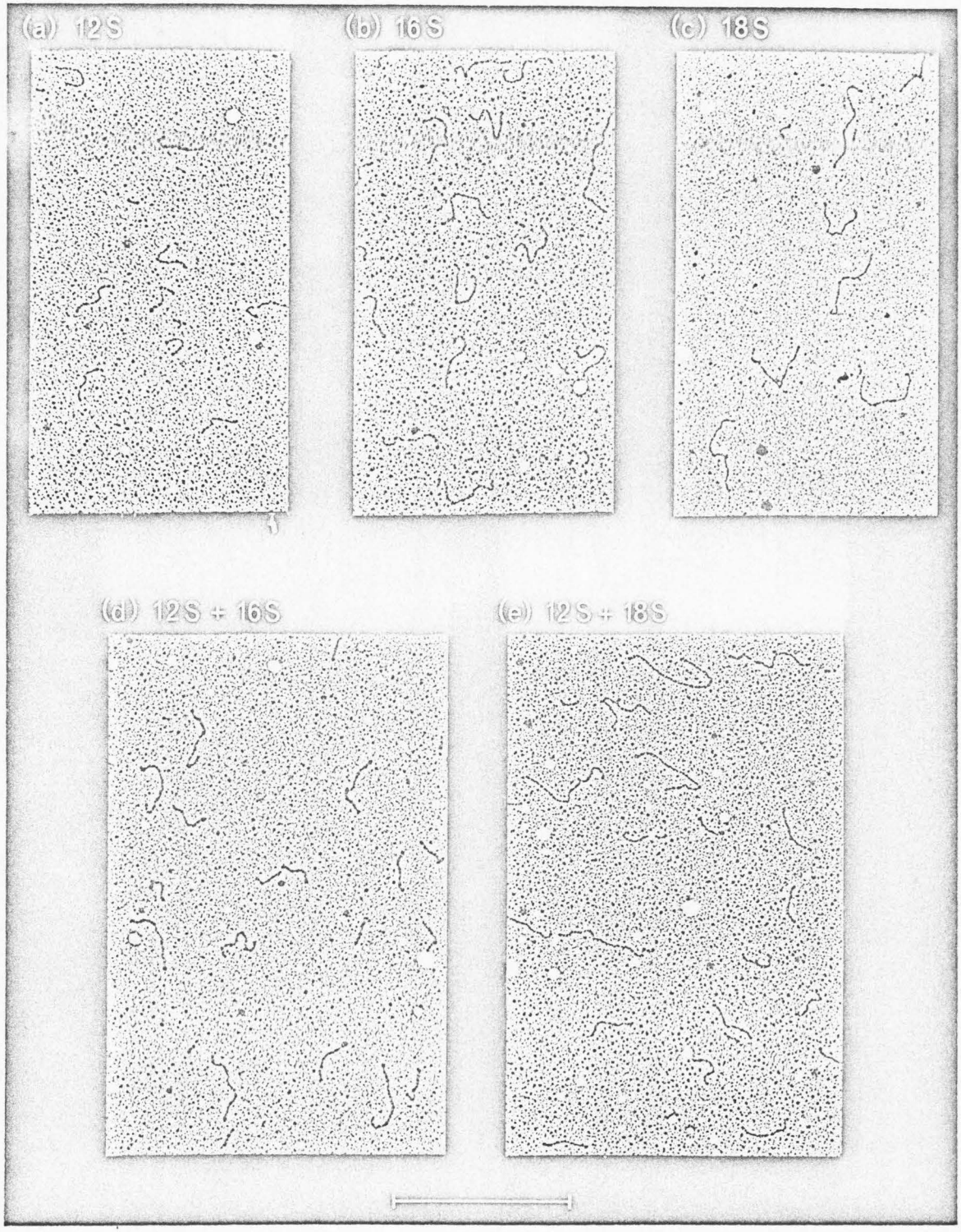

(a) 12S
(b) 16S
(c) 18S
(d) 12S + 16S
(e) 12S + 18S

Fig. 2. Frequency distribution of lengths observed in RNA samples spread by the formamide-urea procedure.

(a) 12 S mitochondrial RNA;

(b) 16 S mitochondrial RNA;

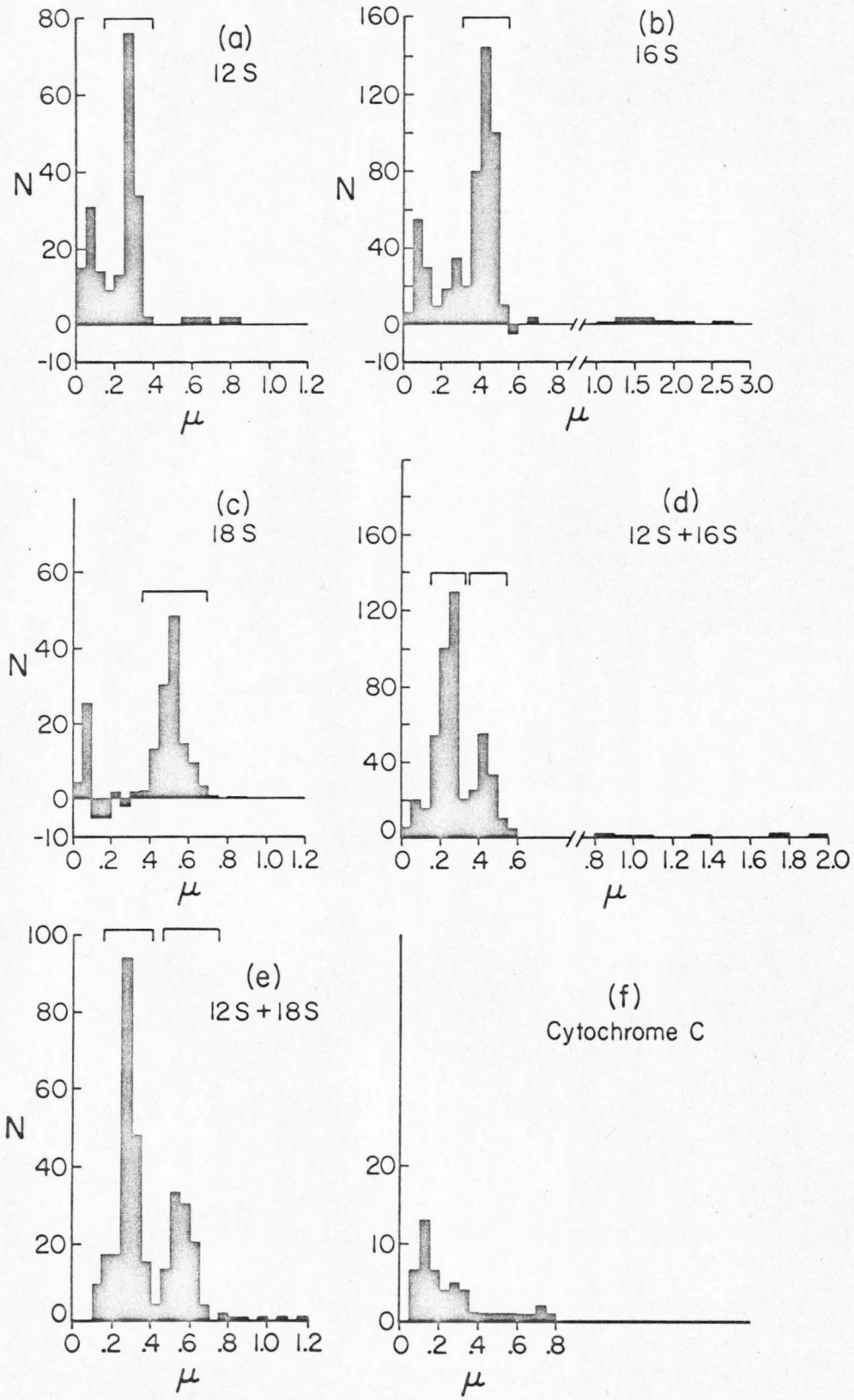
(c) 18 S cytoplasmic rRNA;

(d) mixture of 12 S mitochondrial RNA and 16 S mitochondrial RNA;

(e) mixture of 12 S mitochondrial RNA and 18 S cytoplasmic RNA:

(f) polymeric material observed in cytochrome C sample.

The bar above the major peak on the histograms represents the interval over which the average length has been determined.


(a)
12 S
(b)
16 S
(c)
18 S
(d)
12 S + 16 S
(e)
12 S + 18 S
(f)
Cytochrome C
N
μ

length distribution of a second preparation of 12 S RNA possessed a peak at the same position indicated for the 12 S preparation presented in Figure 2. The distributions have been corrected over each interval for polymeric material that contaminates commercially available 2X crystallized cytochrome C and is indistinguishable from RNA. The frequency distribution of lengths for this material is presented in Figure 2f and is the average of ten frames from two experiments. The peak of this material does not occur at the peak positions of any of the RNA preparations used in this study. Relatively large peaks occurring in the distributions at lengths < 0.2 microns and negative values for the number of molecules probably reflect the large statistical error in scoring these shorter molecules.

The modal lengths and average lengths over the interval indicated in the histograms for the purified components are presented in Table 1. The average lengths for the individual components are generally within 10% of each other from one experiment to another. The number average length and weight average length are about the same (see Table 1). Furthermore, the fractional standard deviations based on weight or number average distributions are approximately the same, about 0.1, for each of the RNA species presented in Table 1. A fractional standard deviation of ~0.1 is routinely observed for single-stranded ϕX DNA spread from formamide solutions, when only molecules

TABLE 1. Length measurements and distribution parameters for mitochondrial RNA[a]

RNA	Number Average Length $L_N(\mu)$	Weight Average Length $L_W(\mu)$	Modal Length (μ)	$T_N(\mu)$[†]	$T_W(\mu)$[‡]	T_W/L_W[‡]	Ratio of Weight Average Lengths	Ratio of Modal Lengths
12S I	.28	.29	.27	.04	.04	.14	18S/12S = 1.79	1.96
12S II	.27	.28	.27	.03	.03	.11		
16S I	.43	.43	.42	.04	.04	.10	18S/16S = 1.20	1.26
16S II	.45	.45	.47	.04	.04	.09		
18S	.51	.52	.53	.06	.06	.11	16S/12S = 1.48	1.56
12S in 12S + 18S	.28	.29	.27	.04	.04		Mixture 18S/12S = 1.90	2.03
18S in 12S + 18S	.54	.55	.55	.04	.04			
12S in 12S + 16S	.27	.27	.27	.02	.02		16S/12S = 1.59	1.56
16S in 12S + 16S	.43	.43	.42	.03	.03		18S/16S = $\frac{18S/12S}{16S/12S}$ = 1.20	1.29

[a] The data presented in this table refer to the length distributions presented in Figure 2, and for 12S II and 16S II, in Figure 4.

[†] Standard deviation based on number average length.

[‡] Standard deviation based on weight average length.

[‡] Fractional standard deviation based on weight average length and standard deviation.

within two standard deviations from the mean for the distribution of all molecules are used in making the analysis. Length measurement of single components requires calibration of the magnification, and this introduces an additional error of 3-5%.

Since the differences observed in these studies were small as compared to the possible errors due to variability from grid to grid, it was considered desirable to mix the 12 S RNA with either the 16 S or 18 S RNAs and to determine the ratio of observed lengths. The length distributions for the mixtures of RNA components are presented in Figure 2d and e. Two distinct peaks can be seen in both mixtures representing the two species of RNA that are present, and the peaks fall at approximately the same position previously observed for the components analyzed separately. The average lengths of the RNA components observed in these mixtures (over the interval indicated by the bar in the histogram) are presented in Table 1. The ratios of modal length for the RNA species in each of the mixtures and in unmixed samples are also presented in Table 1. We note that the ratio of lengths determined in the mixed and unmixed samples are quite similar. We have not included our data on the mixtures of 16 S and 18 S rRNA. Two peaks with the expected maxima were observed, but the resolution between them was poor, as expected.

The 12 S, 16 S, and 18 S RNA species were also mounted for electron microscopy from the formamide solvent regularly

used in this laboratory (Davis, Simon & Davidson, 1971) for obtaining single-stranded DNA in an extended form. By this method of preparation, ϕX174 DNA appears well extended and contrasted, and is a rather smooth filament (Plate II d) with a contour length of about 1.3 μ. (Under these formamide conditions, double-stranded ϕXRF II has a contour length of 1.7 μ.) On the contrary, under the strongly denaturing conditions used in this work for RNA spreading the contour length of single-stranded ϕX is about 1.75 μ.

The appearance of the RNA samples prepared by this procedure are presented on Plate II a, b, and c. The RNA molecules appear as short, linear structures of variable thickness.

The length distributions for the 12 S, 16 S, and 18 S RNA preparations spread from the partially denaturing formamide medium are presented in Figure 3a, b, and c. In all three samples the lengths observed are much shorter than those for the more fully denatured RNA samples. For example, the length of 18 S RNA observed under these partially denaturing conditions is only 0.1 μ, to be compared to the previously determined length of 0.55 μ for the more fully denatured 18 S RNA. In addition, in contrast to the symmetrical distribution for the 18 S RNA, the distributions for the 12 S and 16 S RNA appear to have some molecules of length equal to and greater than the lengths encountered in the 18 S sample. Thus, it is very clear that the

Plate II: Partially denatured RNA as visualized by uranyl acetate staining and dark-field electron microscopy.

(a) 18 S cytoplasmic RNA;

(b) 16 S mitochondrial RNA;

(c) 12 S mitochondrial RNA;

(d) ϕX174 single-stranded DNA.

Some partially denatured RNA molecules are indicated by arrows. A single molecule of duplex DNA appears in the upper part of the 12 S and 16 S pictures. Bar length equals 1 μ.

