Biochemical and Electron Microscopic Characterization of DNA–RNA Complexes from HeLa Cell Mitochondria†

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ABSTRACT: The previous electron microscopic investigations on the occurrence in HeLa cell mitochondria of transcription complexes of mitochondrial DNA [Aloni, Y., and Attardi, G. (1972a), *J. Mol. Biol. 70,* 363–373] have been extended with the aim of obtaining these complexes in a reasonably pure form for biochemical analysis. By using conditions designed to minimize losses of such structures and any possible contamination by nuclear DNA, it has been shown that a substantial fraction (40 to 50%) of mitochondrial DNA can be isolated from exponentially growing HeLa cells in the form of fast-sedimenting complexes with RNA. These complexes have been characterized with respect to density and sedimentation properties, content in newly synthesized RNA, stability of the association of RNA with DNA, presence of different forms of mitochondrial DNA, and electron microscopic appearance. The properties of these complexes, as well as the results of reconstruction experiments, strongly suggest that the majority of such structures represent true transcriptional intermediates. The occurrence in this fraction of replicating or newly replicated mitochondrial DNA molecules has been observed. Although the presence of single-stranded DNA segments makes the replicative intermediates particularly susceptible to aggregation with free RNA, electron microscopic observations point to the possibility that these intermediates may be recruited for transcription.

In previous studies (Aloni and Attardi, 1972a), the isolation and electron microscopic characterization of transcription complexes of HeLa cell mtDNA were described. In that work, the isolation procedure was based on the separation in a sucrose gradient of the fast-sedimenting components of a sodium dodecyl sulfate lysate of the mitochondrial fraction and on their further buoyant density fractionation in a CsCl/ethidium bromide density gradient. Since the identification of the transcription complexes was made essentially by electron microscopy and losses or disruption of the complexes could have occurred easily during the preparation of the samples, no evaluation could be made of the degree of purity of the fraction analyzed in terms of content in true transcription complexes relative to mtDNA–RNA aggregates of unspecific nature; therefore, no quantitation was attempted in the previous study of the proportion of mtDNA which can be isolated in the form of transcription complexes from exponentially growing cells.

The isolation, in reasonably pure form, of the transcriptional intermediates of mtDNA is a prerequisite for their biochemical analysis. The achievement of this isolation would open the way to a detailed study of the mechanism of symmetrical transcription of HeLa cell mtDNA (Aloni and Attardi, 1971b, 1972a; Young and Attardi, 1975; Murphy et al., 1975); in particular, it would make it possible to approach such questions as whether or not the two strands are transcribed concurrently in the same mtDNA molecule, whether there are one or more promoters for transcription on each strand, or whether there is any link at the molecular level between replication and transcription. The possible existence of such a link was suggested earlier by the observation of a time relationship between the two processes with respect to the cell cycle (Pica-Mattoccia and Attardi, 1971, 1972; England et al., 1974).

In the present work, by using conditions designed to minimize losses of the transcription complexes during the preparation and any possible contamination by nuclear DNA, it has been shown that a substantial fraction of HeLa mtDNA (40 to 50%) can be isolated in a form which behaves as true transcription complexes, as judged by a variety of analytical criteria. Furthermore, the occurrence in this fraction of replicating or newly replicated mtDNA molecules has been observed.

Materials and Methods

Media. The media designations are as follows: (1) NKM: 0.13 M NaCl, 0.005 M KCl, 0.001 M $MgCl_2$; (2) TD buffer: 0.025 M Tris[1] buffer (pH 7.5 at 25 °C), 0.14 M NaCl, 0.005 M KCl, 0.0007 M Na_2HPO_4; (3) sodium dodecyl sulfate buffer: 0.01 M Tris buffer (pH 7.0), 0.1 M NaCl, 0.001 M EDTA, 0.5% sodium dodecyl sulfate.

Cell Growth. HeLa cells were grown in suspension in modified Eagle's medium (Levintow and Darnell, 1960) with 5% calf serum. The cultures were free of any detectable contamination by *Mycoplasma.*

Labeling Conditions. (1) Pulse Labeling. In the pulse-labeling experiments, exponentially growing cells were centrifuged at 37 °C and resuspended in fresh medium containing 5% dialyzed serum, at a concentration of 2.5–7.0 × 10^5 cells/mL, 1 h before the pulse. Pulse labeling of mtDNA was carried out with [*5-methyl*-^{3}H]thymidine (0.4–7.5 μCi/mL of medium; 40–56 Ci/mmol); after the pulse, the cells were rapidly collected on crushed frozen NKM in a salt–ice bath, either immediately or after a period of chase with 10^{-5} unlabeled thymidine. Pulse labeling of mitochondrial RNA was performed with [5-^{3}H]uridine (0.6–4 μCi/mL; 25–29 Ci/mmol); for pulses longer than 10 min, actinomycin D at 0.1 μg/mL was added to the culture 30 min prior to the labeling, in order to inhibit selectively the synthesis of cytoplasmic rRNA (Perry, 1964; Dubin, 1967; Penman et al., 1968).

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[1] Abbreviations used are: mt, mitochondrial; H, heavy; L, light; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid.

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(2) Long-Term Labeling. Long-term labeling of mtDNA was carried out by exposing exponentially growing cells for 2 days to [2-^{14}C]thymidine (0.02–0.09 μCi/mL; 59–62 mCi/mmol) or [*5-methyl*-^{3}H]thymidine (0.3–1.0 μCi/mL; 50–56 Ci/mmol) in modified Eagle's medium with dialyzed calf serum.

In order to provide markers for the identification of the different mtDNA species and an internal standard to correct for variations in extent of cell homogenization and efficiency of DNA extraction in different samples, all pulse-labeled cell cultures in each experiment were mixed with a constant amount of cells long-term labeled with [2-^{14}C]thymidine.

Isolation and Analysis of mtDNA and mtDNA–RNA Complexes. mtDNA and mtDNA–RNA complexes were isolated by a modification of the technique of Storrie and Attardi (1972). Unless otherwise stated, the temperature was kept under 4 °C during the purification. A mitochondrial fraction was prepared by differential centrifugation and further purified by treatment with 0.03 M EDTA (Attardi et al., 1969). The mitochondrial pellet was resuspended in 0.25 M sucrose, 0.0015 M $MgCl_2$, 0.05 M NaCl, 0.005 M Tris buffer (pH 7.2) (4 mL of solution/mL of packed cells) and digested for 1 h at 0 °C with 100 μg/mL of RNase-free pancreatic DNase (electrophoretically purified, Worthington or Sigma, batches selected for the lowest RNase contamination). After pelleting and two washings with 0.25 M sucrose in 0.01 M EDTA, 0.01 M Tris buffer (pH 7.4), the mitochondrial fraction was lysed with 1% sodium dodecyl sulfate, 0.01 M EDTA, 0.01 M Tris buffer (pH 7.4) (2.9 mL/mL of packed cells); Pronase (self-digested for 2 h at 37 °C) was then added to a concentration of 75 μg/mL. After incubation at 37 °C for 30 min, the lysate was brought to 1 M CsCl by addition of 7 M CsCl, chilled to 0 °C for 30 min, and centrifuged twice at 12 000 rpm in the Sorvall SS 34 rotor for 15 min. The final supernatant was centrifuged for 12 to 16 h in the Spinco 65 fixed-angle rotor at 38 000 rpm at 4 °C. The pelleted material was dissolved in 0.6 mL of 0.01 M EDTA, 0.01 M Tris buffer (pH 7.4) and analyzed by centrifugation through a two-step CsCl/ethidium bromide gradient (Smith et al., 1971; Storrie and Attardi, 1972) [1.5 mL of saturated (4 °C) CsCl in 0.01 M EDTA, 0.01 M Tris buffer (pH 7.4), containing 100 μg/mL ethidium bromide; 3 mL of CsCl (ρ = 1.40 g/mL), in the same buffer, containing 400 μg/mL ethidium bromide] in the Spinco SW 65 rotor at 38 000 rpm for 5 h at 4 °C. In several experiments, the centrifugation was performed with the SW 41 rotor at 27 000 rpm for 5 h [using gradients consisting of 2 mL of saturated CsCl–EtBr, 4 mL of CsCl (ρ = 1.40 g/mL)/ethidium bromide, and 0.8 mL of the sample]. The gradients were collected dropwise from the bottom of the tube and the 5% trichloroacetic acid insoluble radioactivity was determined on a portion of each fraction; for density determination, the refractive index was measured on 5-μL samples of selected fractions.

In plotting the data of comparative experiments, the ^{3}H radioactivity measured in total mtDNA in the two-step CsCl/ethidium bromide gradient was normalized for variations in cell homogenization and efficiency of mtDNA extraction on the basis of the long-term [2-^{14}C]thymidine radioactivity recovered in total mtDNA. The correction factor did not exceed 20%.