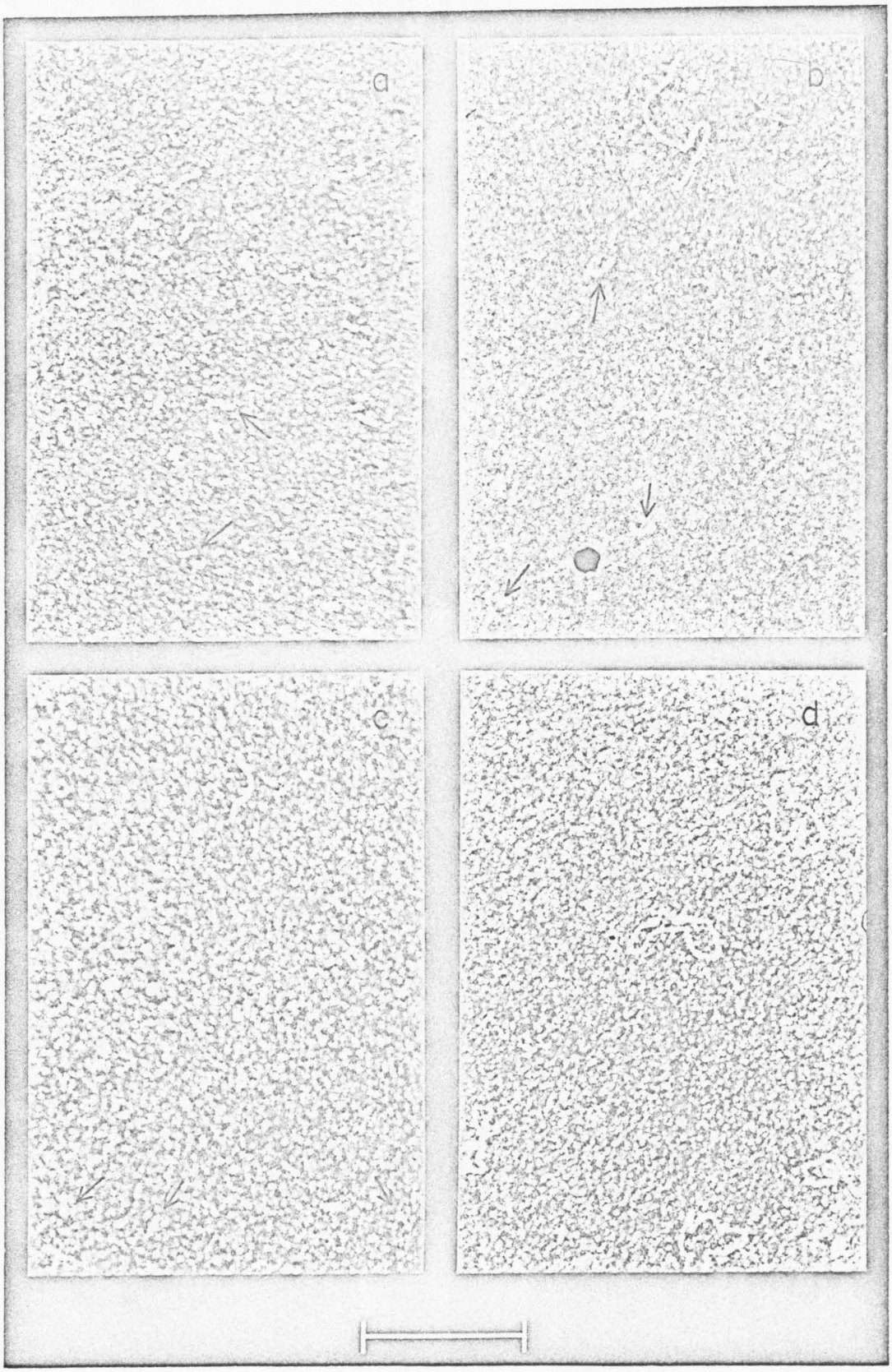
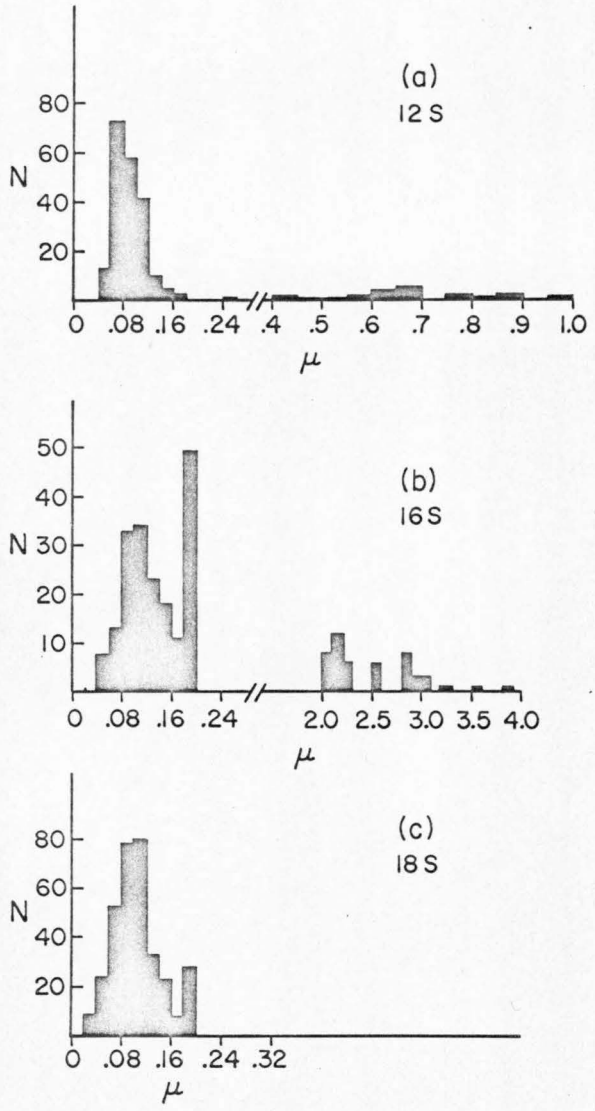

a
b
c
d

Fig. 3. Frequency distributions of length observed in RNA samples spread by the formamide procedure.

(a) 12 S mitochondrial RNA;

(b) 16 S mitochondrial RNA;

(c) 18 S cytoplasmic RNA.


(a)
12 S
N
80
60
40
20
0 .08 .16 .24 .4 .5 .6 .7 .8 .9 1.0
μ
(b)
16 S
N
50
40
30
20
10
0 .08 .16 .24 2.0 2.5 3.0 3.5 4.0
μ
(c)
18 S
N
80
60
40
20
0 .08 .16 .24 .32
μ

formamide conditions which are adequate for extending single-stranded DNA do not suffice to extend the RNAs studied. There is a hint also that the 12 S and 16 S mitochondrial RNA may possess secondary structures which are sometimes partially denatured by the solvents used, although most frequently they are not.

It was conceivable that in these contracted molecules, there were regions that were more extended and other regions that were thicker and more contracted. In order to search for such heterogeneity, the grids were stained with uranyl acetate but were not shadowed. This gives higher resolution in the basic protein film method. In order to achieve sufficient contrast, dark-field microscopy with the beam tilting device available in the Philips 300 electron microscope was used. No strong evidence for variable thickness along a strand was observed.

The analysis of the 12 S and 16 S RNA samples under the partially denaturing formamide conditions was also useful to establish whether any DNA contaminant was present in the preparations. Indeed, as appears from Figure 3, both the 12 S and 16 S preparations were found to contain a significant amount of relatively long double-stranded molecules (longer than 0.2 μ), presumably DNA. This DNA would have been expected to be denatured under the strongly denaturing conditions used for RNA spreading. Although a quantitation of the relative mass

proportion of double-stranded DNA and single-stranded RNA in the formamide treated samples is made difficult by the small size of the RNA molecules which tend to be confused with the background, it appears that the relative amount of long filaments under strongly denaturing conditions (Fig. 1) is considerably less than would be expected, in the absence of single-strand nicking, from the denaturation of the long double-stranded DNA molecules. In order to exclude the possibility that in the previously described analysis short single-stranded DNA fragments had overlapped with, and consequently altered, the distribution of 12 S and 16 S RNA, one investigation was carried out of the length distribution of 12 S and 16 S RNA samples which had been purified by both sucrose gradient centrifugation and polyacrylamide gel electrophoresis (as described in the legend of Fig. 4) and which was free of any detectable DNA contamination as estimated by electron microscopic analysis for double-stranded DNA. As shown in Figure 4 and Table 2, the length distributions of these more purified 12 S and 16 S RNA samples are substantially identical to those determined with the previously analyzed preparations.

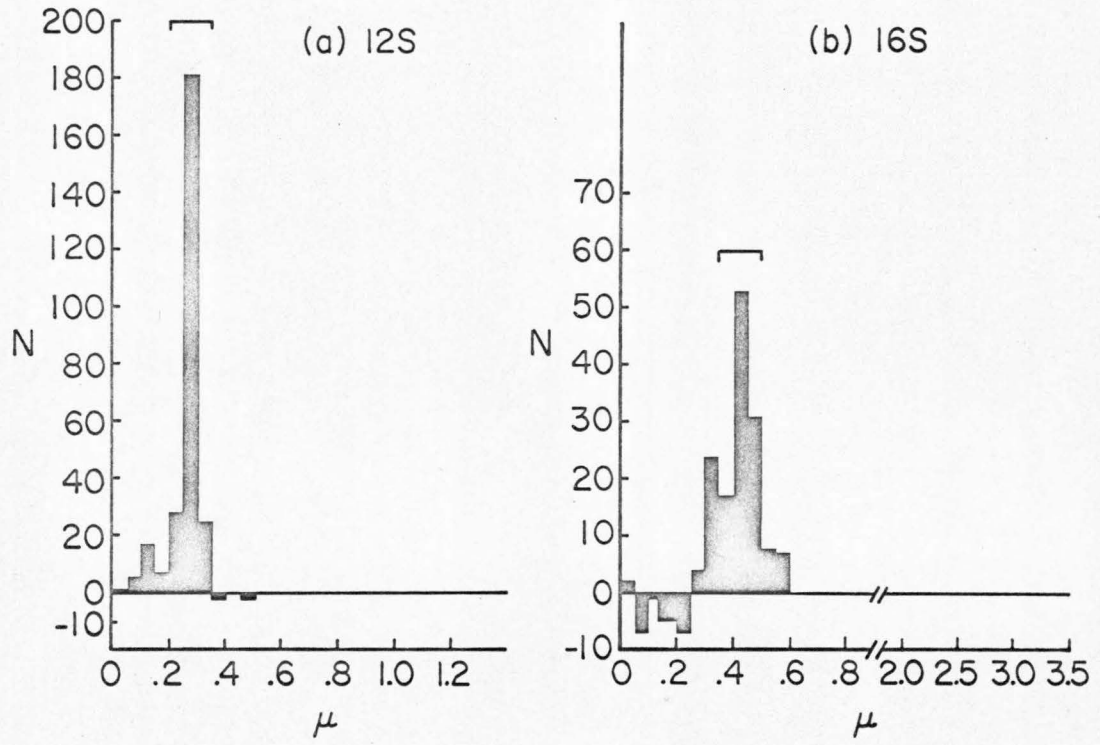
4. Discussion

The development of a spreading procedure for RNA has permitted a measurement of the lengths of the mitochondrial rRNA components relative to 18 S cytoplasmic RNA.

Fig. 4. Frequency distribution of lengths of RNA samples purified by sucrose gradient centrifugation and polyacrylamide gel electrophoresis. The 16 S and 12 S RNA components, run twice through sucrose gradients were further purified by polyacrylamide gel electrophoresis, as described previously (Attardi et al., 1970), and finally rerun through a third cycle of sucrose gradient centrifugation.

(a) 12 S mitochondrial RNA;

(b) 16 S mitochondrial RNA.


(a) 12S
(b) 16S
N
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200
180
160
140
120
100
80
60
40
20
0
-10
70
60
50
40
30
20
10
0 .2 .4 .6 .8 1.0 1.2
0 .2 .4 .6 .8 2.0 2.5 3.0 3.5

The molecular weight of the sodium salt of 18 S cytoplasmic rRNA has been measured as 0.71×10^6 by sedimentation equilibrium (McConkey & Hopkins, 1969). Using an average residue weight of 344, based on the nucleotide composition determined by Amaldi & Attardi (1968), this corresponds to a degree of polymerization of 2060 nucleotides. If the molecular lengths in microns given in Table 1 under these strongly denaturing conditions used for spreading are proportional to the degree of polymerization, this latter quantity is 1010 and 1570 nucleotides per molecule for 12 S and 16 S mitochondrial rRNA, respectively. We calculate an average residue weight of 342 for these molecules from the nucleotide composition reported by Attardi et al. (1970) so the corresponding molecular weights are calculated to be 0.35×10^6 and 0.54×10^6.

The sedimentation velocities of the three species in sucrose gradients in the presence of formaldehyde (Fig. 1) are as 12.6 S to 14.7 S to 18.0 S. According to Boedtker (1968), in formaldehyde, $S \sim M^{0.40}$. This gives molecular weights of 0.29×10^6 and 0.43×10^6 for 12 S and 16 S mitochondrial rRNA measured relative to 18 S cytoplasmic rRNA with a molecular weight of 0.71×10^6.

The relative sedimentation velocities of the three species in 0.1 M salt in the absence of formaldehyde are as 12.5 S to 15.9 S to 18.0 S. These sedimentation coefficients are fairly close to those observed in the presence of formaldehyde, suggesting that the change in secondary structure upon treatment with formaldehyde is about

the same for the three species. If most of the secondary structure is destroyed in formaldehyde, the amount of secondary structure must be about the same for the three rRNAs in 0.1 M NaCl.

Under the conditions of polyacrylamide gel electrophoresis used in this laboratory (0.04 M Tris buffer, 0.02 M sodium acetate, 0.002 M EDTA, 0.5% dodecyl SO_4, pH 7.4, runs at room temperature), the relative mobilities of 12 S, 16 S, 18 S, and 28 S rRNAs have been found to be as 2.78, 2.17, 2.13, and 1.0, respectively (Attardi et al., 1970; Attardi & Attardi, 1971). Assuming a linear relationship between logarithm of S_E and relative electrophoretic mobility, and using the 28 S and 18 S rRNAs as reference values, one can estimate S_E values of 14.3 and 18.2 for the 12 S and 16 S species, respectively. On the basis of a linear relationship between logarithm of mol. wt. and gel mobility (Bishop, Claybrook & Spiegelman, 1967), and using values of 0.71×10^6 and 1.9×10^6 for the mol. wts. of the 18 S and 28 S HeLa cytoplasmic rRNA species, as determined by sedimentation equilibrium (McConkey & Hopkins, 1969), the apparent mol. wts. of the 12 S and 16 S mitochondrial rRNA components can be calculated to be 0.42 and 0.72×10^6, respectively.

It is clear that the effects of secondary structure are different for gel electrophoresis as compared to sedimentation in 0.1 M NaCl plus formaldehyde in sucrose, sedimentation in 0.1 M NaCl in sucrose, and to our electron microscope spreading

procedure. It should be mentioned that a strong temperature dependence of the electrophoretic mobility of animal mitochondrial RNAs relative to that of the cytoplasmic rRNA species, presumably due to differential thermal unfolding of the two types of RNA, has been recently demonstrated (Groot, Aaij & Borst, 1970). In our hands, sedimentation analysis and length measurements by electron microscopy have led to consistent estimates of relative molecular weights. There is no assurance that differential secondary structure effects are not operative in all of these measurements. However, in view of the strongly denaturing conditions used, it is plausible that they are not operative in the relative molecular length measurements by electron microscopy.

If the electron microscope values of relative molecular weights are accepted, the measured molecular lengths correspond to average residue spacing of 2.7 Å per base. For ϕX DNA mounted from 50% formamide, where it appears to be reasonably extended, a value of about 2.9 to 3.0 Å per base is observed, and for double-stranded DNA, the values are usually about 3.3 Å per base pair. Thus, the spacing of the RNA bases appears to be only slightly closer than for single-strand DNA.

It should be mentioned that from measurements of sedimentation rate carried out in the presence of dimethylsulfoxide, Dawid (personal communication) has recently estimated molecular weights of 5×10^5 and 2.7×10^5 for the large (electrophoretic "21 S") and, respectively, the small (electrophoretic "13 S") mitochondrial rRNA species from Xenopus laevis. The corresponding values, estimated from sedimentation rate in the presence of formaldehyde, were $5.1 - 5.7 \times 10^5$ and $3.1 - 3.5 \times 10^5$. These latter values are about the same as those obtained in the present work by electron microscopy of mitochondrial rRNA from HeLa cells.

Granboulan & Scherrer (1969) have already described a procedure for mounting RNA for electron microscopy from aqueous 3 M - 6 M urea, 0.25 M - 0.5 M NH_4Ac. Their results and ours are at least approximately in agreement. For the purpose of guaranteeing full denaturation of the RNA, the strongly denaturing conditions used here appear, however, to be preferable a priori.

The authors quoted above report a residue spacing of 2.45 Å per base for several rRNAs and a spacing of 3.17 Å per base for several viral and messenger RNAs. It should be noted that the GC content of the rRNAs used by these workers ranged from 54% (E. coli) to 71% (HeLa, 325 pre-rRNA) (Table 3, Granboulan & Scherrer, 1969). By contrast, the GC content of

the messenger and viral RNAs ranged from 44% (Reo-virus RNA) to 52% (avian myeloblastosis-virus RNA). If there exists a GC dependence of denaturation of RNA in 3 M - 6 M urea such that GC-rich RNA is denatured to a lesser extent from GC-poor RNA, then one expects length measurements of GC-rich RNAs to lead to residue spacings which are smaller than the residue spacings determined from length measurements of GC-poor RNAs. This could explain the different residue spacings observed by Granboulan & Scherrer (1969) for the two classes of RNA they studied.

Recently, the size of the mitochondria-specific rRNAs from Aspergillus nidulans (23.5 S and 15.5 S components) has been measured by electron microscopy utilizing the Granboulan & Scherrer mounting technique (Verma, Edelman, Herzberg & Littauer, 1970). The molecular weights determined in this study were in better agreement with those estimated from the electrophoretic mobility in polyacrylamide gel than with those estimated from the sedimentation velocity in sucrose gradient. Since these rRNAs are known to possess a low GC content (about 32%) they would be expected to denature more easily and to possess the larger residue spacing of 3.17 Å per base observed by Granboulan & Scherrer (1969). Furthermore, the conditions commonly used for gel electrophoresis of RNA, notably the relatively low ionic strength and room temperature, may lead

to a partial denaturation or unfolding of RNAs low in GC content, which would then migrate more slowly than the native RNA species due to their extended configuration. Measurement of the electrophoretic velocity of the partially denatured low GC RNA relative to that of an undenatured high GC RNA, taken as a mol. wt. reference, would then overestimate the molecular weight of the low GC RNA. In fact, evidence has been reported for this tendency to overestimate the size of RNA species with low GC content by gel electrophoresis (Loening, 1969).

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Footnotes

†rRNA, ribosomal RNA; dodecyl SO_4, sodium dodecyl sulfate

‡ The symbol S_E is used to indicate the S value estimated from the relative electrophoretic mobility of the RNA. The values reported here have been calculated from the original patterns assuming a linear relationship between the logarithm of S_E and gel mobility, as theoretically expected (Borst & Grivell, 1971), and using the 28 S and 18 S cytoplasmic rRNAs as reference values.

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D. Determination of the Relative Positions of the Ribosomal Genes on HeLa Mitochondrial DNA by Electron Microscopy

This section is the preprint of

Expression of the Mitochondrial Genome in HeLa Cells
VIII. The Relative Position of Ribosomal RNA Genes
in Mitochondrial DNA

by Donald Robberson, Yosef Aloni, Giuseppe Attardi and Norman Davidson.

This paper is to be submitted for publication to the
Journal of Molecular Biology.

Expression of the Mitochondrial Genome in HeLa Cells VIII[†]. The Relative Position of Ribosomal RNA Genes in Mitochondrial DNA

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[†] Paper VII in this series is Aloni & Attardi (1971a).

Abstract

Electron microscopy of hybrids between the heavy strand of HeLa mitochondrial DNA and HeLa mitochondrial rRNA demonstrates that the genes for 16 S and 12 S HeLa mitochondrial rRNAs[†] are situated adjacent or very close to each other on mitochondrial DNA. The length of the DNA segment separating the two genes is estimated to correspond to less than 500 nucleotide pairs.

Mitochondrial DNA of HeLa cells codes for several discrete RNA species with sedimentation coefficients of 16 S, 12 S and 4 S (Attardi et al., 1970). The 16 S and 12 S RNA are the structural components of 60 S mitochondria-specific ribosomes (Attardi & Ojala, 1971). The 16 S RNA is contained in the larger mitochondrial ribosomal subunit (45 S) and the 12 S RNA in the smaller ribosomal subunit (35 S) (Attardi & Ojala, 1971). The 4 S components presumably correspond, in part at least, to mitochondria-specific tRNA species, as shown for rat liver (Nass & Buck, 1971).

Hybridization studies indicate that both 16 S and 12 S RNAs are transcribed from the heavy strand of mitochondrial DNA and that there is one gene for each of these species per mitochondrial duplex DNA of molecular weight 9.6×10^6 (Aloni & Attardi, 1971b). In this work, we have studied the relative position of the two rRNA genes on the mitochondrial DNA molecule by electron microscopy.

Thermal hybrids were prepared between each of the rRNA species (16 S and 12 S) separately and in combination and the heavy strand of mitochondrial DNA. In all cases, saturation was achieved in approximately 5 min at 60°C using high concentrations of RNA and DNA (Fig. 1).

The hybrids were treated with RNase and then mixed with double-stranded DNA size markers (ØX RF-II or SV40 DNA) for examination by the basic protein technique. Grids were stained with uranyl acetate and examined by light and dark field microscopy as previously described (Robberson et al., 1971).

The types of molecules which have been scored are depicted in

FIG. 1. Kinetics of hybridization of mitochondrial rRNA to the heavy strand of mitochondrial DNA. Mitochondrial RNA components, purified as previously described (Aloni & Attardi, 1971b), were incubated at 60°C in a volume of 50 µl for the indicated times. The concentration of the heavy strand of mitochondrial DNA was 0.8 µg/ml., and that of RNA, 8 µg/ml. After hybridization, each sample was diluted to 0.35 ml. with 2X SSC containing pancreatic RNase, preheated at 80°C for 10 min, at a final concentration of 5 µg/ml. RNase digestion was carried out for 15 min at 23°C, after which the solutions were filtered through Bac-T-flex type B-6 filters (Schleicher & Schuell), prewashed with 2X SSC. Subsequent washing of the filters was done as previously described (Aloni & Attardi, 1971c).

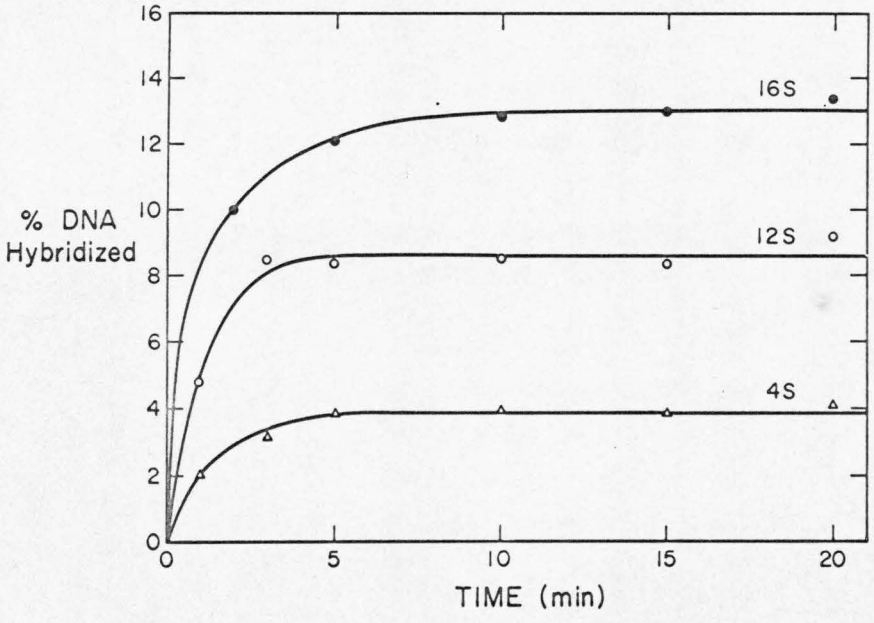

% DNA Hybridized
16
14
12
10
8
6
4
2
0
0
5
10
15
20
TIME (min)
16S
12S
4S

Figure 2a. Only hybrids with clear bushes (>500 bases) at both ends were measured. Examples of the stained hybrids in light field and dark field which have been used for length measurements are presented in Plate I: (1-6), 12 S hybrids; (7-14), 16 S hybrids; (15-24), 12 S + 16 S hybrids. A close examination of the hybrids between the heavy mitochondrial DNA strand and the mixture of 12 S and 16 S RNA reveals no identifiable single-stranded region anywhere in the interior of the hybrid, which would appear as a bush. We believe that any region corresponding to 500 bases or more of single-stranded DNA would be detected by this technique. The basis for this statement is our examination of deletion bushes of size of approximately 500 bases in heteroduplexes between wild-type ØX and a deletion mutant of ØX, missing about 500 base pairs, that has been discovered in this laboratory (Kim, Sharp & Davidson, 1971). The deletion mutant occurs as a monomer and a tandem dimer. Examples of heteroduplexes, showing the deletion bushes, are presented in Plate I, 27, 28. In Plate I, 27, the heteroduplex between a circular dimer ØX RF from which a 500 base portion has been deleted from each of the monomer parts, and a single wild-type ØX single-stranded circle is presented. The structure of this heteroduplex is shown in Figure 2c.

A second example of a ∿500 base bush is presented in Plate I, 28. In this example a monomer ØX RF from which 500 bases has been deleted has hybridized to a wild type ØX single-stranded circle, as shown in Figure 2b.

The majority of hybrids between the heavy strand of mitochondrial

FIG. 2. Illustrations of constructed hybrids. (a) General appearance of hybrids between the heavy strand of HeLa mitochondrial DNA and mitochondrial 16 S and 12 S rRNA that were observed. Hybrids which were observed, but not scored for length measurements, are indicated.

(b) Hybridization of ØX (Δ) complement with wild-type ØX174. The heteroduplex formed by this hybridization appears as a circular DNA duplex molecule upon which there is a "bush" of ∿500 bases, of non-hybridized, collapsed DNA, as indicated by the arrow in the diagram.

(c) Hybridization of dimer ØX (Δ) complement with a single wild-type ØX174 molecule. The heteroduplex formed by this hybridization appears as a circular DNA duplex molecule upon which there are two "bushes", one of ∿500 bases and one of ∿4500 bases, on non-hybridized, collapsed DNA, as indicated by the arrows in the diagram.

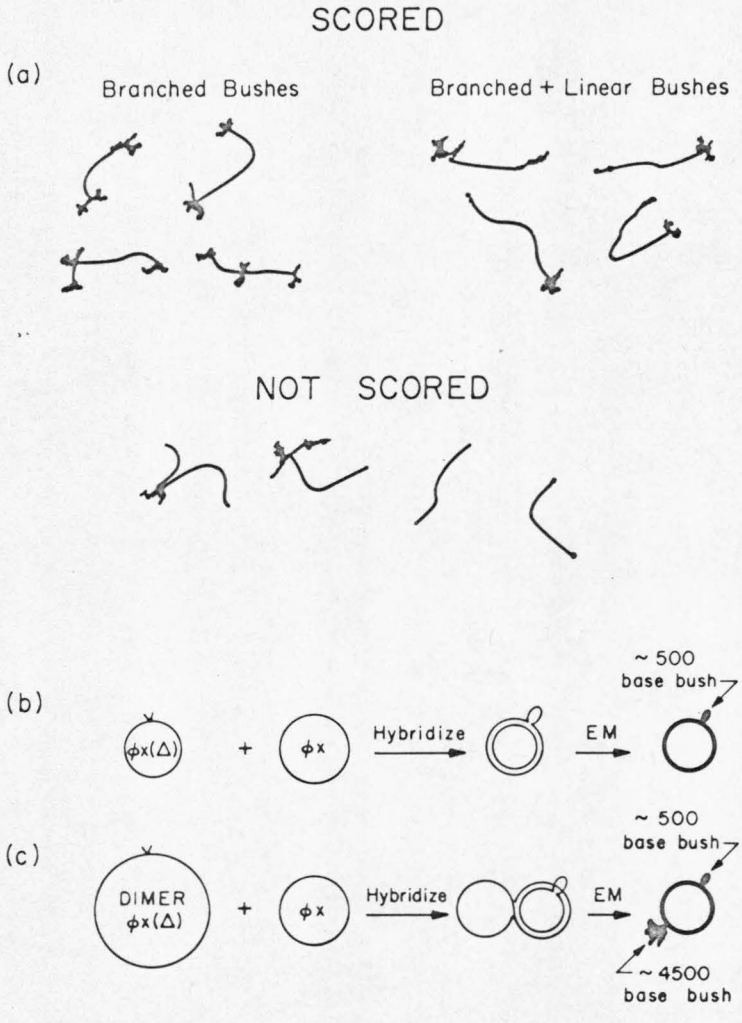

SCORED
(a)
Branched Bushes
Branched + Linear Bushes
NOT SCORED
(b)
~ 500
base bush
φx(Δ)
+
φx
Hybridize
EM
(c)
~ 500
base bush
DIMER
φx(Δ)
+
φx
Hybridize
EM
~ 4500
base bush

Plate I. Selected examples of hybrids stained with uranyl acetate and visualized by light-field and dark-field electron microscopy.

(1-6). Hybrids between 12 S mitochondrial rRNA and the heavy strand of mitochondrial DNA (12 S x H). (See Fig. 2a).

(7-14). Hybrids between 16 S mitochondrial rRNA and the heavy strand of mitochondrial DNA (16 S x H). (See Fig. 2a).

(15-24). Hybrids between the equimolar mixture of 12 S and 16 S mitochondrial rRNA and the heavy strand of mitochondrial DNA ([12 S + 16 S] x H). (See Fig. 2a).

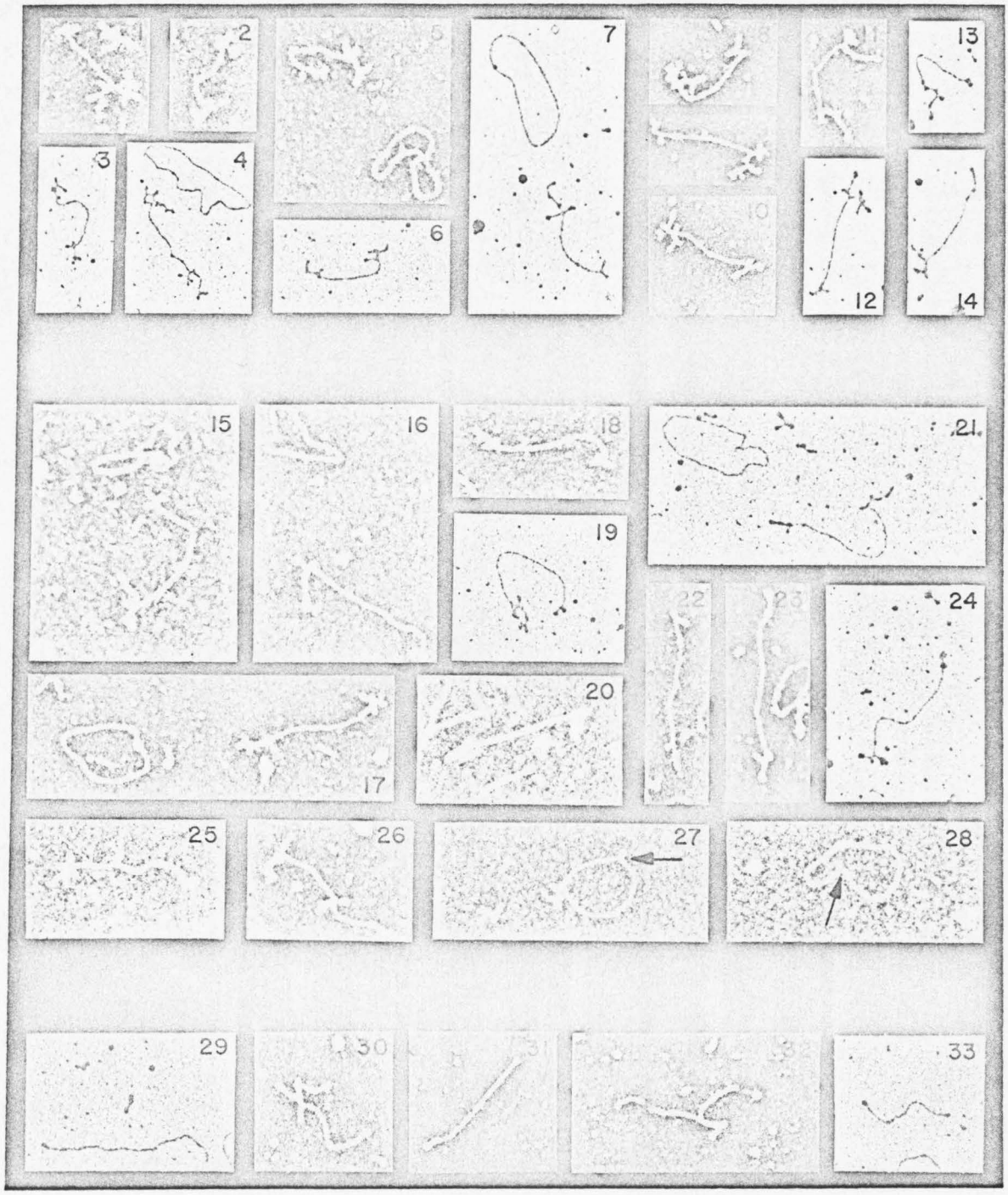
(25, 26). Hybrids between equimolar mixture of 12 S and 16 S mitochondrial rRNA and the heavy strand of mitochondrial DNA that contain an internal bush.

(27). Heteroduplex of dimer ØX (Δ) and a single wild-type ØX174. (See Fig. 2c).

(28). Heteroduplex of ØX (Δ) and wild-type ØX174. (See Fig. 2b).

(29). Hybrids between 16 S or 12 S mitochondrial rRNA and the heavy strand of mitochondrial DNA that were observed but not scored. (See Fig. 2a).