In some experiments, the material from selected portions of the CsCl/ethidium bromide gradient was freed of the ethidium bromide and, after a varying treatment, further analyzed in another two-step gradient or in an equilibrium CsCl/ethidium bromide gradient or in a sucrose–formamide gradient, as detailed in the figure legends.

Triton X-100 Lysis of the Mitochondrial Fraction. In some experiments, the mitochondrial fraction was lysed with Triton X-100, and mtDNA was extracted from the detergent-insoluble components. For this purpose, after DNase treatment, the mitochondrial fraction was washed carefully by centrifugation at 12 000 rpm for 10 min in a Sorvall SS 34 rotor and resuspended in 0.25 M sucrose, 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris buffer (pH 6.7) (twice, 10 mL/mL of packed cells). After the final centrifugation, the pellet was resuspended in a solution of 2% Triton X-100 and 20 μg/mL polyvinyl sulfate (an RNase inhibitor) in the same buffer (2 mL/mL of packed cells). The resulting mitochondrial lysate was centrifuged at 13 500 rpm for 20 min in the SS 34 rotor. The membrane pellet was solubilized in 0.01 M EDTA, 0.01 M Tris buffer (pH 7.4), 1% sodium dodecyl sulfate (2.9 mL/mL of packed cells), and incubated for 30 min at 37 °C with 75 μg/mL Pronase. The purification procedure was then continued as described above.

Separation of Heavy and Light mtDNA Strands. Separation of the H and L strands of mtDNA in an alkaline CsCl gradient was performed according to Aloni and Attardi (1971a).

Electron Microscopy. mtDNA–RNA complexes and the mtDNA molecules isolated from these complexes after RNase treatment were examined in the electron microscope by the basic protein film technique (Kleinschmidt and Zahn, 1959), as previously described (Robberson et al., 1971, 1972a). Ethidium bromide and CsCl were removed from the samples by extraction with isoamyl alcohol, followed by dialysis against 0.2 M NaCl, 0.01 M EDTA, 0.01 M Tris buffer (pH 7.4). The stained and shadowed samples were examined by light-field microscopy in a Philips EM 300 or a 201 C electron microscope. Photographs of the molecules were taken on 35-mm film. The length measurements were calibrated with a diffraction grating replica of 54 864 lines per inch (Ladd).

Results

Isolation of mtDNA–RNA Complexes (Standard Procedure). To separate mtDNA–RNA complexes from clean mtDNA molecules, we used centrifugation through a two-step CsCl/ethidium bromide gradient[2] (Smith et al., 1971; Storrie and Attardi, 1972). Such a gradient separates the molecular species on the basis of both their molecular weight and hydrodynamic parameters and their density in the presence of ethidium bromide. The original conditions for the preparation of the gradient have been modified in the present work in order to obtain a better separation of the fast-sedimenting species in the region of high density.

The profiles in two-step CsCl/ethidium bromide gradients of sodium dodecyl sulfate–Pronase lysates of the mitochondrial fraction from HeLa cells labeled for 48 h with [2-^{14}C]thymidine and either for 10 min with [5-^{3}H]uridine or for 30 min with [*5-methyl*-^{3}H]thymidine are shown respectively in Figures 1a and 1b. All the [^{14}C]- and [^{3}H]thymidine-labeled material was found to be alkali resistant. The DNA pattern can be divided into six main regions. Cuts 2–5 contain almost exclusively mtDNA, as judged from the sensitivity to 1 μg/mL ethidium bromide (94–97%) of its pulse labeling with [^{3}H]-thymidine (Figure 1b). In particular, peaks 4 and 5 (called I_a and II by Storrie and Attardi, 1972) correspond respectively to clean closed- and open-circular mtDNA. Peak 3 (I_b according to Storrie and Attardi, 1972) is a shoulder on the heavy

[2] The expression "two-step CsCl/ethidium bromide gradient" is used in this paper to indicate its initial form; during the centrifugation, a linear gradient becomes established by diffusion.

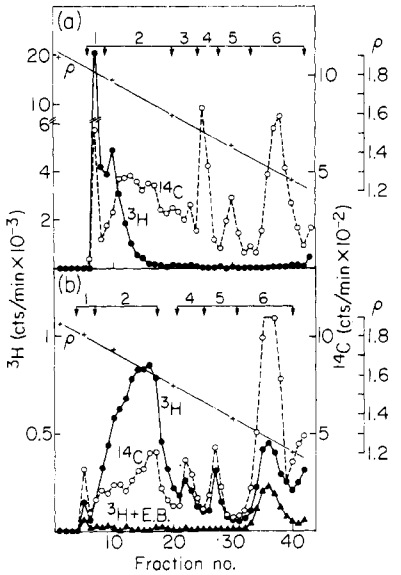

FIGURE 1: Analysis on a two-step CsCl/ethidium bromide gradient of a sodium dodecyl sulfate-Pronase lysate of mitochondria from HeLa cells pulse labeled with [^{3}H]uridine (a) or with [^{3}H]thymidine in the absence or presence of ethidium bromide (b). (a) 2.3 × 10^8 cells were labeled for 10 min with [5-^{3}H]uridine (3.7 μCi/mL) and mixed with an identical culture labeled for 48 h with [^{14}C]thymidine: (●-●) [^{3}H]uridine cpm; (○- -○) [^{14}C]thymidine cpm. (b) 10^8 cells were labeled with [*methyl*-^{3}H]thymidine (0.62 μCi/mL) for 30 min in the absence or in the presence of 1 μg/mL ethidium bromide (added 30 min before labeling) and mixed with a culture of cells labeled for 48 h with [^{14}C]thymidine: (●-●) [^{3}H]thymidine cpm, without ethidium bromide; (▲-▲) [^{3}H]thymidine cpm, with ethidium bromide; (○- -○) [^{14}C]thymidine cpm.

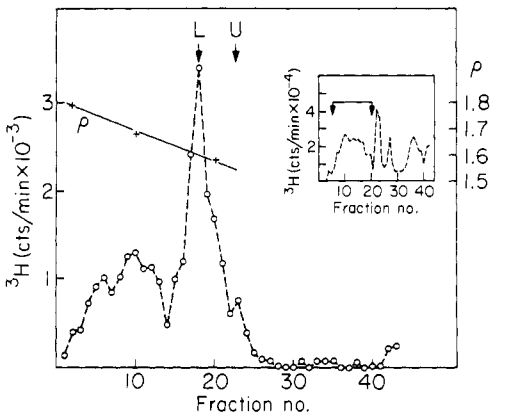

FIGURE 2: Equilibrium density in CsCl/ethidium bromide gradient of the fast-sedimenting mtDNA. The mtDNA was isolated by the standard procedure from 1.6 × 10^8 HeLa cells labeled for 24 h with [*methyl*-^{3}H]thymidine (0.62 μCi/mL). The fractions corresponding to regions 2 and 3 of the two-step CsCl/ethidium bromide gradient were pooled (see insert), the density was adjusted to 1.55 g/cm^3 with solid CsCl, and the ethidium bromide concentration was adjusted to 150 μg/mL (final sample volume = 6.5 mL). The centrifugation was performed in the Beckman SW41 rotor at 29 krpm for 40 h at 20 °C. L (lower band) and U (upper band) indicate the banding position, respectively, of closed- and open-circular mtDNA (peaks 4 and 5) run as markers in parallel tubes.

side of peak 4, more or less distinct from cut 2: it consists presumably mainly of catenated forms of mtDNA (Storrie and Attardi, 1972). Finally, about 45% of the mtDNA migrated in the form of a broad band in the lower part of the gradient (region 2), where the density is higher than the equilibrium density of pure mtDNA; therefore, it must result from the combination of DNA with a component of higher density, presumably RNA.

Peak 6, of very variable size, consists mainly, if not entirely, of contaminating degraded nuclear DNA, as shown by the relatively low inhibition (~40%) of its synthesis by 1 μg/mL ethidium bromide (Figure 1b). The observation that the incorporation of [^{3}H]thymidine into DNA of this peak, during the 30-min pulse, is more inhibited by ethidium bromide than is the incorporation into total cellular DNA (~20%) may either indicate that it contains also fragments of mtDNA or that nuclear DNA extracted by this technique is not representative of total nuclear DNA.

Peak 1 corresponds to a thin layer of pink precipitated material floating in the lower part of the gradient. This material is, most of the times, collected in one or two fractions and gives a sharp peak; in some cases, it sticks to the bottom of the tube and is not collected. It contains most of the labeled RNA and some DNA. The inhibition by ethidium bromide of the incorporation of [^{3}H]thymidine into DNA of this peak is only about 60%; therefore, a substantial portion of it is presumably not mtDNA. In the experiments described in this work, this material was not taken into account. The steady-state distribution of mtDNA in cuts 2-5 of the two-step CsCl/ethidium bromide gradient has been measured in a large number of experiments from the [^{14}C]thymidine radioactivity profiles: 45% (±10%) of mtDNA is found in cut 2, 12% (±5%) in cut 3, 28% (±10%) in cut 4, and 15% (±5%) in cut 5.