Several molecules of ØX RF (Δ) DNA in 12 S x H and 16 S x H or SV40 DNA in (12 S + 16 S) x H are included for size reference.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DNA and the mixture of 12 S and 16 S RNAs possess no internal bushes (>500 bases). Our conclusion is that the genes for 12 S and 16 S RNA are adjacent or very close to each other on the mitochondrial DNA, with a possible separation of less than 500 base pairs.

The length distributions for the hybrids are presented in Figure 3 a, b, and c. A single peak with an approximately Gaussian spread of lengths is observed in each case.

The average lengths and standard deviations are given in Table I. The most important result is that the average double-strand length of the 12 S + 16 S hybrids is equal to the sum of the lengths of 12 S and 16 S hybrids. The standard deviation of the ØX RF lengths was about 8% in these experiments. The standard deviations reported in the table for the hybrids is then about as expected for duplex segments of the given lengths; thus there is no evidence for heterogeneity (Davis, Simon & Davidson, 1970). We have previously estimated mol. wts. corresponding to 1010 nucleotides and 1570 nucleotides for 12 S and 16 S mitochondrial rRNA, respectively. With a duplex length of 0.46 μ for the 16 S mitochondrial rRNA hybrid, this corresponds to a spacing of 2.96 Å/residue. The value observed for DNA under comparable conditions is 3.27 Å/residue.

Quite infrequently one does observe a hybrid which contains an internal bush corresponding to >500 bases, as illustrated in Plate I, 25, 26 observed in the mixture of 16 S and 12 S mitochondrial rRNAs hybridized to the heavy strand of mitochondrial DNA. These hybrids could have arisen from hybridization of either 12 S or 16 S RNA and contaminating messenger RNA transcribed from another section of the

FIG. 3. Length distributions of hybrids between mitochondrial rRNA and the heavy strand of mitochondrial DNA.

(a) Hybrid lengths observed for 12 S mitochondrial rRNA hybridized to the heavy strand of mitochondrial DNA (12 S x H).

(b) Hybrid lengths observed for 16 S mitochondrial rRNA hybridized to the heavy strand of mitochondrial DNA (16 S x H).

(c) Hybrid lengths observed for the equimolar mixture of 12 S and 16 S mitochondrial rRNA hybridized to the heavy strand of mitochondrial DNA ([12 S + 16 S] x H).

The dashed lines represent the length distributions for ØX RF (Δ) duplex DNA in (a) and (b) or SV40 DNA in (c) used as an internal size marker. The length distributions were normalized to the length of ØX RF with an average length of 1.7 μ.

The fraction of total molecules observable as duplex hybrids was 0.63 in 12 S x H, 0.57 in 16 S x H, and 0.62 in [12 S + 16 S] x H. The fraction of storable duplex hybrids with branched and/or linear bushes was 0.20 in 12 S x H, 0.08 in 16 S x H, and 0.09 in [12 S + 16 S] x H.

The average size of the DNA used in constructing these hybrids was estimated from scoring the fraction of intact circular or full-length single strands visualized by the formamide technique (Davis, Simon & Davidson, 1971). On the assumption that random degradation from circular single strands occurred during preparation, the average molecular weight was calculated as $1.0 - 1.4 \times 10^6$.

☐ Hybrids with branched and/or linear bushes.

▨ Hybrids with branched bushes (see Fig. 2a).

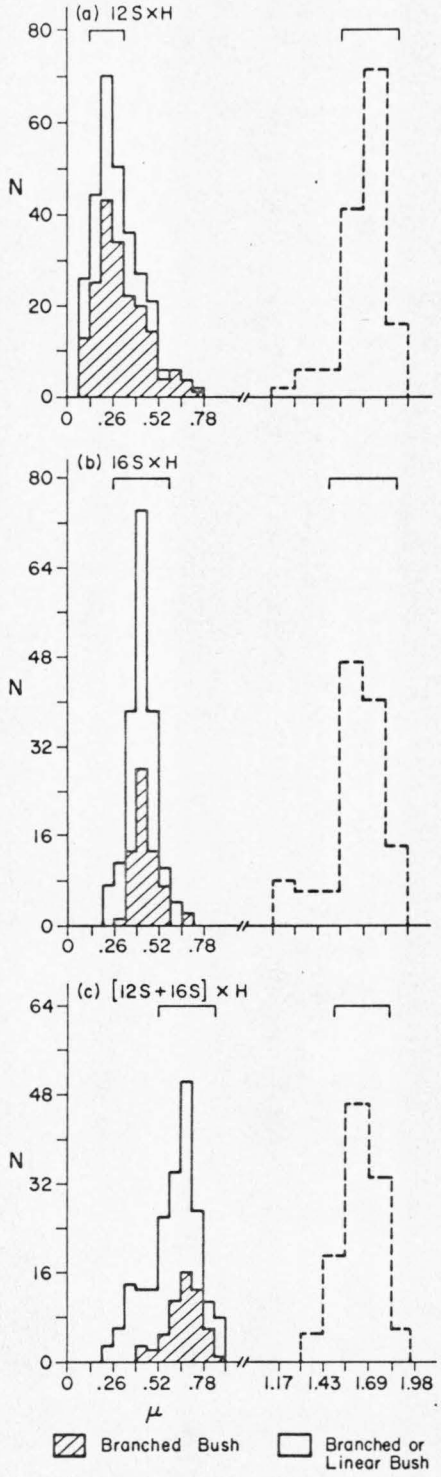

(a) 12S×H
(b) 16S×H
(c) [12S+16S] × H
N
μ
0 .26 .52 .78
1.17 1.43 1.69 1.98
Branched Bush
Branched or Linear Bush

Table 1. Length Measurements of Mitochondrial RNA-DNA Hybrids

Hybrid	Hybrid Length (μ) Observed*	
	$\langle L \rangle_n \pm T_n$	$\langle L \rangle_w \pm T_w$
12 S x H	.25 ± .06	.26 ± .06
16 S x H	.46 ± .08	.47 ± .07
[12 S + 16 S] x H	.73 ± .13	.74 ± .13
12 S x H + 16 S x H	.71 ± .20	.73 ± .19

*$\langle L \rangle_n$ = Number average length

$\langle L \rangle_w$ + Weight average length

T_n = Standard deviation of $\langle L \rangle_n$

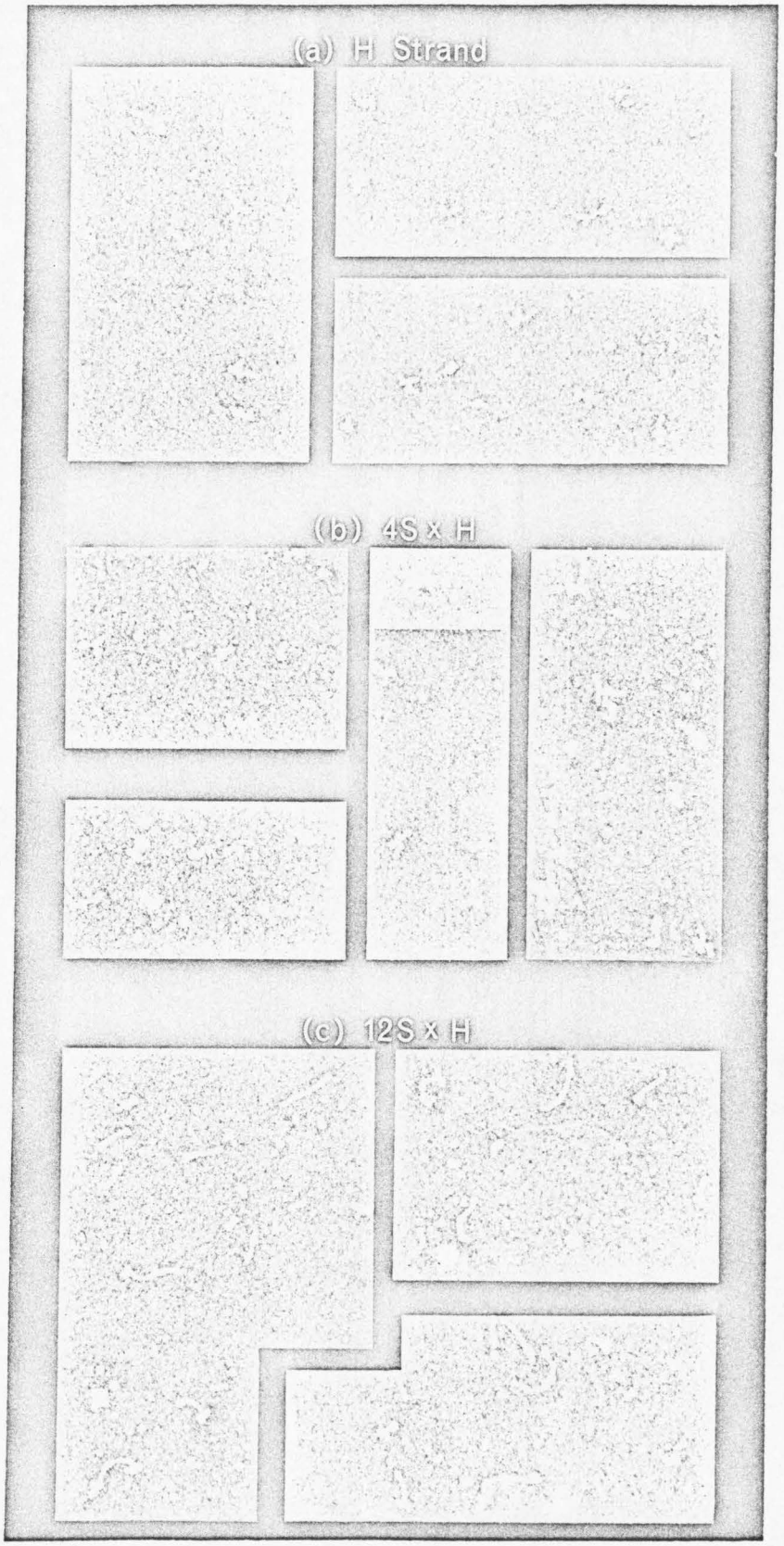
T_w = Standard deviation of $\langle L \rangle_w$

genome removed from the 12 S, 16 S RNA region or from other causes such as contaminating L strand DNA.

The heavy strand of mitochondrial DNA codes for 8 genes of 4 S RNA (Aloni & Attardi, 1971b). If all these genes were clustered in one segment, this segment would be approximately one-half the duplex length of the 12 S hybrid we observe. No duplex region of this size is observed in the hybrids between 4 S RNA and the heavy strand of mitochondrial DNA (Plate II), and we conclude that the 4 S genes are not all clustered in one segment of the mitochondrial DNA.

These investigations were supported by grants GM-10991 and GM-11726 from the National Institutes of Health, by USPH 6M 00086 Training Grant (D.R.) and by a Research Training Fellowship from the International Agency for Research on Cancer by the W. H. O. (Y.A.).

Plate II. Heavy strand of mitochondrial DNA (a) and hybrids between 4 S RNA (b) or 12 S RNA (c) and heavy strand, visualized by dark-field electron microscopy. Bar length represents 1 μ.


(a) H Strand
(b) 4S x H
(c) 12S x H

Footnotes

[†]rRNA, ribosomal RNA.

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E. A General Approach to the Isolation of Specified DNA Sequences

This section is the preprint of

Chemical Coupling of RNA to Hydrazide-Derived Sepharose

by Donald Robberson and Norman Davidson.

This paper is to be submitted for publication.

Abstract: A coupling procedure has been developed for the efficient covalent attachment of periodate oxidized RNA to an insoluble matrix of hydrazide-derived sepharose.

INTRODUCTION

The isolation of large single-stranded DNA complementary, in part, to specific RNAs, should make feasible the physical mapping of genes by electron microscopy of the RNA-DNA hybrids. Although methods have been reported for the isolation of DNA sequences of approximately the same size as the RNA (Kohne, 1968; Marks and Spencer, 1970; Colli, Smith and Oishi, 1971) these DNA sequences are too small for use in physical gene mapping. The development of a coupling procedure for the efficient covalent attachment of RNA to an insoluble matrix should allow purification of DNA sequences complementary to specific RNAs by hybridization of the total DNA sample to the resin-attached RNA.

MATERIALS AND METHODS

Activation of Sepharose: Sepharose was activated by cyanogen bromide by the following procedure in order to render it sensitive to nucleophilic attack by ε-amino caproic acid methyl ester.

One hundred ml of Sepharose 4B-200 (Sigma) was first washed by filtration on a coarse sintered glass funnel with several changes of doubly distilled water and suspended to a total volume of 200 ml in 2X distilled water. Next a solution of ε-amino caproic acid methyl ester-HCl (Cyclo) was prepared by dissolving 9 g of the salt in 0.1 M Na bicarbonate buffer, pH 9. The solution was then brought to pH 9 by the addition of 4 N NaOH. The solution was kept ice cold

as much as possible. Ten gm of cyanogen bromide, dissolved in 200 ml H_2O, was added to the 200 ml suspension of sepharose in distilled water which was stirred magnetically at room temperature. The suspension was immediately adjusted to pH 11 with 4 N NaOH and maintained at pH 11 for 9 minutes, after which it was washed on a coarse sintered glass funnel with 2 ℓ of ice cold 0.1 M Na bicarbonate buffer, pH 9. The activated sepharose was suspended to a volume of 170 ml in 0.1 M Na bicarbonate buffer, pH 9. The solution of the amino ester prepared as described above was then added to the activated sepharose and again kept in suspension with magnetic stirring. The solution was gently stirred at 4°C for 24 hr. The cyanogen bromide procedure is similar to one previously reported (Cuatrecasas, Wilchek and Anfinsen, 1968).

Hydrazinolysis. The ester function of the ε-amino-caproic acid methyl ester which had been linked to the sepharose was converted to the hydrazide by the following procedure. After the sepharose was reacted for 24 hrs at 4°C with the ε-amino-caproic methyl ester, the resin was washed with 2 ℓ of ice cold 2X distilled water and suspended to a total volume of 200 ml. Approximately 20 ml of this suspension was removed for control experiments and the remainder of the resin was resuspended in water to a total volume of 160 ml in a 500-ml Erlenmeyer flask. The solution was placed on ice and 140 ml of hydrazine hydrate 99-100% (Matheson, Coleman and Bell) was added slowly. The suspension was then placed in a preheated water

bath and incubated at 70°C for 15 min keeping the resin suspended by gently swirling the reaction mixture. A thermometer was placed in the reaction vessel for the purpose of monitoring the reaction temperature. The mixture was then cooled in an ice bath to 25-30°C, and washed on a sintered glass funnel with 2 ℓ (distilled water and 1-2 ℓ) 2X distilled water. (A suspension of the resin in water should now be ∿pH 7). The resins were suspended to a volume (generally a total volume equal to twice the resin volume) convenient for pipetting and stored at 4°C for several weeks without significant loss of RNA coupling capacity.

Blocking of the Carboxyl Groups: The procedure we describe is adapted from earlier work on peptides (Hoare and Koshland, 1967). Approximately 1/3 of the hydrazide sepharose prepared as described above (∿30 ml settled resin) was washed with 500 ml 2X distilled water and resuspended in 2X distilled water to a total volume of 67 ml in a 150-ml beaker and stirred magnetically at room temperature. Next, 5.3 g of glycine amide·HCl (Cyclo) was added and the pH adjusted to 4.75 with 1 N NaOH after which 0.67 g 1-ethyl-3-(dimethylaminopropyl) carbodiimide·HCl was added. The pH was maintained at 4.75 with 1 N HCl for 2 hr. The blocked hydrazide resin was washed at room temperature with 1.5 ℓ of 2X distilled water and then suspended in a convenient pipetting volume of 5×10^{-4} M EDTA, pH 7.2 and stored at 4°C. It should be noted that the blocking reaction described herein introduces a functional

group(s), in addition to the amide that blocks free carboxyls, which we have not identified but which is capable of nonspecifically binding polynucleotides to the surface of the resin (see text). This binding capacity is lost over a period of a few months on storage at 4°C.

Sources of RNA: t-RNA and synthetic polyribonucleotides were purchased from Schwarz Bioresearch; a portion of the 16S and 23S ribosomal RNA used in this study was prepared from subunits by centrifugation of the subunits in sucrose gradients containing SDS (Jeanteur, Amaldi and Attardi, 1968). Another portion of 16S r-RNA was isolated by phenol extraction of subunits of E. coli B ribosomes and was the kind gift of T. Yamane.