After a 30-min [^{3}H]thymidine pulse, the labeled mtDNA contained in cut 2 has a higher specific activity than the mtDNA of the other regions of the two-step gradient (Figure 1b). This phenomenon will be analyzed in more detail in a following section.

The profile of the RNA labeled during a 10-min [^{3}H]uridine pulse shows a peak coinciding with the DNA peak 1, with a shoulder on the light side (more or less resolved) tailing in the region of band 2 (Figure 1a). All [^{3}H]uridine-labeled material in the gradient was alkali sensitive. It should be recalled that the RNA analyzed in the two-step gradient contained only the faster-sedimenting components (>16 S), which had pelleted during the overnight centrifugation at 38 k rpm in the Spinco 65 fixed-angle rotor.

A series of experiments was carried out to investigate the influence of the preparative procedure on the amount of the material sedimenting in region 2 of the two-step gradient. Isopycnic centrifugation of the mitochondrial fraction on a two-step sucrose gradient (Hare et al., 1978) was found to decrease the yield of both the total and the fast-sedimenting mtDNA. If the mitochondrial fraction was treated with RNase prior to the lysis, the fast-sedimenting complexes disappeared (see, for example, Figure 4 in Storrie and Attardi, 1972). If the lysis was performed without incubation with Pronase, the yield of total mtDNA was lowered by about 30% and that of fast-sedimenting material by 65%. When the overnight and 5-h centrifugations were performed at 20 °C instead of 4 °C, the proportion of DNA in band 2 diminished by 15%.

Analysis of the Fast-Sedimenting mtDNA

Equilibrium Density of the Fast-Sedimenting mtDNA (Band 2) in a CsCl/Ethidium Bromide Gradient. The density of the fast-sedimenting long-term labeled mtDNA was analyzed by equilibrium centrifugation in a CsCl/ethidium bromide gradient (at 20 °C). Fractions corresponding to regions 2 and 3 of a two-step gradient were used for this experiment. In the profile shown in Figure 2, one can see a prominent peak corresponding to the lower band material (L, closed-circular mtDNA) and a small shoulder at the position of the upper band material (U, open-circular mtDNA), as well as a broad band of higher density ($\rho \simeq 1.65$–1.80 g/cm^3), representing about

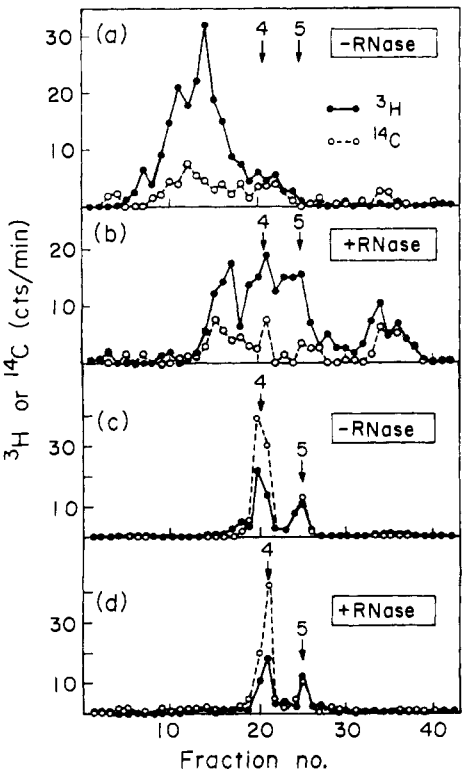

FIGURE 3: Effect of RNase digestion on the centrifugation properties in a two-step CsCl/ethidium bromide gradient of band 2 mtDNA (a,b) and of closed- and open-circular mtDNA (c,d). mtDNA was purified by the standard procedure from a mixture of 8×10^7 cells labeled for 10 min with [^{3}H]thymidine (15 μCi/mL) and cells long-term-labeled with [^{14}C]thymidine. The fast-sedimenting mtDNA (band 2 in the two-step CsCl/ethidium bromide gradient) and the closed- and open-circular mtDNA (peaks 4 and 5) were isolated. Ethidium bromide was removed by extraction with isoamyl alcohol (three times) and CsCl by dialysis against 2×500 mL of 0.01 M Tris buffer (pH 7.4), 0.2 M NaCl, 0.01 M EDTA at 4 °C. One-half of each sample was digested for 30 min at 0 °C with 100 μg/mL pancreatic RNase (heated at 80 °C for 15 min). RNase-treated and -untreated DNA samples were run on two-step CsCl/ethidium bromide gradients under standard conditions: (●-●) ^{3}H cpm; (○- -○) ^{14}C cpm.

46% of the analyzed DNA. One can thus estimate that 40 to 50% of the DNA from cut 2 has an equilibrium density in a CsCl/ethidium bromide gradient higher than the clean closed-circular mtDNA marker and must therefore be complexed with some component of higher density, presumably RNA. These denser components have exactly the density distribution previously described (Aloni and Attardi, 1972a) for mtDNA-RNA complexes in HeLa cells. Partial dissociation of these unstable complexes during the centrifugation at 20 °C may account for the appearance of closed- and open-circular mtDNA.

Effect of RNase Digestion on the Distribution of Band 2 mtDNA in a Two-Step CsCl/Ethidium Bromide Gradient. Long-term and pulse-labeled mtDNA was run in a two-step CsCl/ethidium bromide gradient, and the material from band 2 was freed of ethidium bromide, digested with pancreatic RNase under conditions described in the legend for Figure 3, and rerun in the same type of gradient. As shown in Figure 3b, both the [^{14}C]- and the [^{3}H]thymidine-labeled DNA migrate now in the region corresponding to cuts 3 to 5, giving a poorly resolved pattern; some material of low molecular weight is present at the top of the gradient. In contrast, a non-RNase-treated sample of band 2 mtDNA migrates to the original position (Figure 3a). On the other hand, the migration pattern of the mtDNA from cuts 4 and 5 is not modified after RNase treatment (Figure 3c,d). This shows that, under the conditions used in our experiment, RNase has no effect on clean mtDNA molecules [although they are known to contain a few RNase-sensitive sites (Wong-Staal et al., 1973)]. The striking change in the distribution in a two-step gradient of band 2 material after RNase digestion is in agreement with the idea that this material consists of mtDNA-RNA complexes.

Separation of mtDNA-RNA Complexes by Sedimentation through a Sodium Dodecyl Sulfate-Sucrose Gradient. To investigate the possibility that the material in band 2 results from aggregation of free RNA with DNA during exposure to high ionic strength in the early steps of the isolation procedure, mtDNA-RNA complexes from a mixture of cells long-term labeled with [^{14}C]thymidine and cells pulse labeled (30 min) with [5-^{3}H]uridine were separated from most of the free RNA by sedimentation through a sodium dodecyl sulfate-sucrose gradient (Aloni and Attardi, 1972a), prior to the two-step CsCl/ethidium bromide gradient analysis. As previously reported, fast-sedimenting DNA and RNA species accumulated in or near the dense sucrose cushion. Material sedimenting faster than 28 S was pooled and analyzed on a two-step CsCl/ethidium bromide gradient. The profile obtained showed mainly fast-sedimenting components, forming a broad band in the region 2, as observed in two-step gradient patterns when the sodium dodecyl sulfate-sucrose gradient fractionation was omitted; in addition, one could see a small amount of closed- and open-circular mtDNA. By comparison with the results of the fractionation by the standard procedure used in this work, the yield in fast-sedimenting mtDNA-RNA complexes was little affected by the addition of this purification step to the procedure.

Lysis of the Mitochondrial Fraction with Triton X-100. As a further approach to exclude trapping of free RNA by mtDNA molecules during the sodium dodecyl sulfate-Pronase treatment or in the subsequent steps of purification, all soluble mitochondrial components were eliminated by lysing the mitochondrial fraction with Triton X-100 after DNase treatment and recovering by centrifugation the detergent-insoluble structures: the DNA and DNA-RNA complexes associated with these structures were analyzed in a two-step CsCl/ethidium bromide gradient after solubilization of the pellet components by sodium dodecyl sulfate and Pronase. Under our conditions of Triton X-100 lysis, most of the mitochondrial polysomes and free RNA are released (Ojala and Attardi, 1972). The profiles in the two-step gradient of [^{3}H]thymidine long-term-labeled DNA extracted by the usual procedure and by the Triton X-100 lysis method were qualitatively similar, with the fast-sedimenting mtDNA components (band 2) representing 36% of the total mtDNA obtained by the Triton X-100 procedure; however, the yield of both total and fast-sedimenting mtDNA by the Triton X-100 procedure was only about 50% of that obtained by the usual procedure. The recovery of closed-circular DNA was especially low (25% of the control). This was very likely due to its sensitivity to traces of DNase. Washing of the mitochondrial fraction after DNase treatment is very critical in the Triton X-100 procedure, since the residual DNase is not immediately inactivated by sodium dodecyl sulfate and Pronase as in the usual technique. The action of DNase made it difficult to detect the missing DNA in the supernatant fraction of the Triton X-100 lysate.

Dissociation Treatments. To investigate the nature of the association of RNA with mtDNA in the mtDNA-RNA complexes, complexes isolated by the standard procedure were brought to low ionic strength by dialysis against 0.01 M Tris buffer (pH 7.4), 0.01 M EDTA (two changes of 500 volumes,

overnight at 4 °C), and rerun in a two-step CsCl/ethidium bromide gradient. The resulting profile of mtDNA indicated that the complexes had broken down, as after RNase treatment (not shown). By contrast, when the overnight dialysis was carried out against buffer containing 0.1 M NaCl, no effect whatsoever on the stability of the mtDNA-RNA complexes was observed.

The above results suggested that RNA and DNA were held together in the heavy complexes by hydrogen bonds. However, these complexes were found to be rather resistant to mild denaturing agents, as shown by their behavior in a sucrose gradient in the presence of 47% formamide. In these experiments, mtDNA-RNA complexes were prepared from HeLa cells labeled for 48 h with [^{14}C]thymidine and for 10 min with either [^{3}H]thymidine (Figure 4a) or [^{3}H]uridine (Figure 4b). Forty to fifty percent of both the [^{14}C]thymidine long-term labeled and the [^{3}H]thymidine pulse-labeled DNA from band 2 was found to sediment in a formamide–sucrose gradient faster than the closed- and open-circular mtDNA markers, accumulating in part at the dense sucrose cushion; the remainder migrated as the closed and open circular mtDNA markers (Figure 4a). Under the same conditions, about one-third of the 10-min uridine pulse-labeled RNA contained in band 2 material sedimented faster than the closed-circular mtDNA marker, while the remainder formed a broad band in the upper third of the gradient (Figure 4b). When the sample was treated with RNase-free DNase, the totality of the RNA was found on the contrary in a rather sharp peak at the top of the gradient, with an advancing edge extending to the middle of the gradient (Figure 4c).

Reconstruction Experiments. In order to exclude the possible artifactual nature of the DNA–RNA complexes isolated on two-step gradients, several reconstruction experiments were carried out. Purified [^{3}H]thymidine-labeled mtDNA, either in toto (after RNase digestion) or after removal of mDNA–RNA complexes, was added to a sodium dodecyl sulfate–Pronase lysate of [^{14}C]thymidine long-term-labeled mitochondria and copurified with the [^{14}C]thymidine-labeled mtDNA.

In the first two experiments, total mt[^{3}H]DNA, purified by the standard procedure from HeLa cells long-term labeled (48 h) (Figure 5a and 5b) or pulse-labeled (15 min) with [^{3}H]-thymidine (Figure 5c and 5d) and treated with pancreatic RNase to destroy the mtDNA-associated RNA, was used. The two-step CsCl/ethidium bromide gradient patterns obtained with the reconstructed mixtures (Figure 5b and 5d) should be compared with the patterns of purified RNase-treated mt[^{3}H]DNA alone (Figure 5a and 5c). While the controls show only closed- and open-circular mtDNA and a shoulder of concatenated forms, without any evidence of fast-sedimenting components, a nonnegligible fraction of the mt[^{3}H]DNA copurified with the [^{14}C]thymidine-labeled mtDNA migrates with the mt[^{14}C]DNA band 2. This fraction amounts to 17 and 60% of the added mt[^{3}H]DNA for the long-term and the pulse-labeled DNA, respectively. These values must be compared to the initial proportions of fast-sedimenting DNA contained in the [^{3}H]DNA preparations before RNase treatment, i.e., 42 and 80%, respectively.

In the third experiment, long-term-labeled mt[^{3}H]DNA was first purified by a two-step gradient (insert a in Figure 5e) and then freed of mtDNA–RNA complexes by running it to equilibrium in a CsCl/ethidium bromide gradient (insert b in Figure 5e) and removing the mtDNA species denser than closed-circular mtDNA. In this experiment, the proportion of upper plus lower band mt[^{3}H]DNA which migrates with band 2 of the marker, when added to a [^{14}C]thymidine-labeled mitochondrial lysate and copurified with the mt[^{14}C]DNA, is less than 6% of the total mt[^{3}H]DNA added.

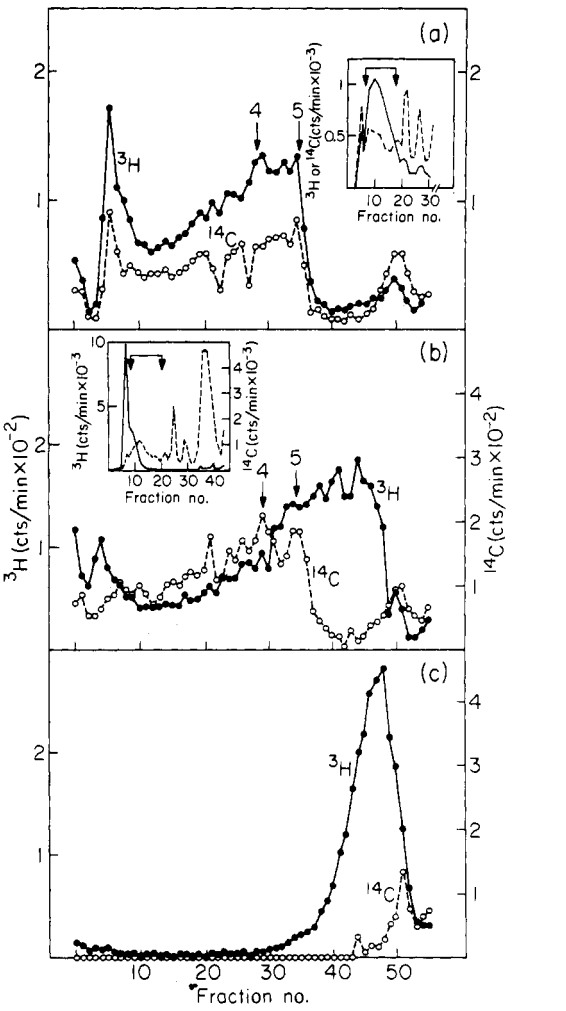

FIGURE 4: Sedimentation profiles of mtDNA-RNA complexes in a 47% formamide–sucrose gradient. Cells labeled for 10 min either with [^{3}H]-thymidine (a) or with [^{3}H]uridine (b,c) were mixed with cells long-term labeled with [^{14}C]thymidine, and mtDNA was extracted and fractionated by the standard procedure. The mtDNA–RNA complexes (band 2) were pooled (see inserts in a and b), and the ethidium bromide and CsCl were removed, respectively, by extraction with isoamyl alcohol and by dialysis against 3 × 500 mL of 0.4 M NaCl, 0.04 M EDTA, 0.02 M Tris buffer (pH 7.4) at 4 °C. Recrystallized formamide was added to a final concentration of 47%, and the samples (3 mL) were layered on gradients of 5 to 20% sucrose in 0.01 M Tris buffer (pH 7.4), 0.2 M NaCl, 0.02 M EDTA, containing 47% formamide (made over a 5-mL 50% sucrose cushion in 47% formamide). The samples were centrifuged in a Beckman SW27 rotor (large buckets) for 7.5 h at 20 °C at 27 krpm. Closed- and open-circular mtDNA (peaks 4 and 5), freed of ethidium bromide and CsCl as described above, were run as markers in parallel tubes. One-half of the [^{3}H]uridine pulse-labeled material was, after removal of the ethidium bromide, dialyzed for 3 h against distilled water at 4 °C. The sample was made 0.005 M $MgCl_2$, and digested with 100 μg/mL RNase-free pancreatic DNase at 37 °C for 30 min; it was thereafter treated as the other samples (c): (●-●) ^{3}H cpm; (○- - -○) ^{14}C cpm.