Alkaline Phosphatase Treatment: A stock solution of alkaline phosphatase was prepared by diluting 20 λ of concentrated enzyme suspension into 5 ml of 0.2 M tris acetate, .01 M Mg acetate, pH 7.7. The solution was then heated at 90°C for 10 min. To one volume of RNA (.5-3 mg/ml) in .2 M tris acetate, .01 M Mg acetate, pH 7.7 was added an equal volume of the diluted alkaline phosphatase solution. The solution was then incubated at 37°C for 1 hr. The RNA was then chilled in ice and precipitated with 2 volumes ethanol. The precipitate was collected by centrifuging 5' at 8000 rpm in the SS-34 rotor of the Sorvall centrifuge. The pellet of RNA was dissolved in an amount of 0.1 M Na acetate buffer, pH 5, equivalent to its initial volume and stored at -20°C. The t-RNA used in these experiments was not treated with alkaline phosphatase.

Oxidation: To 1 ml of alkaline phosphatase treated RNA or t-RNA (.5-3 mg/ml) was added 0.14 ml of a fresh solution of 0.2 M $NaIO_4$ (meta) dissolved in doubly distilled water. The oxidation proceeded for 1 hr at room temperature (23-25°C) in the dark after which time it was stopped by the addition of .08 ml ethylene glycol followed by incubation in the dark for an additional 15' at room temperature. The oxidized RNA was then either precipitated with ethanol as described above or was dialyzed against several changes of 0.1 M sodium acetate buffer, pH 5, at 4°C over a 24-hr period. The oxidized RNA samples were stored at -20°C and used over a three-day period. This oxidation procedure is similar to previously reported methods (Hunt, 1965; McIlreavey and Midgely, 1967). Control samples of unoxidized RNA were carried through all of the above steps except for the addition of $NaIO_4$ in which case distilled water was substituted.

Coupling of RNA to Resins: Small portions of the resin suspensions prepared as described above were washed several times in 0.1 M Na acetate, pH 5, prior to the addition of RNA. These washings are conveniently carried out in 13 x 100 mm Kimax screwcap glass culture tubes. Separation of resins from the washing can be obtained by centrifuging 1-2' in a clinical centrifuge. For resins which did not exhibit nonspecific binding of polynucleotides, oxidized RNA and control (unoxidized) samples were added to resin suspensions washed as described above.

After appropriate incubation times at 25°C, the resin was centrifuged as described and the absorbance for the supernatant

at 260 nm minus the absorbance at 330 nm was determined. Coupling of the oxidized RNA was indicated by a decrease in this absorbance from the initial (t = 0) value. After taking the absorbance of the supernatant, the supernatant was returned to the reaction tube and the resin resuspended. Reaction mixtures were kept in suspension by gentle rocking on a wrist-action shaker. Conditions for the reported experiments were determined by varying the concentrations of RNA and resins so that reactions would be essentially complete within 2 hrs. After completion of the coupling reaction to the unblocked resins, the resins were washed exhaustively with 0.1 M Na acetate buffer, pH 5.0, and then digested with pancreatic RNase at 50 μg/ml in SSC 1 hr at 25°C. The supernatant absorbance at 260 nm was monitored to determine the amount of coupled RNA released by the RNase digestion. In all cases there was good agreement between the amount of RNA coupled as estimated by observing the supernatant absorbance as a function of time and the amount of coupled RNA estimated by RNase digestion of the resin.

For experiments in which the polynucleotide was bound to the surface of the resin for coupling of the RNA (the blocked hydrazide sepharose), the time course of reaction was followed by dilution with Na bicarbonate buffer, pH 9, which proved adequate for releasing uncoupled, nonspecifically bound RNA from the surface of the resin. The absorbance of these supernatants indicated an amount of RNA coupled to the resin in the specified time period which was found

to be in good agreement with samples which had been reacted for specified time periods, quenched, and washed exhaustively in 0.1 M Na bicarbonate buffer at pH 9 and subsequently digested with RNase as described above. The amounts of RNA coupled to the resin were corrected for hyperchromicities accompanying RNase digestion of the RNA. These hyperchromicities at 260 nm in SSC at 25°C were found to be 21.6% (t-RNA), 6.4% (pr-U), 46.0% (p-rC), and 11% (16S r-RNA).

Coupling of Small Molecular Weight Aldehydes: The resin content of hydrazides was checked by performing coupling reactions with benzaldehyde dissolved in 0.1 M Na acetate, pH 5.0. Supernatant absorbance at 248 nm was determined as a function of time.

Solutions of the mononucleotide UMP were oxidized with equivalent amounts of $NaIO_4$ as previously described (Hunt, 1965). The coupling to the various resins was followed by determining the absorbance at 260 nm of the resin supernatant as a function of time as described for the polynucleotides. The coupling of UMP is a measure of the coupling capacity of a small charged molecular weight moiety, in contrast to the uncharged benzaldehyde molecule. Spectra were also taken throughout the course of all the coupling reactions to verify that the UV absorbance was associated with the compound of interest.

RESULTS AND DISCUSSION

Sepharose 4B can be activated by cyanogen bromide (Axen, Porath and Ernback, 1967) to nucleophilic attack by ε-amino-caproic acid methyl ester and subsequently reacted with hydrazine to produce a hydrazide sepharose. This hydrazide sepharose (HS) is capable of reacting with small molecular weight aldehydes such as benzaldehyde and periodate oxidized UMP. The coupling of t-RNA and especially 16S r-RNA is less effective and occurs at a slower rate (see Table 1). The number of moles of RNA coupled at saturation, presented in Table 1, demonstrate that the larger the RNA the smaller the amount of RNA coupled per unit amount of resin. This effect might be the result of having negative charges on the surface of the resin produced perhaps by the hydrolysis of the caproic acid ester. The hydrazide sepharose was therefore treated with glycine amide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide to block these acid functions as amides (Hoare and Koshland, 1967). The resulting blocked hydrazide sepharose (BHS) now exhibits nonspecific binding of polynucleotides. This nonspecifically bound polynucleotide can be released from the surface of this resin by raising the pH of the suspension. The manner in which RNA bound at pH 5 is released from the resin surface as the pH is raised is similar to the titration of a monobasic acid with an apparent pK_a of 7.5. This pK_a is defined as the pH at which 1/2 of the total bound t-RNA has been

Table 1

Properties of the Coupling Reaction of Various Compounds to Hydrazide-Derived Sepharoses

Compound	Initial Concentration (Molarity)	Resin	Volume of settled resin (ml)	Reaction volume (ml)	$t_{1/2}$ * (min)	Moles coupled per ml settled resin at saturation
Benzaldehyde	1×10^{-4}	Sepharose (unreacted)	0.13	2.00	∞†	0
"	1×10^{-4}	Hydrazide sepharose (HS)	0.13	2.00	65	1.1×10^{-6}
Unoxidized UMP	1×10^{-4}	"	0.26	2.00	∞	0
Oxidized UMP	1.04×10^{-4}	"	0.26	2.00	60	0.6×10^{-6}
Unoxidized t-RNA	2.64×10^{-6}	"	0.26	1.52	∞	0
Oxidized t-RNA	2.42×10^{-6}	"	0.26	1.52	65	8.0×10^{-9}
Unoxidized 16S r-RNA	1.09×10^{-7}	"	0.26	1.52	∞	0
Oxidized 16S r-RNA	1.00×10^{-7}	"	0.26	1.52	70	0.1×10^{-9}
Benzaldehyde	1×10^{-4}	Sepharose (untreated)	0.13	2.00	∞	0
"	1×10^{-4}	Blocked hydrazide sepharose	0.13	2.00	120	0.8×10^{-6}
Unoxidized UMP	1×10^{-4}	"	0.26	2.00	∞	0
Oxidized UMP	1×10^{-4}	"	0.26	2.00	120	1.1×10^{-6}
Unoxidized t-RNA	6.8×10^{-6}	"	0.026	0.52	∞	0
Oxidized t-RNA	6.8×10^{-6}	"	0.026	0.52	18	87×10^{-9}
Unoxidized 16S r-RNA	2.8×10^{-7}	"	0.021	0.52	∞‡	0
Oxidized 16S r-RNA	2.8×10^{-3}	"	0.021	0.52	9	3.5×10^{-9}

All reactions were carried out at ambient temperature (25 ± 1°C).

*Reaction time required to attain one-half the maximum amount of RNA coupled for the reaction conditions indicated.

†Symbol to indicate that no coupling was observed.

‡A low level of unoxidized r-RNA (<10% of the input r-RNA) bound to the surface of this resin, was not released by washing at pH 9 (see Text).

released to the supernatant. More than 95% of the bound t-RNA is released at pH 9.

The quantity of RNA, expressed in moles, that was nonspecifically bound per ml of settled resin was observed to be inversely proportional to the hydrodynamic radius of the RNA when the RNA is viewed as a non-free-draining ideal random coil and followed the relation, $n \sim 6 \times 10^{-8}/S_{20,w}$. It has not been possible to determine the chemical nature of this binding group(s) that is formed in the blocking reaction. Amino acid analysis of the blocked resin showed that a net increase of 243 millimicromoles of glycine per ml of settled resin did result from the blocking reaction.

The rates and saturation levels of coupling for benzaldehyde and UMP to this blocked hydrazide sepharose were similar and corresponded approximately to the values obtained with unblocked hydrazide sepharose (HS) (Table 1); however, when periodate oxidized RNA was bound to the surface of BHS for a specified amount of time and then the reaction quenched with 0.1 M Na bicarbonate buffer at pH 9, it was found that significant quantities of t-RNA and 16S r-RNA had been coupled to the resin. The amount of RNA coupled to the resin at each time point, as indicated by pH 9 release of uncoupled RNA to the supernatant, was found to be in good agreement with the amount of RNA coupled estimated by exhaustively washing with 0.1 M Na bicarbonate, pH 9, and subsequently digesting with

RNase in SSC. The saturation levels of coupling presented in Table 1 for t-RNA and 16S r-RNA have increased ∿11X and ∿30X, respectively. Furthermore, the rate of coupling for these polynucleotides has also markedly increased as evidenced by the time required to attain 1/2 of the maximum amount coupled (Table 1). This phenomenon might be explained in terms of a reduction in dimensionality for this coupling reaction for binding the polynucleotide to the surface of the resin (Adam and Delbrück, 1968).

We were also able to demonstrate that RNA coupled to BHS is available for hybridization with complementary sequences. This is illustrated by the hybridization of p-rA from aqueous solutions (Fig. Ia) or from solutions containing DMSO (Fig. Ib) to p-rU which had been coupled to BHS. The controls in this experiment were p-rC attached to BHS and BHS treated with unoxidized p-rU carried through all steps in the coupling of oxidized p-rU. If the p-rU resin with p-rA hybridized to it is then incubated in increasing concentrations of DMSO, at constant ionic strength, there is a release of the p-rA from the complex of p-rU resin (Fig. Id) at approximately the same point at which a 1:1 complex of p-rU and p-rA denatures in solution (Fig. Ic). This experiment infers that a correct base-paired polynucleotide had been formed in the resin hybridization.

FIGURE LEGEND

Fig. I. Hybridization of p-rA to resin-coupled p-rU.

(a) Hybridization of p-rA to p-rU which had been coupled to BHS. The concentration of p-rA, indicated by the absorbance at 256 nm in the supernatant, was determined as a function of time (-o-o-). The buffer used was 0.1 M NaCl, .01 M Na cacodylate, pH 6.98. The hybridization was carried out at 25°C in a total volume of 1.0 ml of which 0.13 ml represented the settled resin to which polynucleotide had been coupled. One control sample consisted of p-rC coupled to BHS (a total of .45 A_{268} absorbance units) (-□-□-). The other control sample consisted of BHS resin incubated with unoxidized p-rU which was subsequently washed as described in Materials and Methods (-Δ-Δ-). This resin contained <.06 A_{260} absorbance units of p-rU. The BHS sample to which p-rU had been coupled contained .78 A_{260} absorbance units of p-rU.

(b) Same as for (a) except that the hybridization buffer is .1 M NaCl .01 M Na cacodylate, pH 6.98, 50 volume percent DMSO.

(c) Solution denaturation as evidenced by the hyperchromicity (h_{260}) of a 1:1 complex of p-rA and p-rU by increasing concentrations of DMSO (volume %) at constant ionic strength (0.1 M NaCl, 0.01 M Na cacodylate, pH 6.98) at 25°C. The decrease indicates in hyperchromicity at concentrations of DMSO greater than 80% probably

(continued)

(Legend to Fig. I, continued)

indicates precipitation of the polynucleotide(s). The midpoint of the denaturation corresponds to 65.6% DMSO.

(d) DMSO denaturation at constant ionic strength (0.1 M NaCl, 0.01 M Na cacodylate, pH 6.98) of the complex formed between p-rA and BHS-release of p-rA coupled p-rU, resulting in release of p-rA to the supernatant. The samples used here were derived from part (b) after exhaustive washing with the hybridization buffer. The midpoint for release of p-rA to the supernatant corresponds to 66.5% DMSO.

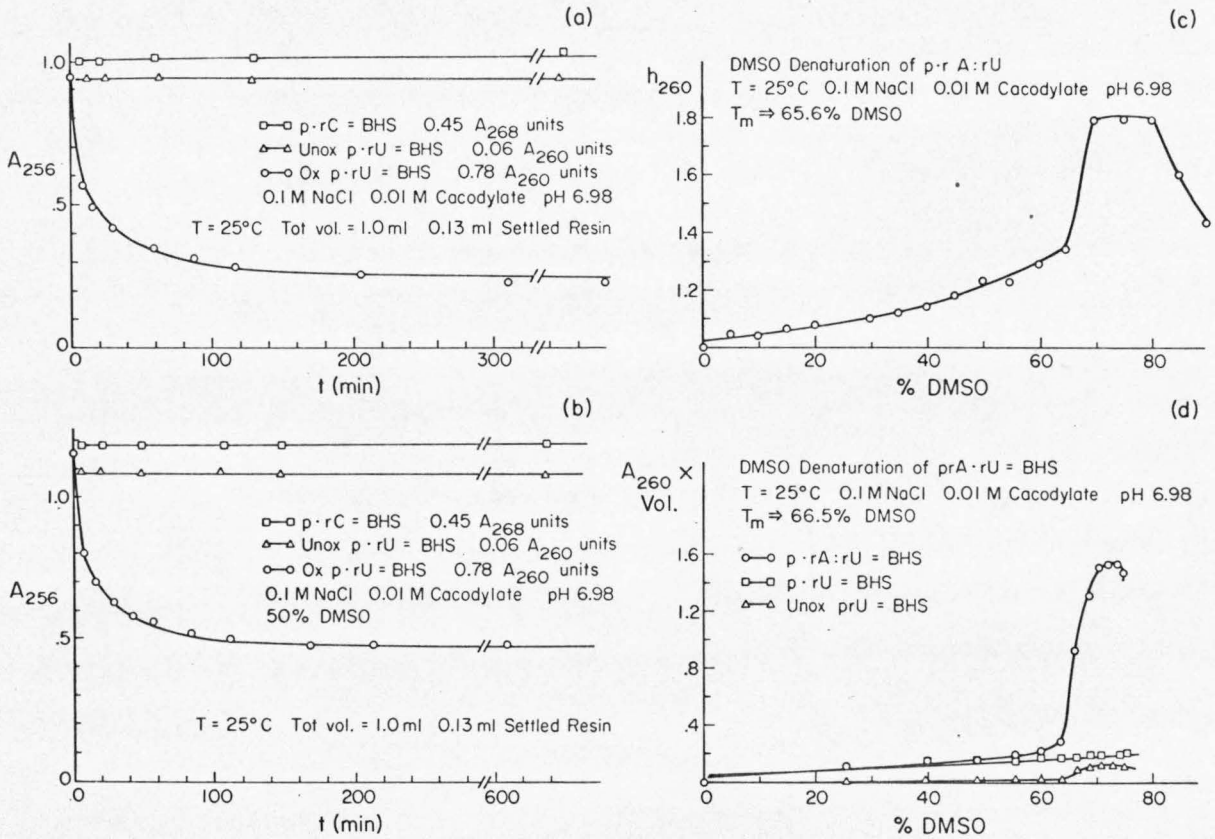
(a)
p·rC = BHS 0.45 A_{268} units
Unox p·rU = BHS 0.06 A_{260} units
Ox p·rU = BHS 0.78 A_{260} units
0.1 M NaCl 0.01 M Cacodylate pH 6.98
T = 25°C Tot vol. = 1.0 ml 0.13 ml Settled Resin
A_{256}
t (min)
(b)
p·rC = BHS 0.45 A_{268} units
Unox p·rU = BHS 0.06 A_{260} units
Ox p·rU = BHS 0.78 A_{260} units
0.1 M NaCl 0.01 M Cacodylate pH 6.98
50% DMSO
T = 25°C Tot vol. = 1.0 ml 0.13 ml Settled Resin
A_{256}
t (min)
(c)
DMSO Denaturation of p·r A:rU
T = 25°C 0.1 M NaCl 0.01 M Cacodylate pH 6.98
$T_m \Rightarrow$ 65.6% DMSO
h_{260}
% DMSO
(d)
DMSO Denaturation of prA·rU = BHS
T = 25°C 0.1 M NaCl 0.01 M Cacodylate pH 6.98
$T_m \Rightarrow$ 66.5% DMSO
p·rA:rU = BHS
p·rU = BHS
Unox prU = BHS
A_{260} × Vol.
% DMSO

Fig. 1

Coupling of specific RNAs to these resins should allow, in principle, the isolation of the correct complementary DNA sequences.