Analysis of the DNA Involved in mtDNA–RNA Complexes

Buoyant Density in a CsCl/Ethidium Bromide Gradient. The fast-sedimenting mtDNA–RNA complexes (cut 2 in a two-step gradient) were isolated from a mixture of cells long-term labeled with [^{14}C]thymidine and cells pulse labeled (10 min) with [^{3}H]thymidine and digested with pancreatic RNase to destroy the RNA, and the extracted DNA was then run to equilibrium in a CsCl/ethidium bromide gradient (Figure 6) mtDNA from cuts 3–5 of the same two-step gradient was similarly treated with RNase and run in a CsCl/ethidium

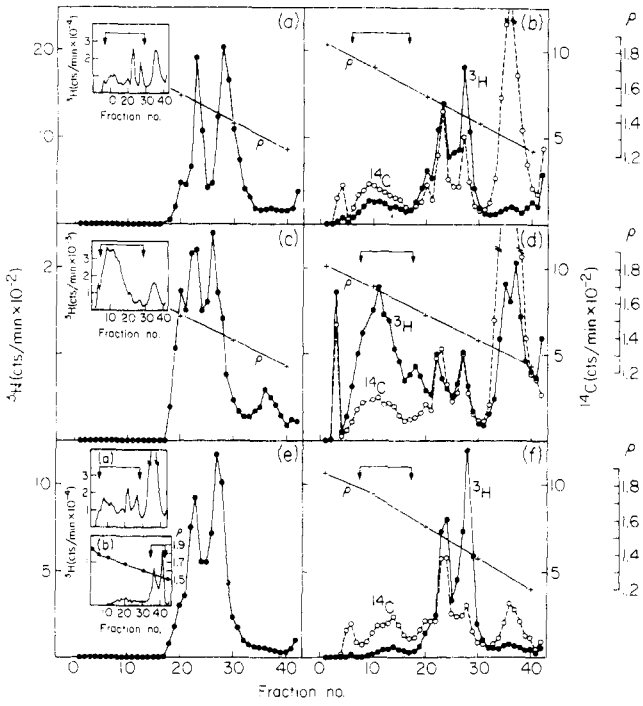

FIGURE 5: Reconstruction experiments carried out by mixing [^{3}H]thymidine-labeled purified mtDNA with [^{14}C]thymidine-labeled mitochondrial lysates. (a,b) mtDNA prepared by the standard procedure from 3.2×10^8 cells long-term labeled with [^{3}H]thymidine was run on a two-step CsCl/ethidium bromide gradient; the fractions corresponding to all forms of mtDNA were pooled (see insert), freed of ethidium bromide and CsCl as explained in the legend for Figure 3, and incubated for 1 h at room temperature with 100 μg/mL heated pancreatic RNase. After phenol extraction and dialysis against 1% sodium dodecyl sulfate in 0.01 M Tris buffer (pH 7.4), 0.01 M EDTA, one-half of the sample was rerun on a two-step gradient (a); the other half was mixed with the sodium dodecyl sulfate–Pronase lysate of the mitochondrial fraction from 1.6×10^8 cells long-term labeled with [^{14}C]thymidine; the mixture was then subjected to the standard purification procedure and run on a two-step gradient (b). (c,d) mtDNA prepared from 3.6×10^8 cells labeled for 15 min with [^{3}H]thymidine was subjected to the same treatment described above. (e,f) Long-term [^{3}H]thymidine-labeled mtDNA from 8×10^8 cells was run on a two-step gradient and then the fractions indicated by arrows in insert a were pooled and centrifuged to equilibrium (SW65 rotor, 48 krpm, 27 h) in a CsCl/ethidium bromide density gradient (ρ_{av} = 1.65 g/cm^3): the closed- and open-circular mtDNA were pooled, and one-half was run on a two-step CsCl/ethidium bromide gradient (e); the other half was mixed with the sodium dodecyl sulfate–Pronase lysate of the mitochondrial fraction from [^{14}C]thymidine long-term-labeled cells and treated as samples in panel b and d (f): (●–●) ^{3}H cpm; (○- - -○) ^{14}C cpm.

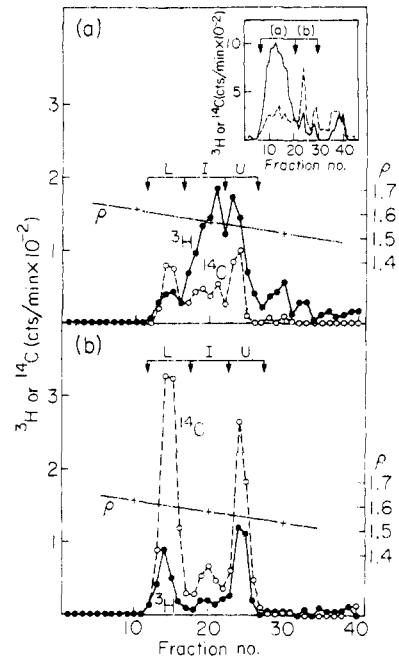

FIGURE 6: Density distribution in a CsCl/ethidium bromide gradient of the DNA isolated from mtDNA–RNA complexes. mtDNA was isolated by the standard procedure from a mixture of 4×10^8 cells labeled for 10 min with [^{3}H]thymidine (3.7 μCi/mL) and cells long-term labeled with [^{14}C]thymidine. Fractions corresponding to band 2 (a) and to peaks 3–5 (b) in the profile of the two-step gradient were pooled as indicated in the insert. After removal of ethidium bromide by extraction with isoamyl alcohol and of CsCl by dialysis, the samples were treated with 100 μg/mL heated RNase at 0 °C for 30 min and purified by filtration on a Sephadex G-100 column. The solutions were brought to a density of 1.55 g/mL with solid CsCl and to a concentration of 150 μg/mL of ethidium bromide and centrifuged for 48 h at 39 krpm in the rotor 65 at 20 °C: (●–●) ^{3}H cpm; (○- - -○) ^{14}C cpm.

bromide gradient. In such a gradient, according to Berk and Clayton (1974), the lower band (L) contains closed-circular DNAs: D-loop mtDNA and clean closed-circular mtDNA. The upper band (U) contains clean open-circular mtDNA, nicked D-loop mtDNA, gapped-circular mtDNA with small gaps, and linear DNA. The region intermediate between the two main bands (I) contains catenated molecules of mixed closed- and open-circular composition, expanded-D molecules [Exp-D and Exp-D-(1)] (in the latter type, in contrast to the Exp-D molecules, L-strand synthesis has already begun) and gapped-circular molecules with large gaps. Fully closed catenated oligomers band in the lower band and fully open catenated oligomers in the upper band.

Figure 6a shows the distribution in a CsCl/ethidium bromide gradient of the population of [^{14}C]thymidine long-term-labeled and [^{3}H]thymidine pulse-labeled mtDNA molecules deriving, after RNase treatment, from the mtDNA–RNA complexes (cut 2). The [^{14}C]DNA banding in the region of intermediate density represents 33% of total labeled DNA and seems to be composed of two peaks; the lower and upper bands contain about 36 and 31% of mtDNA, respectively. In the [^{3}H]thymidine pulse-labeled DNA, the proportions of the different peaks are very different: the lower band contains only 14% of the label, the material banding in the region of intermediate density about 44% and the upper peak 28%. Some material can also be found at a lower density.

The DNA extracted from cuts 3–5 of the two-step gradient pattern shows a different distribution (Figure 6b). In the ^{14}C profile, the lower and upper bands contain the great majority of the radioactivity (~52 and 32%, respectively), the remainder (~14%) being in the intermediate region. The [^{3}H]thymidine pulse-labeled mtDNA contains a higher proportion of molecules banding in the upper band (~44%) than in the lower band (35%). The slight difference in density between the [^{14}C]- and [^{3}H]DNA in the lower band is significant and has also been reported by Berk and Clayton (1974), who interpreted it as being due to the existence of a transient form of newly replicated closed-circular mtDNA lacking D-loop.

Determination of the Content in Labeled H and L Strands of the Different Forms of mtDNA. The results of the experiments described in the preceding sections indicated that the mtDNA–RNA complexes banding in the region 2 of the two-step gradient included the majority of the replicating and newly replicated mtDNA molecules. In order to investigate whether there was a fractionation, within the region 2 of the gradient, of DNA–RNA complexes involving different replicative forms of mtDNA and newly replicated molecules, the proportion of pulse (10 min) and long-term-labeled H and L strands was measured in mtDNA from different portions of

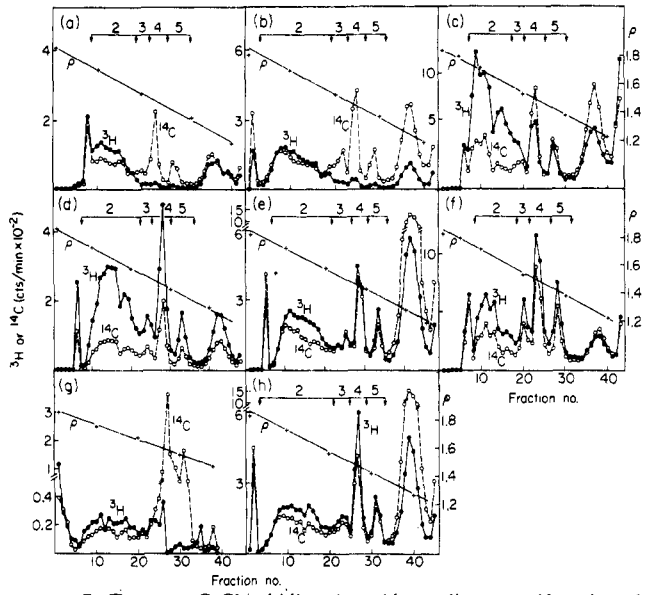

FIGURE 7: Two-step CsCl/ethidium bromide gradient centrifugation of mtDNA labeled after a [^{3}H]thymidine pulse or a pulse followed by a chase: (a) 2-min pulse (2.5 μCi of [^{3}H]thymidine/mL); (d) 2-min pulse and then 1-h chase with 10^{-5} M unlabeled thymidine; (b) 10-min pulse (1.25 μCi of [^{3}H]thymidine/mL); (e) 10-min pulse and then 1-h chase; (h) 10-min pulse and then 4-h chase; (c) 30-min pulse (0.625 μCi of [^{3}H]-thymidine/mL); (f) 30-min pulse and then 15-h chase. (g) A portion of the material corresponding to cuts 2 to 5 in the profile shown in panel a (2-min [^{3}H]thymidine pulse) was centrifuged to equilibrium in a CsCl/ethidium bromide density gradient as described in the legend for Figure 2. In the three different pulse-chase experiments (2-, 10-, and 30-min pulse), different amounts of ^{14}C-labeled cells were used; however, in each experiment, equal amounts of ^{14}C radioactivity were added to the various samples.

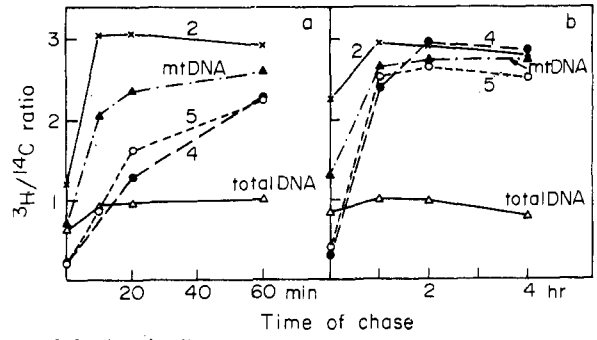

FIGURE 8: Ratios of radioactivity in pulse and in long-term-labeled DNA [^{3}H/^{14}C] in the different mtDNA species, as a function of the length of the chase, in the pulse-chase experiments. These data are derived from the two-step CsCl/ethidium bromide gradient patterns of the experiments shown in part in Figure 7a,d and 7b,e,h. The numbers refer to the various peaks in the two-step gradient profiles shown in that figure. The ratios were normalized by taking the [^{3}H/^{14}C] ratio of total cell DNA at equilibrium (1-h chase) as unit: (a) [^{3}H]thymidine 2-min pulse; (b) [^{3}H]thymidine 10-min pulse; mtDNA = sum of cuts 2 to 5 in the profiles; total DNA = total cell DNA.

cut 2 and from cuts 3–5, after separation of the strands on alkaline CsCl gradients.

The H/L ratio for the [^{14}C]thymidine long-term-labeled mtDNA was found to be about 0.85 in all mtDNA fractions from the two-step CsCl–ethidium bromide gradient. The lower ratio than expected from the relative thymine content of the H and L strand [1.26 (Brown, 1976)] may be due to differential losses of the two strands (differential adsorption to the walls of tubes?). More striking, however, was the observation that the H/L ratio was even lower for the [^{3}H]thymidine pulse-labeled mtDNA, in particular 0.43 in mtDNA from cut 2 and about 0.57 in mtDNA from cuts 3–5. This relative deficiency in H strands in the pulse-labeled material may indicate a slowing down of the initiation of replication relative to the elongation of molecules already initiated (with a resulting predominant extension of L chains, because of the asymmetry of replication [Robberson et al., 1972b; Berk and Clayton, 1974)]. That this phenomenon may be related to the culture conditions before pulse labeling, namely, to the change from normal serum to dialyzed serum in the medium (see Materials and Methods), is suggested by control experiments in which the cells were pregrown in the presence of dialyzed serum and labeled in the same medium either directly or after a centrifugation–resuspension step: in both cases, the overall H/L ratio in the pulse-labeled mtDNA was found to be identical or almost to that in long-term labeled DNA (1.1 and 1.2 in pulse-labeled mtDNA vs. 1.2 in long-term labeled mtDNA, in the two above mentioned experiments), whereas it was again lower (0.7 vs. 1.0) under the usual conditions of serum change. This phenomenon has not been investigated further.

Kinetics of Labeling of the Different mtDNA Species. Pulse-chase experiments were performed to determine the kinetics of labeling of the different forms of mtDNA and to obtain information on the possible relationship between transcription and replication of this DNA. Suspensions of exponentially growing HeLa cells were labeled for 2 min with [^{3}H]thymidine and harvested either immediately after the pulse or after different periods of chase (10, 20, and 60 min) with an excess of cold thymidine. The mtDNA from each sample was analyzed on a two-step gradient. Figures 7a and 7d show the patterns obtained, respectively, after a 2-min [^{3}H]thymidine pulse and after a 2-min pulse and a 60-min chase. The results of the complete experiment are illustrated in Figure 8a. An experiment using a 10-min pulse followed by a 0-, 1-, 2-, or 4-h chase (Figure 7b,e,h; Figure 8b), and another one using a 30-min pulse followed by a 0- or 15-h chase (Figure 7c and 7f) were also performed.

After a 2-min [^{3}H]thymidine pulse, the radioactivity was found essentially in the fast-sedimenting DNA fraction of the two-step gradient (band 2) and in nuclear DNA (Figure 7a). When the cells, after the pulse, were incubated in the presence of 10^{-5} M thymidine, there was a residual incorporation of [^{3}H]thymidine for as long as 10 min in total cell DNA and as long as 1 h in total mtDNA, as shown by the increase in the ^{3}H/^{14}C ratios in Figure 8a. The radioactivity incorporated into mtDNA of band 2 reached a maximum after 10 to 20 min of chase, and then showed a very slow decline. On the other hand, the radioactivity incorporated into closed-circular (component 4) and open-circular mtDNA (component 5) increased, following the 2-min pulse, up to at least 1 h of chase (Figure 8a). Similar results were obtained in the 10-min pulse–chase experiment (Figures 7b,e,h and 8b). The proportions of the different DNA species in the total [^{3}H]thymidine-labeled mtDNA were estimated using the [^{14}C]thymidine long-term-labeled DNA as an internal standard. Band 2 mtDNA was the main labeled component after the 2- or 10-min pulse; its proportion in total mtDNA decreased with the time of chase, reaching the value found in the long-term-labeled DNA by 1 to 2 h. Reciprocally, the proportion of the closed- (component 4) and open-circular form (component 5) of mtDNA, as well as of catenated oligomers (component 3), increased with time, reaching the equilibrium values in 1 h for the open-circular DNA and catenated oligomers, and 1 to 2 h for the closed-circular DNA. The proportions of the different forms of [^{3}H]thymidine pulse-labeled mtDNA appeared to be unchanged after a 15-h chase (Figure 7f).

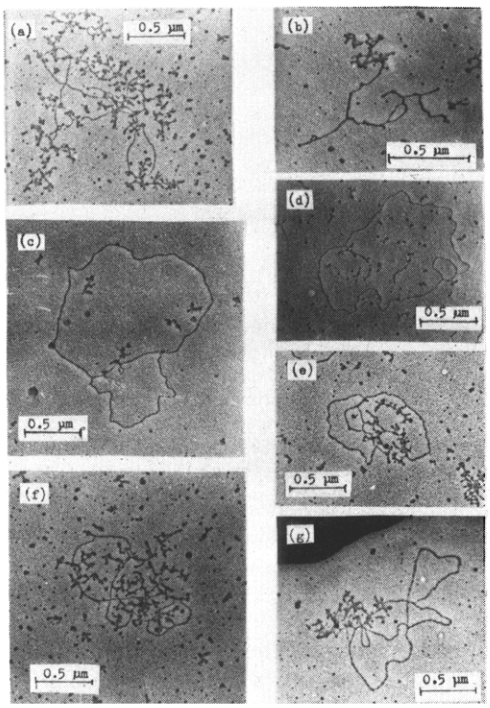

FIGURE 9: Electron micrographs of transcription complexes and replicative intermediates of mtDNA: (a) open-circular mtDNA molecule (4.8 μm), with attached RNA bushes around the whole contour; (b) highly twisted circular mtDNA molecule with RNA bushes; (c) expanded D-molecule (exp-D) carrying small bushes of collapsed single-stranded DNA in the displaced strand of the loop; (d) exp-D molecule with an RNA bush in the segment diametrically opposed to the expanded loop; (e) gapped circular molecule (~3.8 μm) carrying a bush of collapsed DNA in the center, and other bushes, presumably of RNA, in the upper and lower right quadrants of the contour; (f) open-circular mtDNA, possibly gapped, with many RNA bushes; (g) mtDNA molecule (appearing as a double-forked replicative intermediate) with a large bush consisting presumably of RNA. The average size of the measurable monomers was 4.8 ± 0.2 μm (107 molecules).