ACKNOWLEDGMENT

We express our gratitude to Dr. Michael Raftery for the helpful discussions and assistance in the preparation of this work.

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Part II

REPLICATION OF CLOSED CIRCULAR DNA IN MOUSE L CELLS

This section is the preprint of

A Novel Closed Circular Mitochondrial DNA
with the Properties of a Replicating Intermediate

by Harumi Kasamatsu, Donald L. Robberson, and Jerome Vinograd.

This paper has been submitted for publication to the
Proceedings of the National Academy of Sciences.

Biological Sciences: Biochemistry

A Novel Closed Circular Mitochondrial DNA with the Properties of a Replicating Intermediate

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Communicated June , 1971

Running title: Displacement Loops in Mitochondrial DNA

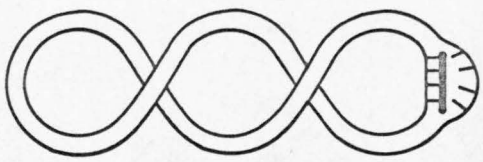
Abbreviations: TE, 10 mM Tris, 1 mM EDTA; EB, ethidium bromide; H, heavy; L, light.

ABSTRACT A fraction of the covalently closed mitochondrial DNA in mouse L cells contains a replicated heavy strand segment that is hydrogen-bonded to the circular light strand. The inserted single strand is dissociable from the circular duplex at elevated temperature.

Introduction

Mitochondrial DNA from animal cells is known to occur in the form of closed circular molecules with a molecular weight of about 10 million daltons (1). In the course of a study of the mechanism of replication of this DNA in two strains of mouse L cells, we have found that approximately one half of the covalently closed molecules in exponentially growing cells contain a short three-stranded DNA region which we have called a D-loop, or displacement loop (Fig. 1). The D-loop appears to have been formed in the closed DNA by displacement synthesis of a short progeny strand with a specific region of the light mitochondrial strand serving as a template. This communication presents the results of experiments designed to elucidate the structure and properties of this new kind of closed circular DNA and considers its role as an intermediate in the replication process.

Figure 1. Diagrammatic representation of closed circular D-mtDNA containing a displacement loop. The displacing strand is represented by a heavy line and the displaced strand by a curved line with attached bars.



MATERIALS AND METHODS

The two cell lines chosen for this study were the LD line (our designation) obtained from C. Schildkraut, Albert Einstein College of Medicine, New York, and the LA9 line isolated by Littlefield (2) and obtained from L. Crawford, Imperial Cancer Research Fund, London. Mitochondrial DNA (mtDNA) in the LD cells occurs in the form of circular dimers with a contour length of 10 μ. The LA9 cells contain monomeric mtDNA. The spinner-adapted cells were grown in Dulbecco's modified Eagle's phosphate medium containing 10% calf serum. The generation times of the LA9 and LD cells were 22 and 20 hr, respectively. Exponentially growing cells, about 2×10^5 cells/ml, were labeled with 0.5 μCi/ml of [^{3}H] thymidine (Schwarz/Mann), 15.6 Ci/mmole, for 3 hr prior to harvest.

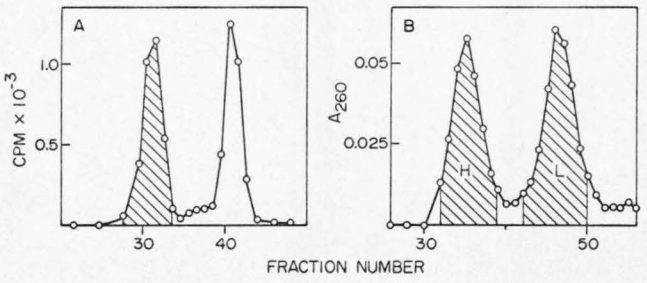
Isolation of crude mitochondria. Labeled cells were harvested at 0–4° C by centrifugation at 1500 *g*, washed with 0.14 M NaCl, 5 mM KCl, 7 mM Na_2HPO_4, 25 mM Tris, pH 7.5, and resuspended in a 10-fold volume of TE (10 mM Tris, 1 mM EDTA), 10 mM NaCl, pH 7.5. After 10 min, the cells were disrupted with 7–10 strokes in a Dounce homogenizer. After addition of 1.5 M sucrose to 0.25 M, approximately 20 ml of the homogenate was layered onto 15 ml of 1.5 M sucrose containing TE, pH 7.5, and centrifuged for 30 min at 25,000 rpm, 4° C, in a Spinco SW27 rotor. The crude mitochondrial fraction withdrawn from the interface was diluted 6-fold with 10 mM Tris, 2 mM $MgCl_2$ (pH 7.5), centrifuged for 15 min at 20,000 *g*, and resuspended in 0.25 M sucrose, 5 mM $MgCl_2$, 5 mM NaCl, 10 mM Tris (pH 7.2). The mitochondrial suspension was incubated with 25 μg/ml DNase I and 250 μg/ml RNase A (both from Sigma

Chemical Company) for 45 min at 37° C. The suspension was chilled to 0° C, EDTA was added to a concentration of 25 mM, and the mitochondria were washed twice by centrifugation with MS buffer (3).

Isolation and purification of mtDNA. MtDNA was extracted from the washed mitochondria, banded in ethidium bromide-CsCl, and purified by velocity sedimentation as described elsewhere (4, Methods *a*(i)–(iii)), except that the incubation with SDS was performed at 37° C. The band velocity sedimentation was performed by layering the sample after dialysis against 10 × TE, 0.1 M NaCl, pH 8.5, onto 4 ml of CsCl (3.5 ml, 1.4 g/ml; 0.5 ml, 1.7 g/ml) containing 100 μg/ml ethidium bromide (EB) (Boots Pure Drug Co. Ltd), and centrifuging for 5 hr at 38,000 rpm, 20° C, in an SW50.1 rotor. The total mtDNA was collected and rebanded in EB-CsCl at 30,000 rpm, 20° C, for 40 hr in an SW50.1 rotor. 10-drop fractions were collected from the bottom of the tube and aliquots of the fractions were counted in a scintillation spectrometer (Fig. 2*A*). The DNA samples were dialyzed in the dark against 2 ml of Dowex 50 resin suspended in 25 ml of 10 × TE, 0.1 M NaCl, pH 8.5.

Preparation of the complementary strands of mtDNA. MtDNA isolated from lower bands in EB-CsCl gradients was obtained from LA9 cells labeled for 24 hr with [^{14}C]thymidine (Schwarz/Mann) (0.04 μCi/ml, specific activity 50.8 mCi/mmole. Nonlabeled lower-band mtDNA was added to adjust the specific activity of the DNA to 100 cpm/μg. The mixture was dialyzed into TE, 10 mM NaCl (pH 7.5) containing Dowex 50 resin, then into 95% formamide in 1 mM Tris, 1 mM EDTA (pH 7.5) at room temperature for 40 min, and finally into TE, 10 mM NaCl for 1 hr.

Figure 2. Buoyant sedimentation patterns. *A*, total purified mtDNA in an EB-CsCl density gradient. *B*, the separated complements from closed mtDNA in an alkaline CsCl density gradient. The field is directed to the left.


A
B
CPM x 10^-3
1.0
0.5
A260
0.05
0.025
H
L
30
40
30
50
FRACTION NUMBER

The DNA was sedimented through 1.4 g/ml CsCl, 100 μg/ml EB, to remove 7S displacing strands from the closed circular DNA. The complementary DNA strands were separated in an alkaline CsCl gradient (50 mM K_3PO_4, 1.750 g/ml CsCl, pH 12.4, at 30,000 rpm, 20° C, for 60 hr in an SW39 rotor). 5-drop fractions were collected into 0.4 ml of 20 mM Tris, 1 mM EDTA, pH 7.5, and the absorbances at 260 nm were measured.

Electron microscopy. Samples for electron microscopy were prepared by the formamide basic protein method essentially as described previously (5). In a typical preparation, 2 μl of 1.0 M Tris-base, 30 mM disodium EDTA, pH 8.4, and 3–5 μl of H_2O were added to 8 μl of 99% formamide. The solution was chilled to 0° C, and 2–4 μl of DNA in TE, 0.1–0.5 M NaCl, pH 8.0, and 3 μl of cytochrome *c* (1 mg/ml in TE, pH 8.0–8.5) were added. The solution was layered onto a hypophase of 10% formamide, 10 mM Tris, 3 mM EDTA (pH 8.4). Films were picked up on parlodion-coated screens and rotary-shadowed with Pt-Pd wire or stained with uranyl acetate (6). Measurements were made from tracings of negatives enlarged on a Nikon 6F projection comparator.

Hybridization of 7S DNA to heavy and light strands. The fractions containing the heavy (H) and light (L) mtDNA strands from the alkaline CsCl gradients were appropriately pooled (Fig. 2*B*) and concentrated by evaporation. The 7S single-stranded DNA was obtained from labeled closed mtDNA by heat treatment and two sucrose gradient sedimentations. The sample was evaporated to about 0.05 μg/ml, an estimate based on the assumption of complete recovery of 7S DNA. A portion of each of the

complements was mixed with 7S DNA so that the final concentrations were 3.0 μg/ml H or L strand, 0.01 μg/ml 7S DNA, and 2.0 M CsCl. A control sample contained only 7S DNA in the same solvent. The samples were heated at 75° C for 8.5 hr. It was calculated (7) that more than 90% of the 7S DNA should have reannealed with a mitochondrial complement under these conditions. The hybridized samples were centrifuged in 3.0 ml of CsCl with a density of 1.710 g/ml for 72 hr at 30,000 rpm, 20° C, in an SW50 rotor.

RESULTS

Displacement loops are readily seen in the electron microscope on specimen grids prepared by the formamide basic film method (Fig. 3). That the D-loop region contains both a duplex DNA segment and a looped-out single-strand DNA segment may be concluded from an inspection of the four examples shown. The single-strand region is thinner and has a less regular contour than the duplex region. It is similar in appearance to the viral ϕX DNA added as a marker. The single-strand region must be DNA rather than RNA, because RNA under these spreading conditions is not fully denatured and collapses with an approximate five-fold shortening of the length (8). The lengths of the single-strand and the duplex parts of the D-loop are approximately equal (Table 1). Moreover, the frequency of D-loops in the purified DNA is unaffected by a preincubation of the DNA with RNase A (Table 2).

The duplex region, indistinguishable in appearance from the whole circular duplex, is considered to be a DNA-DNA duplex rather than a DNA-RNA hybrid. Chemical experiments presented below show that heating the sample to 90° C in 0.03 M NaCl causes the release of a 7S alkaline-resistant single-stranded DNA (Fig. 4*F*) with the simultaneous loss of displacement loops.

The relative frequency of molecules containing D-loops (D-mtDNA) in the closed DNA isolated from lower bands in EB-CsCl buoyant gradients was 34% and 51% in two separate preparations of mtDNA from LA9 cells (Table 2). Early in this study it was observed that exposure of the closed DNA to low ionic strengths (*e.g.*, 20 mM NaCl) results in a loss

Figure 3. Electron micrographs of closed circular mtDNA forms and purified displacing strands. *A* and *B*. Closed mtDNA from LA9 cells was prepared for electron microscopy by the formamide technique, and stained and shadowed. Displacement loops are indicated by arrows. A single-stranded ϕX DNA is present in *A*. *C*, 7S DNA isolated from a sucrose gradient after sedimentation of heat-treated closed mtDNA from LD cells and prepared for electron microscopy as in *E*. *D*. The thin region between the arrows in an enlargement of a D-loop in the molecule in *E* indicates that the duplex portion of the D-loop appears to be single-stranded near the upper fork. The contrast has been reversed in printing. *E*, a closed mtDNA from LD cells with two displacement loops. The nonshadowed specimen grid was stained and visualized by dark-field electron microscopy.

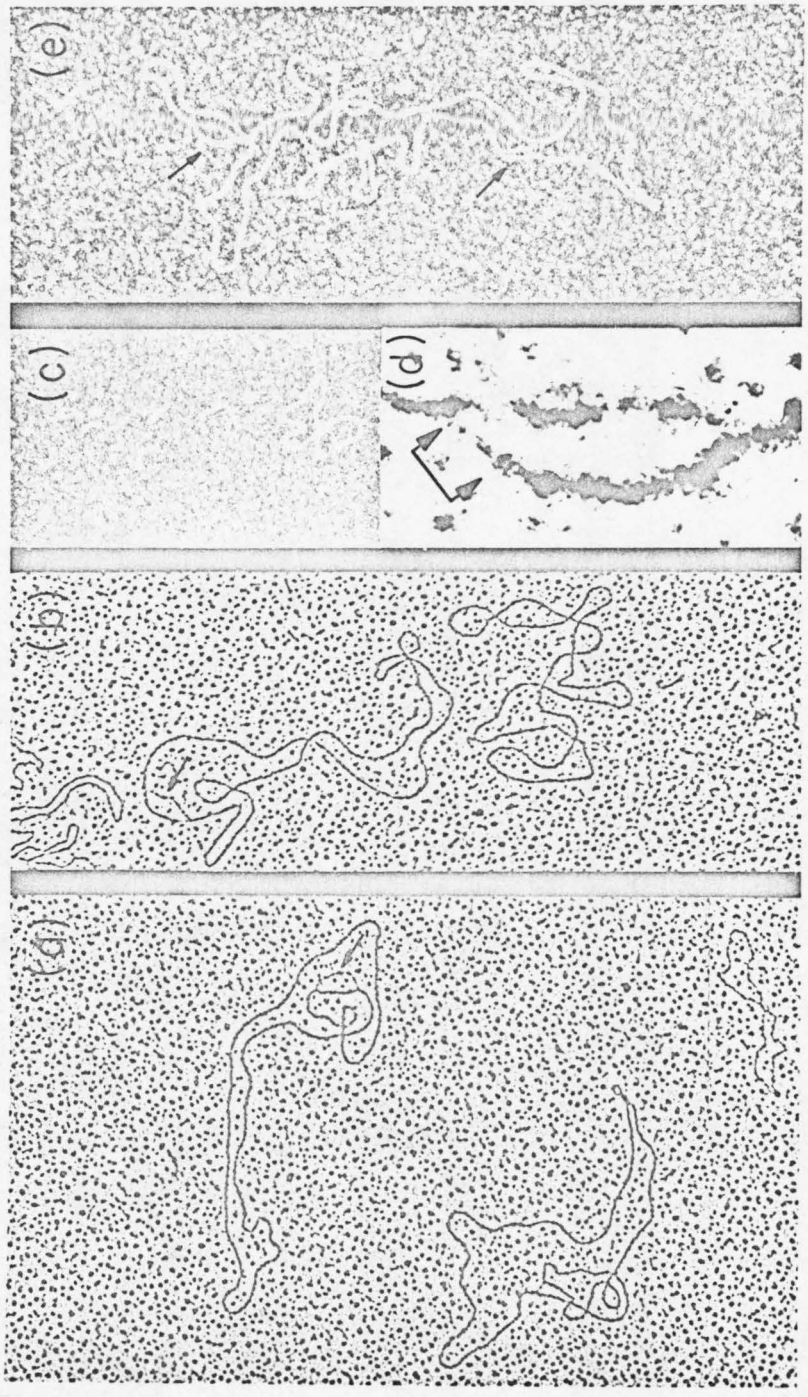

(e)
(c)
(d)
(b)
(a)

TABLE 1. The size of the duplex and single-stranded regions in the D-loops of closed mtDNA from LA9 and LD cells and the size of 7S DNAs dissociated from the D-mtDNAs

	(Wt. av. length/Monomer length) 100	
	LA9	LD
Duplex region	3.5 ± 0.7 (38)	3.2 ± 0.8 (60)
Single-stranded region	3.5 ± 1.0 (38)	3.2 ± 1.0 (60)
7S single strands	3.1 ± 0.5 (351)	2.3 ± 0.5 (365)

The measured fork-to-fork distances were normalized by the mean length of the circular duplex DNA on the same specimen grid and, in the case of single-stranded DNA, by the contour length of viral ϕX DNA. The values of 1.7 and 9.6×10^6 were taken as the molecular weights of ϕX DNA and mtDNA, respectively. The number of molecules measured is given in parenthesis.

of D-loops, presumably by the process of branch migration (9). In this case progressive pairing of homologous nucleotides in the circular strands displaces the short single strand. Incomplete branch migration would result in the formation of a circular molecule with a single-stranded tail. If the latter form is taken to be indicative of D-mtDNA, the frequency of D-mtDNA rises to 49% and 56% in the two preparations. The fluorescence photographs of the EB-CsCl gradients and the distribution of radioactivity (Fig. 2*A*) indicated that approximately one half of the mtDNA was in the lower band; we may, therefore, conclude that about one fourth of the total mtDNA in our preparations contains displacement loops. This surprisingly large frequency in nonsynchronized cells implies that D-mtDNA accumulates and may constitute a "hold point" in the replication process.