To investigate the possibility of a contamination of the DNA in band 2 by large-size catenated oligomers not associated with RNA, a portion of the mtDNA from cuts 2 to 5 of the two-step gradient in the 2-min pulse experiment was pooled and run in a CsCl/ethidium bromide equilibrium density gradient. The $^{3}H/^{14}C$ ratio in the mtDNA associated with RNA, banding at a density higher than 1.62 g/cm^3, was found to be identical to that of the mtDNA in band 2 in the two-step gradient (Figure 7g).

Electron Microscopic Examination of the mtDNA–RNA Complexes. Since [^{3}H]thymidine pulse-labeled mtDNA isolated immediately after labeling is found in the region of mtDNA–RNA complexes, one would expect to find, in electron microscope preparations of these complexes, mtDNA replicative intermediates among the mtDNA molecules bearing RNA bushes. To avoid dissociation of the DNA–RNA complexes, the aqueous spreading technique was chosen. Unfortunately, this technique does not allow a good display of the mtDNA replicative intermediates. Only large exp-D and exp-D(1) mtDNA molecules can be clearly identified; gapped molecules would appear as undersized molecules with a region of collapsed single-stranded DNA. D-mtDNA and small exp-D molecules would be hardly recognizable, especially if the molecules are also bearing RNA bushes. In the present work, samples of RNase-treated mtDNA–RNA complexes were also examined for comparison.

In the untreated preparations, bushes of collapsed RNA attached to mtDNA molecules appeared very often as complex and highly branched structures (see, for example, Figure 9a,f). By contrast, the segments of collapsed single-stranded DNA, as observed after RNase treatment, appeared most frequently as small curls or knots. Another criterion for distinguishing RNA bushes from DNA bushes was based on the known sequence of events in the replication of mammalian cell mtDNA (Robberson et al., 1972b; Berk and Clayton, 1974). Accordingly, any bush attached to an exp-D or exp-D(1) mtDNA molecule outside of the region of the loop was interpreted as RNA; similarly, any bush attached to a double-stranded DNA segment of a presumptive gapped mtDNA molecule was considered as consisting of RNA. On the basis of the above-mentioned criteria, the distinction between RNA and DNA bushes was in most cases relatively clear.

The proportion of molecules bearing RNA bushes, in our preparations of mtDNA–RNA complexes, varied from sample to sample between 17 and 45%. In most cases, there were only a few bushes per molecule; however, molecules with a great number of bushes, distributed all along the contour, were occasionally seen. As previously described by Aloni and Attardi (1972a), we observed the different types of mtDNA molecules involved in complexes with RNA (Figure 9): completely or partially relaxed circular DNA (Figure 9a), twisted circular DNA (Figure 9b), and what appeared to be catenated oligomers. In the untreated, as well as in the RNase-treated samples, about 2% of the molecules were clearly recognizable exp-D or exp-D(1) mtDNA. Most of these replicative intermediates exhibited no (Figure 9c) or only a few bushes interpretable as RNA (Figure 9d). About 10% of the molecules looked like gapped molecules; some of them were clearly bearing RNA bushes, as the complex represented in Figure 9e. The identification of molecules bearing many RNA bushes as replicative intermediates was made difficult by the complexity of the structures, particularly since in many cases the molecules in question were apparently concatenated.

Discussion

The present work has extended the previous observations on the occurrence in HeLa cell mitochondria of transcription complexes of mtDNA (Aloni and Attardi, 1972a). By using improved conditions of preparation, a substantial fraction (40–50%) of mtDNA has been isolated from exponentially growing cells in a form which exhibits properties expected of bona fide transcription complexes. These results imply that a substantial proportion of mtDNA molecules in these cells is at any given time engaged in transcriptive activity. This is not surprising, if one considers the very high rate of mtDNA transcription per unit of DNA length in HeLa cells, which has been estimated to be 10 to 20 times as high as the average rate of transcription of nuclear DNA (Attardi and Attardi, 1969).

Isolation Procedure. After preliminary trials, the procedure finally adopted involved purification of mitochondria from contaminating nuclear DNA by treatment with electrophoretically purified DNase, lysis of the organelles with sodium dodecyl sulfate, digestion with Pronase, and analysis of the lysate on a two-step CsCl/ethidium bromide gradient, under conditions which provided a good separation of mtDNA from nuclear DNA fragments and a satisfactory fractionation of the different mtDNA species, in particular the resolution of the mtDNA–RNA complexes from other forms of mtDNA. Of crucial importance for obtaining an appreciable yield of these complexes proved to be the omission of RNase treatment in the purification of the mitochondrial fraction [a step present in the original procedure for total mtDNA extraction (Smith et al., 1971)] and the digestion of the mitochondrial lysate with

Pronase. Failure to observe either one or both of these precautions probably accounts for the fact that these complexes have escaped attention by the previous authors who investigated mtDNA structure and replication in mammalian cells. The importance of Pronase treatment for releasing newly synthesized mitochondrial RNA from the fast-sedimenting components of a sodium dodecyl sulfate lysate of mitochondria has been repeatedly documented (Aloni and Attardi, 1971b, 1972a,b). The recent evidence concerning the attachment of HeLa cell mtDNA to the inner mitochondrial membrane (Albring et al., 1977) is in agreement with the possibility of association with the membrane of mtDNA transcription complexes. The use of low temperatures whenever possible, and in particular in the overnight and in two-step CsCl/ethidium bromide centrifugation, was also found to have a positive effect in increasing the yield of the mtDNA-RNA complexes.

Nature of the mtDNA-RNA Complexes. The observation that the heavy mtDNA fraction migrated in a region of the two-step CsCl/ethidium bromide gradient where the density is higher than the equilibrium density of pure mtDNA indicated that it must result from the association of DNA with a component of higher density, presumably RNA. This conclusion was confirmed by the observation that a substantial portion of this material banded in a CsCl/ethidium bromide gradient at buoyant densities from 1.65 to 180 g/cm^3, intermediate between the density of closed-circular mtDNA and that of RNA. The behavior of the heavy complexes after RNase digestion indicated that RNA was indeed the component responsible for the sedimentation and density properties of these complexes.

The association of RNA with mtDNA in these complexes is not covalent, since the RNA can be released by prolonged exposure to low ionic strength. Since the mtDNA-RNA complexes are extracted by sodium dodecyl sulfate-Pronase treatment of mitochondria, it is not very likely that RNA is bound to DNA through a protein component: rather, it seems more probable that this association involves local base pairing (Aloni and Attardi, 1972a). The complexes studied here are rather stable. Under mild denaturing conditions, like centrifugation in sucrose gradient in the presence of 47% formamide at 2 °C or in sucrose-formaldehyde gradient (Attardi and Attardi, 1971) after treatment of the sample with 10% formaldehyde for 16 min at 37 °C (unpublished observations), about one-half of the mtDNA originally present in these complexes and at least one-third of the RNA still sediment faster than closed-circular mtDNA. It seems possible that the relative stability of the mtDNA-RNA complexes observed after sodium dodecyl sulfate-Pronase treatment is due to the phenomenon described by Richardson (1975) for complexes between phage PM2 superhelical DNA and nascent RNA. This author observed that treatment with sodium dodecyl sulfate or other agents that dissociate proteins stabilizes these complexes, by increasing the length of the base-paired RNA segments from less than 20 up to as many as 600 nucleotides. These results were interpreted to indicate the spontaneous formation of a hybrid helix on superhelical DNA after denaturation of the RNA polymerase: the unwinding of the DNA required to form the RNA-DNA helix would presumably relieve some of the strain of the supercoiled DNA and would thus be energetically favored. According to this interpretation, the native enzyme would prevent the formation of the hybrid helix.

The electron-microscopic evidence presented by Aloni and Attardi (1972a) clearly indicated that the mtDNA-RNA complexes studied in that work contained transcription complexes. Similar electron-microscopic observations have been made in this work on material isolated by a different procedure. However, this electron-microscopic analysis was not apt to reveal whether the properties of the molecules analyzed reflected those of the bulk of the material. Thus, both in this work and in the earlier work by Aloni and Attardi (1972a), it could not be excluded that bona fide transcription complexes were copurified with structures of different nature, in particular with artificious complexes of RNA and mtDNA formed during the isolation procedure; such aggregates could conceivably share hydrodynamic and density properties with the transcription complexes. In principle, more than one mechanism exists whereby these aggregates could arise. Uptake of homologous single-stranded DNA fragments by superhelical DNA has been reported by Holloman et al. (1975). However, such complexes form slowly at low temperature and are unstable in the presence of even low concentrations of ethidium bromide, and thus would not have survived the isolation procedure used here. Replicating mtDNA molecules, which contain single-stranded segments, could be expected to form easily aggregates with RNA due to base pairing of homologous regions, in particular during the digestion at 37 °C of the mitochondrial lysate with Pronase: this tendency to aggregation of replicative intermediates has indeed been confirmed by the reconstruction experiment using pulse-labeled mtDNA. In the present work, the great majority of replicating mtDNA molecules was recovered in the mtDNA-RNA complexes. The proportion of replicative molecules in the HeLa cell mtDNA population is not known; however, if it is similar to that found in mouse cells (10–15%) (Robberson et al., 1972b, Robberson and Clayton, 1972), the binding of free RNA molecules to replication intermediates could not account for the proportion of total mtDNA found in the form of mtDNA-RNA complexes, i.e., about 45%. Even if the mtDNA molecules containing D-loop were involved in this aggregation phenomenon, they would not change the above conclusion, since the frequency of D-loops in HeLa cell mtDNA is low (Flory, 1974).

Several experiments were carried out to investigate whether phenomena of aggregation of mtDNA with free RNA were responsible for the observations reported here. The addition to the purification procedure of a step of centrifugation of the mitochondrial lysate through a sodium dodecyl sulfate-sucrose gradient prior to the two-step CsCl/ethidium bromide gradient analysis, for the purpose of separating the bulk of free RNA from mtDNA very early, i.e., before exposure of the lysate to high-salt environment (which would favor the uptake of single-stranded RNA on superhelical DNA and other aggregation phenomena), did not affect appreciably the yield of the DNA-RNA complexes. Furthermore, when mitochondria were lysed with Triton X-100, most of the mtDNA and mtDNA-RNA complexes were recovered in the membrane fraction, while most of the RNA-containing structures and free RNA remained in the supernatant. These results suggest that aggregates probably do not form by slow interaction of mtDNA with the bulk of RNA during the purification of the complexes; however, they could form immediately after lysis of mitochondria with a fraction of the RNA.

In previous work (Aloni and Attardi, 1972a), attempts to reproduce in vitro mtDNA-RNA complexes by mixing 45S nuclear rRNA precursor molecules with closed-circular mtDNA molecules gave negative results. More extensive reconstruction experiments were carried out in the present study by extracting mtDNA, after RNase treatment, from mtDNA-RNA complexes isolated from cells either long-term labeled or pulse-labeled with [^{3}H]thymidine and mixing it with mitochondria from an equivalent number of [^{14}C]thymidine-labeled cells. After reisolating the mtDNA-RNA com-

plexes from the mixture, about 17% of [^{3}H]thymidine long-term-labeled mDNA and 60% of the [^{3}H]thymidine pulse-labeled mtDNA were recovered in the region of the heavy mtDNA fraction. Although these figures are substantially lower than the proportions of heavy complexes present in the original [^{3}H]thymidine-labeled preparations (42 and 80%, respectively), they do show that a portion of mtDNA isolated from these complexes can reassociate with RNA to form fast-sedimenting structures. By contrast, when clean closed- and open-circular mtDNA from [^{3}H]thymidine long-term labeled cells was used, only a small proportion (6%) of it was recovered in the region of the heavy complexes. The interpretation of these results is not simple, although they do indicate that the mtDNA from the fast-sedimenting complexes differs from clean closed- and open-circular mtDNA in its capacity to form complexes. The involvement of the replicating molecules in this phenomenon seems to be excluded by the quantitative arguments presented above. A possible interpretation of these observations is that, after RNase treatment, stretches of base-paired RNA survive in the original mtDNA–RNA complexes, forming displacement loops. These loops, which could be as long as 600 nucleotides if the situation described for PM2 transcription complexes apply here, could be the site of binding, by an exchange process with the bound RNA segment, of free RNA having regions of complementarity to the base-paired DNA strand. The complete symmetrical transcription of HeLa cell mtDNA (Aloni and Attardi, 1971b, 1972; Murphy et al., 1975), which involves copying of the H and L strands at approximately equal rates (Aloni and Attardi, 1971b), and the occurrence in HeLa cell mitochondria of appreciable amounts of symmetrical transcripts (Aloni and Attardi, 1971b; Young and Attardi, 1975) would also conceivably allow a base-pairing of complementary RNA sequences with the displaced strands in the loops or with free tails of the bound RNA segments.

Whatsoever the mechanism of association with free RNA may be, the present results indicate clearly that, in contrast to clean closed- and open-circular mtDNA, the RNase-treated mtDNA extracted from the mtDNA–RNA complexes retains some intrinsic structural property which makes it susceptible to re-form complexes behaving similarly to the original ones. The simplest interpretation is that this structural property is directly related to the transcriptive activity of mtDNA. If this interpretation is correct, it implies that the isolation procedure employed here has effectively separated transcription complexes of mtDNA from other forms of mtDNA. The occurrence of replicating molecules in this mtDNA fraction is discussed below.

If the mtDNA–RNA complexes detected here represent transcription complexes, they should contain nascent RNA molecules which can be labeled during a [^{3}H]uridine pulse. Indeed, in the present experiments, after a 10-min [^{3}H]uridine pulse, the sharp peak of labeled RNA in the dense portion of the two-step CsCl/ethidium bromide gradient exhibited a shoulder on the light side tailing in the region of the mtDNA–RNA complexes. The sedimentation analysis in a formamide–sucrose gradient of these complexes revealed more clearly an appreciable amount of labeled RNA cosedimenting with mtDNA. A larger proportion of labeled RNA was found to be associated with the mtDNA complexes in the earlier work (Aloni and Attardi, 1972a), after a shorter [^{3}H]uridine pulse.

Forms of Mitochondrial DNA Involved in the Formation of mtDNA–RNA Complexes. The long-term-labeled mtDNA obtained from the complexes after RNase digestion and reextraction showed, besides forms behaving as closed- and open-circular monomers, a substantial proportion (20–30%) migrating in the position expected for catenated oligomers in a two-step CsCl/ethidium bromide gradient (Figure 3) and banding in the intermediate region between closed- and open-circular mtDNA in a CsCl/ethidium bromide density equilibrium run (Figure 6). This material is mostly represented by concatenated molecules of mixed closed- and open-circular configuration. Electron microscopic analysis of this mtDNA has confirmed the high proportion of catenated oligomers of mixed composition.

The mtDNA labeled during a short [^{3}H]thymidine pulse migrated in its great majority in the region of the heavy mtDNA fraction in the two-step CsCl/ethidium bromide gradient; the pulse-labeled mtDNA isolated from these complexes by RNase digestion and gel filtration banded in a CsCl/ethidium bromide equilibrium density gradient mainly in the intermediate region (I) and in the upper band (U), as expected for replicative intermediates of monomers and oligomers. The presence of such replicative forms in the fraction of mtDNA–RNA complexes indicates that replicative intermediates, as isolated here, are associated with RNA. This interpretation has been confirmed by the RNase sensitivity and the stability characteristics of the complexes involving these intermediates, which were indistinguishable from those of the bulk of mtDNA–RNA complexes.

The observed kinetics of labeling of the different forms of mtDNA in the pulse–chase experiments are in agreement with a flow of radioactivity from mtDNA present in heavy complexes to open-circular and, with a slight delay, to closed-circular monomer mtDNA.

As to the nature of the DNA–RNA complexes involving replicative intermediates, the reconstruction experiment using pulse-labeled mtDNA indicated clearly the possibility of an aggregation phenomenon, during the isolation procedure, between free RNA and, presumably, single-stranded DNA segments of the intermediates. On the other hand, the electron-microscopic analysis carried out here showed the occurrence of replicative molecules, of the exp-D, exp-D(1), or gapped-molecule type, with RNA bushes attached to double-stranded DNA segments, an observation which pointed to the transcriptive activity of these molecules. This was not, however, a frequent finding; the majority of the replicative intermediates had, in fact, no bushes recognizable unambiguously as RNA on the basis of their site of attachment to the DNA and of their complex branched structure. In view of the density of the DNA–RNA complexes, one has to assume that most of the RNA became detached from DNA during preparation for electron microscopy; on the other hand, the recognition of molecules bearing many bushes as replicative intermediates was made difficult by the complexity of the structures. Because of these factors, it was not possible to correlate quantitatively the electron-microscopic observations with the biochemical finding of a general association with RNA of the replicating mtDNA molecules. Therefore, although there is the suggestion from the electron-microscopic observations that replicative intermediates *can* serve as templates for transcription, one cannot say whether this phenomenon is of occasional or frequent occurrence. Likewise, no conclusion can be derived from the present results as to whether a necessary coupling exists between transcription and some step in mtDNA replication in HeLa cells. Further work is needed to clarify this important question.

It has been shown that transcription of mtDNA can proceed in animal cells, at early stages of development, in the apparent absence of replication (Piko, 1970; Craig, 1970; Chamberlain and Metz, 1972). There is also evidence of incorporation of

[^{3}H]thymidine into mtDNA (which may reflect true replication), when RNA synthesis is inhibited by cordycepin (our unpublished results) or by berenil, a drug which modifies in vivo the tertiary structure of mtDNA (Rastogi et al., 1975). On the other hand, in another system, the SV40 DNA, it is known that DNA replication is a prerequisite for late transcription, and the hypothesis has been advanced that replicative intermediates may be used for late transcription (Girard et al., 1974; Laub and Aloni, 1976; Graessman et al. 1977).

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