The possibility arose that D-mtDNA was formed during the incubation of mitochondria at 37°C with DNase I—the only step in the preparative procedure prior to lysis in which the temperature rose above 4°C. One half of the partially purified mitochondria obtained in preparation 2 was therefore processed in the normal way, while the other half was lysed directly with SDS. The unchanged frequency of D-mtDNA (Table 2) demonstrates that any DNA synthesis that might occur during the incubation does not contribute significantly to the frequency of D-mtDNA. Incubation of a portion of the purified closed DNA with 5 μg/ml RNase A had no significant effect on the frequency of D-mtDNA (Table 2).

Fractionation and heat treatment of closed mtDNA

From our knowledge of the effects of superhelical turns on the

TABLE 2. Frequency of D-mtDNA molecules in closed mtDNA isolated from LA9 cells growing exponentially in suspension culture

	Preparation			
Duplex circles, %	1	2	2a*	2b†
With D-loops	34	54	51	58
With single-stranded tails	15	1	6	2
Clean	50	45	48	40
Molecules classified	125	207	189	163

* A part of the crude mitochondria used in preparation 2 was processed with the omission of the DNase treatment as described in the text.

† A part of the closed DNA in preparation 2 was treated with RNase as described in the text prior to preparation of the specimen grid.

sedimentation velocity properties of closed duplex DNA, we anticipated that the D-mtDNA containing a displacing strand with approximately 3% of the genome length might have a significantly lower sedimentation velocity than the mature DNA. This proved to be the case, as shown in sedimentation velocity experiments performed in 0.5 M NaCl at 20°C (Fig. 4*A* and *B*). The peak fraction, 23, from the leading 38S band contained 13% D-mtDNA as compared with 82% and 60% from selected fractions in the 27S band (Table 3).

Brief heating of unfractionated closed DNA, as well as dialysis against 90% formamide, resulted in the formation of a new species of DNA with a sedimentation velocity of about 7S in sucrose gradients containing 0.5 M NaCl. The 7S component was resistant, as evidenced by acid-precipitable radioactivity, to the action of 0.5 M NaOH for 3 hr at room temperature. To clarify the origin of the 7S DNA, the material obtained upon sedimenting 2 μg of closed mtDNA was pooled as indicated to form the fast (F) and the slow (S) samples (Fig. 4*B*). The separate samples were dialysed into 30 mM salt, heated to 90°C for 30–40 sec, and chilled in ice. The sedimentation velocity experiments demonstrate that the 7S DNA derives from the S sample. Dissociation of the displacing strand from the 28S D-mtDNA results in the formation of a closed DNA (C′-mtDNA) with a sedimentation coefficient of 38S. This is the sedimentation coefficient of the major D-loop–free DNA (C-mtDNA) which, prior to heating, was present in the fast sample (Fig. 4*C*), but was substantially absent in the slow sample (Fig. 4*D*). A portion of the heated S sample still sediments as an intermediate band with a sedimentation coefficient of about 28S. This band, fraction 27 (Table 3),

Figure 4. Sedimentation patterns of LA9 mtDNA from the lower band of an EB-CsCl buoyant density gradient (Fig. 2). The DNA was sedimented in 5–20% sucrose gradients containing TE, 0.5 M NaCl, pH 8.0, at 20° C for 130 min, 36,000 rpm in an SW50.1 rotor. The arrow ↓ indicates the center of a band of ^{14}C-labeled T7 DNA. The arrow ↑ indicates a fraction taken for analysis by electron microscopy (Table 2). Sedimentation proceeds to the left. (*A*) and (*B*), patterns obtained with 1.2-μg and 3-μg samples, respectively. (*C*) and (*D*), patterns obtained with the F and S samples pooled as indicated in (*B*). (*E*) and (*F*), the F and S samples dialysed against TE, 10 mM NaCl, pH 7.5, were heated at 90° C for 40 sec and quenched in ice prior to the centrifugation.

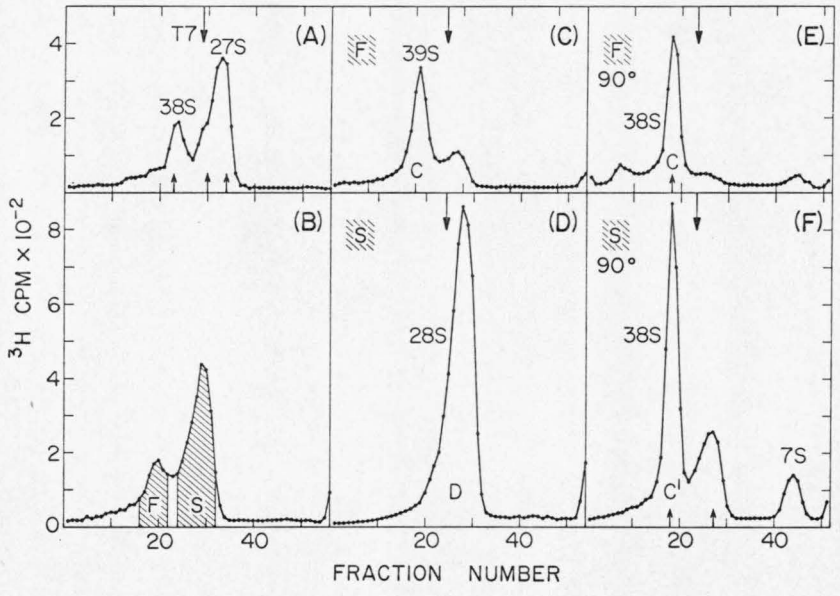

T7
27S
(A)
38S
(C)
F
39S
C
(E)
F
90°
38S
C
(B)
F
S
(D)
S
28S
D
(F)
S
90°
38S
7S
C'
^{3}H CPM X 10^{-2}
FRACTION NUMBER

TABLE 3. Frequency of D-loop molecules and other forms of mtDNA obtained upon partial purification and thermal treatment of closed mtDNA from LA9 cells

	Unheated			Heated		
	4*A**			4*E*	4*F*	
Duplex circles (%)	23†	30	34	18	18	27
With D-loops	13	82	60	0	0	9
With single-stranded tails	1	1	2	0	1	3
Clean	85	18	39	100	99	87
Molecules classified	196	133	164	109	112	79

* Figure no. † Fraction no.

contains E-mtDNA, a closed duplex with a very low superhelix density, as well as some single-stranded DNA.

Size of the D-loops and the thermally dissociated single strands

The duplex and the single-stranded regions measured on enlarged tracings of the electron micrographs appeared to be the same size and to correspond to 3.2–3.5% of the genome size (Table 1). The mean length of 7S single-stranded DNA obtained on heating D-mtDNA from LA9 cells corresponds to 3.1 ± 0.5% of the genome length. The double-strand portion of the D-loop often appears to be thin at one or both of the forks (Fig. 3*D*), as though a portion of the duplex adjacent to the D-loop had been denatured by the spreading forces during specimen preparation. We therefore accept the smaller measured length of the 7S single-stranded DNA as the more reliable estimate of the length of the D-loop. The standard deviation (±18%) of the length measurements is smaller than that expected (±27%) for a homogeneous DNA of this size (5). The mean size corresponds to a molecular weight of $1.5 \pm 0.25 \times 10^5$ daltons and to a molecule with 450 ± 80 nucleotides. The D-loops in the monomer mtDNA from the LA9 cells appear to be somewhat larger than in the dimer mtDNA from the LD cells.

Strand specificity in D-loop formation

To determine whether strand specificity is involved in the formation of the D-loop, the purified 7S displacing strands were self-annealed and were annealed with an excess of the separated H and L complements of mtDNA obtained from an alkaline buoyant density gradient (12). The

weight-average molecular weights of the separated complements prepared and heated as in these experiments have been reported to be approximately 1×10^6 daltons (6).

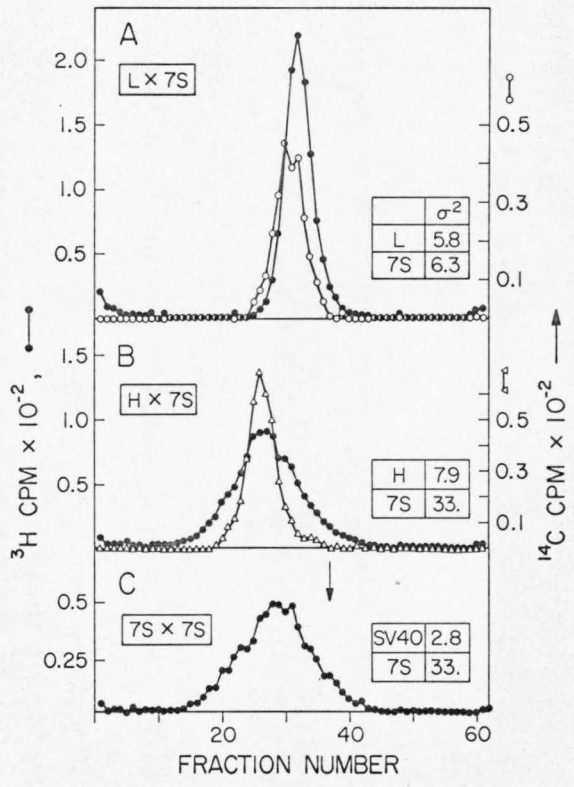
The 7S DNA upon self-annealing (Fig. 5*C*) formed a band with a variance of 33(fraction units)^2. The reciprocal of the variance may be taken to be a rough measure of the molecular weight. The band width of the 7S DNA did not change when the annealing was performed with the H strands having a variance of 8(f. u.)^2 (Fig. 5*B*). The variance, however, narrowed to 6(f. u.)^2 (Fig. 5*A*) when the annealing was performed with the L strands which, in turn, had a variance of 6(f. u.)^2. The above results alone show that 7S DNA is largely, if not completely, complementary to the L strand. The absence of a detectable shoulder of [^{3}H]-thymidine counts that would have been formed by nonhybridized 7S DNA in the experiment in Fig. 5*A* indicates that at least 85% of the 7S DNA anneals with the L strand. The 7S DNA is, therefore, considered to be a segment of the H strand.

The center of the hybrid band formed in the 7S × L annealing experiment is one fraction lighter than the center of the band formed principally by the L fragments free of the sequence complementary to the 7S DNA. The 2 mg/ml buoyant shift is the shift expected upon the formation of a duplex between an L strand fragment with a molecular weight of 1×10^6 and 7S DNA with a molecular weight of 1.5×10^5.

Superhelix densities of closed mtDNA from LA9 cells

The superhelix densities (superhelical turns/10 base pairs) of the closed mtDNAs separated in the sucrose gradients (Fig. 4) were determined by

Figure 5. Strand specificity of the isolated 7S DNA. The ^{3}H 7S DNA was annealed with the separated L and H complements of ^{14}C mtDNA and was self-annealed as described in the text. The reaction products were centrifuged to equilibrium in a CsCl density gradient. Density increases to the left. The variance of the bands, σ^2, was calculated with the relation $\sigma^2 = \Sigma c_i f_i^2 / \Sigma c_i - (\Sigma c_i f_i / \Sigma c_i)^2$, where f and c represent the fraction number and the radioactivity corrected for background, respectively. In a separate experiment, the non-annealed 7S DNA formed a band superimposable on the band in *A*.


A
L × 7S
2.0
1.5
1.0
0.5
0.5
0.3
0.1
σ²
L 5.8
7S 6.3
B
H × 7S
1.5
1.0
0.5
0.5
0.3
0.1
H 7.9
7S 33.
C
7S × 7S
0.5
0.25
SV40 2.8
7S 33.
20
40
60
FRACTION NUMBER
³H CPM × 10⁻², ●—●
¹⁴C CPM × 10⁻² ○—○

the buoyant separation method in EB-CsCl gradients (Table 4). The superhelix density of the C-mtDNA in the F sample did not change significantly upon heating. The sample C′, obtained as a fast-sedimenting species (Fig. 4*F*) after heating the slow component in Fig. 4*B*, had a similar superhelix density. These results, together with the sedimentation velocity and the electron microscope analyses, are consistent with the view that the C-mtDNA is the precursor of D-mtDNA. Heating the D-mtDNA melts out the displacing strand and allows the circular duplex on cooling to rewind to the superhelix density of C-mtDNA.

A second closed mtDNA free of D-loops (E-mtDNA) was also present initially in small amounts in all of the unfractionated closed mtDNA preparations examined in this work. This mtDNA, isolated as the slow-sedimenting component after heating sample S, has a low superhelix density, $-1.0 \pm 0.3 \times 10^{-2}$. The E-mtDNA could have formed from C-mtDNA by the action of a nicking-closing cycle during the isolation procedure. Alternatively, it may be a significant intermediate in the formation of mature mtDNA.

The apparent superhelix density calculated from the relative buoyant separation obtained with the S sample (Fig. 4*B*) was -1.2×10^{-2}. The sample contained D-mtDNA, as well as about 10% E-mtDNA. The buoyant position of D-mtDNA in EB-CsCl gradients is not expected to be given by the previously derived relation between the buoyant separation and the superhelix density. The duplex region in the D-loop is free to rotate at the forks (Fig. 1) and to bind EB without restriction. The displaced strand should bind EB with the binding constant of nicked DNA (11). In addition, the single strand can also wind about the duplex and

TABLE 4. Superhelix densities and sedimentation coefficients of closed mtDNAs from LA9 cells

Sample	Sedimentation coefficient, svedbergs	Superhelix density $-\sigma_0 \times 10^2$	
		Unheated	Heated
F (Fig. 4*B*)	38	2.9	—
S (" ")	27	(1.2)	—
C (" 4*E*)	39	—	2.4
C′ (" 4*F*)	38	—	2.8
E (" ")	28	—	1.0

The superhelix densities were determined by the buoyant separation method (10, 11) in an EB-CsCl density gradient. A mixture of closed and nicked ^{32}P viral SV40 DNA was added as a marker. The centrifugation conditions were those described by Eason and Vinograd (11). The estimated measuring error is $\pm 0.3 \times 10^{-2}$ units. The number in parenthesis is to be regarded as an apparent value only. The sample C was a pool of fractions 17–20 (Fig. 4*E*). The sample C′ was a pool of fractions 16–20 (Fig. 4*F*). The sample E was a pool of fractions 24–30 (Fig. 4*F*).

permit an increased binding of EB at high concentration. Nevertheless, the D-mtDNA still behaves as a low superhelix density DNA and may be expected to be enriched on the dense side of the band formed by C-mtDNA.

The buoyant separation experiments provided the further information that we encountered relatively little adventitious nicking while processing the closed DNAs. For example, in the analyses of the C-mtDNA only 8% of the radioactive label is in the upper band which contains nicked DNA. Similar results were obtained with the other samples analysed (Table 4).

Unique site for the formation of the displacement loop

It has previously been shown that human dimer mtDNA possesses a head-to-tail arrangement of two monomer genomes (12). The LD line of mouse cells with >99% circular dimer mtDNA was, therefore, examined for its content of D-loops. The fraction of closed dimer molecules that contain at least one D-loop is again very high, approximately 42%. Dimers containing two D-loops were also observed at a frequency of approximately 15%. We would expect that the two D-loops in the dimer molecules would be diametrically opposed if a unique site existed for the initiation of the replicative events that give rise to the D-loops. The two lengths of DNA which separate the D-loops in the dimers were therefore measured. The means and the standard deviations of the shorter and the longer lengths between the forks, expressed as fractions of the total of these lengths, were 0.48 ± 0.03 and 0.51 ± 0.02 in the 25 molecules measured. We conclude that the dimer D-loops are diametrically opposed and that there is, indeed, a unique site on the genome for the formation of the displacement loop.

DISCUSSION

The experiments presented in this communication demonstrate that a substantial fraction of the covalently closed mtDNA isolated from exponentially growing mouse L cells contains a small, unique segment of the H strand inserted into the closed duplex. Upon brief heating of the isolated displacement DNA, the noncovalently attached H strand segment dissociates and leaves a closed DNA with a sedimentation coefficient and superhelix density indistinguishable from that of the major D-loop–free DNA originally present in the preparation. The physical–chemical results are summarized in functional terms:

$$\text{Displacement DNA} \triangleq \text{Parental DNA} + \text{7S Displacing Strand.}$$

The displacement loops have been shown in the experiments with dimer DNA from LD cells to occur at a unique site on the circular genome. This result rules out the notion that the D-loops are intermediates in a random repair process. While it is likely that the synthesis of the 7S DNA represents the first stage in the formation of progeny molecules, we cannot at present exclude the possibility that D-loops are formed for some other purpose. The fact that larger displacement loops with various sizes up to the genome size have been observed in the mtDNA from the upper band in EB-CsCl gradients (unpublished observations) has suggested to us that 7S DNA in nicked parental molecules is, indeed, extended to form the progeny H strand.

Among the possibilities for the formation of displacement DNA, we describe here a simple mechanism consistent with the experimental

results. The proposed mechanism has two facets. 1. Initiation and synthesis of the 450 ± 80 nucleotide H strand occurs in closed DNA in the absence of nicking-closing cycles. 2. The mature DNA has a zero superhelix density in the cell and acquires its negative superhelix density upon purification and removal of materials that modify the average pitch of the duplex (4). The displacement DNA experiences the same pitch change. A consequence of the above mechanism is that the displacement replication would have to stop when the positive free energy of superhelix formation, ΔG_τ, associated with the topologically required development of a superhelix, is just equal to the negative free energy available from the synthesis reaction. The value ΔG_τ in cal/mole of mtDNA is given by Eq. (24) in reference 13, $\Delta G_\tau = 170\tau^2$, where τ is the number of superhelical turns per molecule. The coefficient in Eq. (24) has been multiplied by 0.32, the ratio of molecular weights of SV40 DNA and mtDNA. From the expression for the derivative $(d\Delta G_\tau/d\tau)$ at $\tau = 45 \pm 8$, we find that 1.5 ± 0.3 kcal/mole is then required to unwind the duplex by 36° and to extend the displacing strand by one nucleotide. This free energy is in a reasonable range for the free energy of the synthesis reaction.

Alternative mechanisms for the displacement synthesis, consistent with the results presented here, can be formulated with different, but more complex, coupled assumptions regarding the termination mechanism, the origin of supercoiling, and the occurrence of nicking-closing cycles.

After this work was completed, we were informed by J. ter Schegget and P. Borst that they had interpreted the results of their study of the incorporation of labeled DNA precursors into mtDNA in preparations of

chick liver mitochondria as indicating the formation of a complex of short product DNA hydrogen-bonded to closed duplex circles (14).

Acknowledgments

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APPENDIX A

Preparation of *E. coli* Ribosomal RNA

(1) Grow overnight a 10-ml culture of bacteria in T3a medium.

T3a (from R. L. Sinsheimer):

per liter

NaCl	0.5 g
KCl	8.0 g
NH_4Cl	1.1 g
Tris-base	12.1 g
KH_2PO_4	1.0 g
Sodium pyruvate	0.8 g
Amino acid mixture (Nutritional Biochemical Co.)	2.7 g
1 M $MgCl_2 \cdot 6H_2O$	1.0 ml
1 M $CaCl_2 \cdot 2H_2O$	0.1 ml
0.16 M Na_2SO_4	1.0 ml
Thiamine	20 mg
Thymidine (if required for growth)	10 mg

The pH is adjusted at approximately half the final volume to 7.3–7.4 by addition of concentrated HCl. The solution is then diluted to 1.0 l and autoclaved. After autoclaving add 1.0 ml of 0.1 mg/ml $FeCl_3 \cdot 6H_2O$ (sterile) and 20.0 ml of 10% (w/w) glucose (sterile).

The concentration of bacteria in the overnight culture is determined by measuring the absorbance at 600 nm (A_{600}) corrected to a blank of T3a medium. An absorbance of 0.16 indicates an E. coli concentration of 1×10^8 cells/ml. The following procedures are presented for preparation of cells to be labeled with [^{14}C]uracil, typically carried out on a small scale. The procedure has also been used for the preparation of unlabeled cells (several liters) grown to a final concentration of 1×10^9 cells/ml.

(2) Labeling of cells.

To 51 ml of T3a containing $FeCl_3 \cdot 6H_2O$ and glucose, add enough of the overnight culture to bring the starting cell density to ~1×10^7 cells/ml. Incubate 10 min at 37°C with aeration. Add 150 μCi of a sterile aqueous solution of uracil-2-C^{14}, 51.5 mCi/mM (Schwarz/Mann), to 2 ml of T3a containing $FeCl_3 \cdot 6H_2O$ and glucose, then add the [^{14}C]-uracil–T3a to the 51-ml culture of bacteria. Allow growth to continue for several generations to a final density of ~3×10^8 cells/ml.

Centrifuge the cells at 10,000 rev./min for 5–10 min in polycarbonate centrifuge tubes in the SS34 rotor of the Sorvall centrifuge. Resuspend the cells in T3a* and add to 100 ml T3a*. Allow the cells to grow for approximately one generation. This growth in unlabeled medium chases uracil label from the messenger RNA fraction without seriously altering the specific activity of ribosomal RNA.

(3) Lysis procedure (modified from N. Godson and R. L. Sinsheimer, Biochim. Biophys. Acta, 149, 476 (1967)).

The cells are next chilled at 0–4°C and centrifuged as described above. The cell pellet, corresponding to ~1.5×10^{10} cells, is suspended in 2 ml of 25% sucrose, 0.04 M Tris, pH 8.1. While keeping the cells on ice, add Bentonite to a final concentration of 2 mg/ml. Then add 0.2 ml of a lysozyme solution at a concentration of 6.4 mg/ml dissolved in 0.25 M Tris, pH 8.1. Next add 0.2 ml of an EDTA solution containing 20 mg/ml disodium EDTA, pH unadjusted. Allow spheroplast formation to continue for approximately 5 min on ice.

Add 0.3 ml of 0.1 M $MgSO_4$ and 0.3 ml of a 5% Brij 58 solution

made up in 0.01 M Tris, pH 7.2. The suspension is allowed to stand at room temperature for approximately 15 min to effect lysis, and then chilled on ice. High molecular weight DNA and membranes are removed by centrifuging the lysate for 10 min at 43,000 rev./min (5-min acceleration time) in the Ti50 rotor of a Beckman ultracentrifuge using screwcap polycarbonate tubes. The supernatant is decanted and ribosomes are pelleted from this supernatant by centrifuging 90 min (5-min acceleration time) as described above. Gently resuspend pellets of ribosomes in a total of 2 ml of TMM (5 mM Tris, pH 7.3, 10 mM magnesium acetate, 2 mM mercaptoethanol).

The minimum volume of TMM to be used in resuspension is 0.2 times the volume of 0.25 M sucrose used in the lysis procedure. Add a sufficient volume of a DNase I solution (1 mg/ml in TMM) to bring the final concentration to 10 μg/ml. Keep the solution on ice for 15 min, then centrifuge out the ribosomes at 43,000 rev./min for 90 min as described above. Rinse the surface of the ribosome pellet with 1 ml TMM, then redissolve the pellet of ribosomes in 1 ml TMM. E. coli 70S ribosomes have been isolated and may be kept frozen in TMM at less than -10°C. It is convenient to determine the specific activity and yield of the isolated ribosomes at this point.

(4) Separation of ribosomal subunits.

The 70S ribosomes isolated as described above are dialyzed against two changes of 250 volumes each of TMM* (TMM*: 5 mM Tris, pH 7.3, 0.1 mM magnesium acetate, 2 mM mercaptoethanol). The dialysate is layered up to 1.0 ml per tube onto 28 ml of a 5–30% linear

sucrose gradient containing TMM* and centrifuged 14 hr at 22,000 rev./min, 4°C, in the SW25.1 rotor. The gradient is fractionated and portions containing the ribosomal subunits are pooled and stored at -15°C.

(5) Isolation of ribosomal RNA (adapted from P. Jeanteur, F. Amaldi and G. Attardi, J. Mol. Biol., 33, 752 (1968)).

Isolated ribosomal subunits kept at 0°C are precipitated with 2 volumes of ethanol after addition of NaCl to a final concentration of 0.2 M. The precipitate is centrifuged at 10,000 rev./min for 10 min at 0–4°C, and the resulting pellet is taken up in 1.0 ml of 2.5% SDS dissolved in 10 mM Tris, 1 mM EDTA, pH 7.0. The solution is allowed to stand for 15 min at room temperature and then layered onto a 28-ml, 5–30% linear sucrose gradient containing 0.5% SDS, 0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.0. The sample is sedimented in the 25.1 rotor at 22,000 rev./min for 22 hr and at a temperature of 15–20°C. Fractions containing rRNA are pooled and precipitated with 2 volumes of ethanol to remove SDS. The RNA can be stored as the ethanol precipitate at -20°C. Alternatively, the RNA precipitate can be centrifuged out after standing several hours at -20°C, and the RNA redissolved in 0.1 M sodium acetate buffer, pH 5.0, which can then be stored at -20°C. In general, two precipitations are required to effectively remove the SDS.

APPENDIX B

Electron Microscope Techniques

(1) Aqueous basic protein technique.

Stock solutions:

(i) 5 M ammonium acetate, 10 mM Tris-base, 3–5 mM disodium EDTA, pH 8.0–8.4 unadjusted.

(ii) Cytochrome *c* at 1 mg/ml in 10 mM Tris, 1 mM EDTA, pH 8.0–8.5.

(iii) Concentrated uranyl acetate stain consisting of 50 mM uranyl acetate dihidrate, 50 mM HCl in 95% ethanol. Prepare 50–100 ml. Stirring is required for solution of the uranyl acetate, during which time the solution is kept in the dark. The solution may be stored at 4–25° C in the dark.

Spreading procedure:

Dilute 7.5 ml of concentrated ammonium acetate into 150 ml of doubly distilled H_2O. Pour this solution into the dish described in Chapter C of this thesis. Clean the surface of the liquid with the teflon bar, then place teflon bars symmetrically on the dish so that the spread film will be confined to an area of approximately 100 cm^2.

A glass slide cleaned in a 1:1 concentration ratio of nitric acid to H_2O is flushed with doubly distilled H_2O and placed in the hypophase, resting against one of the teflon bars. Pour more of the hypophase solution over the glass slide surface to neutralize any remaining acid and, also, to wet the surface of the slide with ammonium acetate.

Prepare the spreading solution:

5 λ Concentrated ammonium acetate (i)
30 λ H_2O
5 λ 5 μg/ml DNA
10 λ 1 mg/ml cytochrome *c* (ii).

Dust talc onto the hypophase surface, but leave 2–3 cm^2 area in front of the slide free of talc.

Layer the spreading solution over 1 cm^2 of the glass slide just above the interphase and allow it to drain onto the hypophase (1–2 min).

After approximately 2 min, grids may be taken from the surface and dehydrated and stained in 90% ethanol by the following procedure. Dispense 5 ml of 90% ethanol into each of two vials. Add 10 λ of stock uranyl acetate solution (iii) to one vial. Immerse grids first in nonstain ethanol for 5 sec, then in stain ethanol for 30 sec, then again in nonstain ethanol for 1 sec. The isopentane rinse is not needed in this procedure.

It has also been found that Kleinschmidt-spread DNA can be stained with phosphotungstic acid in the manner described for uranyl acetate. The stock concentrated solution is, however, made up in H_2O rather than in 95% ethanol.

An example of PTA staining of DNA is presented in Plate I. A mixture of double-stranded SV40 DNA and single-stranded φX DNA was spread from 50% formamide, 0.1 M Tris, 10 mM EDTA, pH 8, onto a hypophase of 17% formamide, 10 mM Tris, 1 mM EDTA, pH 8. Note that the φX single-stranded DNA is considerably less contrasted than the duplex SV40 DNA.

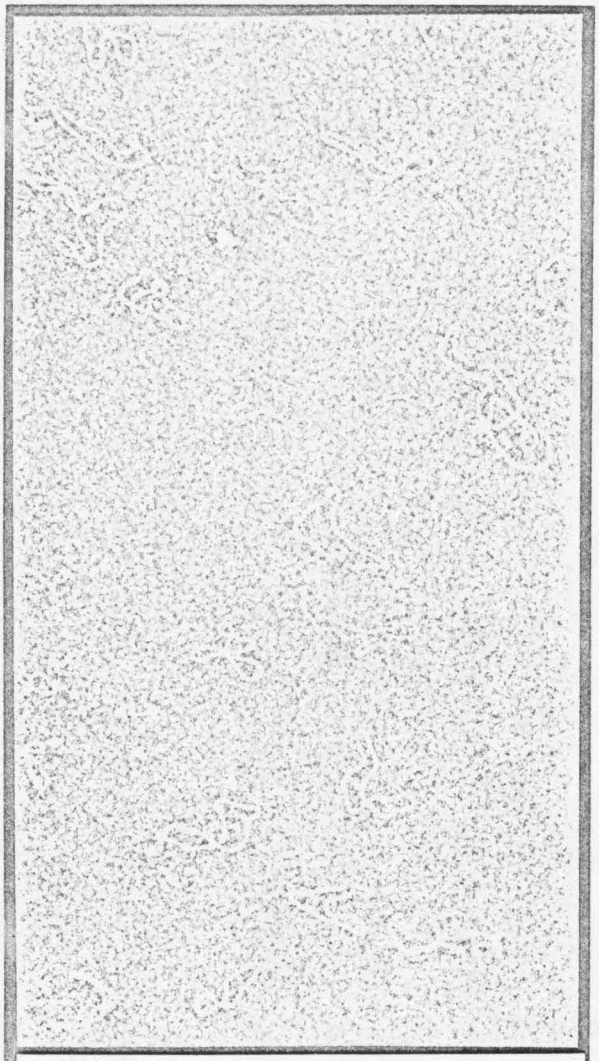

Plate I: PTA-stained ϕX and SV40 DNAs.

(2) Dark-field electron microscopy.

The technique of dark-field electron microscopy is quite advantageous for raising the level of contrast of stained specimens and may be easily applied if a Phillips 300 electron microscope is available. Essentially, one follows the manual and first activates the beam tilt and selects a tilt setting of "2". The amplitude should be set between positions "3" and "4". It is necessary to recenter the beam with the deflection controls and to refocus the objective lens. It may also be necessary to raise the intensity by increasing the setting of the emission current or by going to higher accelerating voltages.