

Association of a protein structure of probable membrane derivation with HeLa cell mitochondrial DNA near its origin of replication

(membrane attachment/electron microscopy)

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Communicated by Norman Davidson, November 29, 1976

ABSTRACT Almost all (about 95%) of the mitochondrial DNA molecules released by Triton X-100 lysis of HeLa cell mitochondria in the presence of 0.15 M salt are associated with a single protein-containing structure varying in appearance between a 10–20 nm knob and a 100–500 nm membrane-like patch. Analysis by high resolution electron microscopy and by polyacrylamide gel electrophoresis after cleavage of mitochondrial DNA with the endonucleases *EcoRI*, *HindIII*, and *Hpa II* has shown that the protein structure is attached to the DNA in the region of the D-loop, and probably near the origin of mitochondrial DNA replication. The data strongly suggest that HeLa cell mitochondrial DNA is attached *in vivo* to the inner mitochondrial membrane at or near the origin of replication, and that a membrane fragment of variable size remains associated with the DNA during the isolation. After sodium dodecyl sulfate extraction of mitochondrial DNA, a small 5–10 nm protein is found at the same site on a fraction of the mitochondrial DNA molecules.

The attachment of animal cell mitochondrial DNA (mtDNA) to the inner mitochondrial membrane has been surmised in the past on the basis of electron microscopy (EM) observations (1); however, nonspecific adsorption phenomena could not be excluded in these early observations because of the lack of suitable position markers. In the present work, by applying to mtDNA extracted under mild conditions EM techniques designed to visualize DNA-protein complexes, we have made observations that support the idea of a specific membrane attachment of mtDNA. In particular, we have found that almost all (about 95%) circular mtDNA molecules released by Triton X-100 lysis of mitochondria in the presence of low salt exhibit a single protein structure associated with them, which varies in appearance between a 10–20 nm knob and a 100–500 nm membrane-like patch. We also report on the unique location of the protein structure near the origin of mtDNA replication and on the occurrence of protein(s) resistant to sodium dodecyl sulfate (NaDodSO_4) at the same site on a fraction of the mtDNA molecules.

MATERIALS AND METHODS

Growth in Suspension and Subcellular Fractionation of HeLa Cells. These were carried out as previously described (2, 3).

Isolation of mtDNA. mtDNA was isolated from a DNase-treated and NaDodSO_4 -solubilized crude mitochondrial fraction by banding in a CsCl/ethidium bromide (EtdBr) gradient (4); the material in the upper and lower bands was pooled and pelleted, and the total mtDNA was separated from degraded nuclear DNA by centrifugation through a 2-step CsCl/EtdBr gradient (5).

Abbreviations: mtDNA, mitochondrial DNA; NaDodSO_4 , sodium dodecyl sulfate; EM, electron microscopy; EtdBr, ethidium bromide; RF, replicative form.

Isolation of mtDNA-Protein Complexes. A crude mitochondrial fraction from 50 ml of packed cells was resuspended in 18 ml of 0.25 M sucrose/0.01 M Tris-HCl at pH 8.0 (25°)/0.01 M EDTA, and lysed by addition of 18 ml of 2% Triton X-100/0.4% sodium deoxycholate in 0.01 M Tris, pH 8.0/0.3 M NaCl/0.01 M EDTA. (Omission of sodium deoxycholate reduced somewhat the yield of the mtDNA-protein complexes solubilized in this step.) After 10 min at 2°, the crude lysate was centrifuged (65 Spinco rotor, 20,000 rpm, 20 min) to remove the contaminating nuclear chromatin. Aliquots (3 ml) of the supernatant were immediately layered over preformed 10–40% gradients of Metrizamide (6) (Nyegaard & Co. A/S, Oslo, Norway) in 0.01 M Tris-HCl buffer, pH 8.0/0.01 M EDTA/0.15 M NaCl/0.25% Triton X-100 (TENT), and centrifuged 17 hr at 40,000 rpm (2°) in an SW 41 rotor. The mtDNA-containing fractions were pooled and diluted with an equal volume of TENT; 9 ml portions of the diluted sample were then layered over 3 ml of saturated solution of CsCl in 0.01 M Tris, pH 8.0/0.01 M EDTA/0.02 or 0.2% sodium dodecyl-*N*-sarcosinate (Sarkosyl, Ciba-Geigy Corp.) and centrifuged in an SW 41 rotor at 39,000 rpm for 14 hr at 5°. The mtDNA in the CsCl cushion solution was then banded to equilibrium in an SW 50.1 rotor by centrifugation at 35,000 rpm for 60 hr at 5°, collected, diluted with 2 volumes of 0.01 M Tris, pH 8.0/0.01 M EDTA/0.02 or 0.2% Sarkosyl, pelleted, and dissolved in a suitable buffer.

In some experiments, the mtDNA-protein complexes were crosslinked by treating the crude or cleared lysate with formaldehyde and glutaraldehyde, as described elsewhere†.

Restriction Endonuclease Cleavage of mtDNA-Protein Complexes. Digestion with the restriction enzymes *EcoRI*, *HindIII*, and *Hpa II* was carried out in the appropriate buffer (7, 8), in the presence of 0.25% Triton X-100.

Polyacrylamide Gel Electrophoresis of Restriction Digests. Electrophoresis through polyacrylamide gradient slab gels was carried out as described by Studier (9).

Electron Microscopy. mtDNA complexes isolated from either formaldehyde-and-glutaraldehyde-treated or untreated lysates were prepared for EM by two different techniques. In one, the x-ray relaxed (7) samples were mounted onto glow-charged carbon supports (10); the samples were diluted to 0.1 $\mu\text{g}/\text{ml}$ in 2 mM spermidine/0.15 M NaCl/0.01 M Tris-HCl, pH 7.5, just prior to their adsorption, and tungsten shadowed with rotation (10). In the alternate procedure, the complexes were mounted onto freshly cleaved mica in the presence of EtdBr and replicated with Pt-Pd and carbon (11). Micrographs were taken with 35 mm film on a Philips EM 300 electron microscope. Molecule lengths were measured with tracing devices similar to those described elsewhere (12). Replicative form (RF)

† Christiansen, G., Landers, T., Griffith, J. & Berg, P. (1977) *J. Virol.* in press.

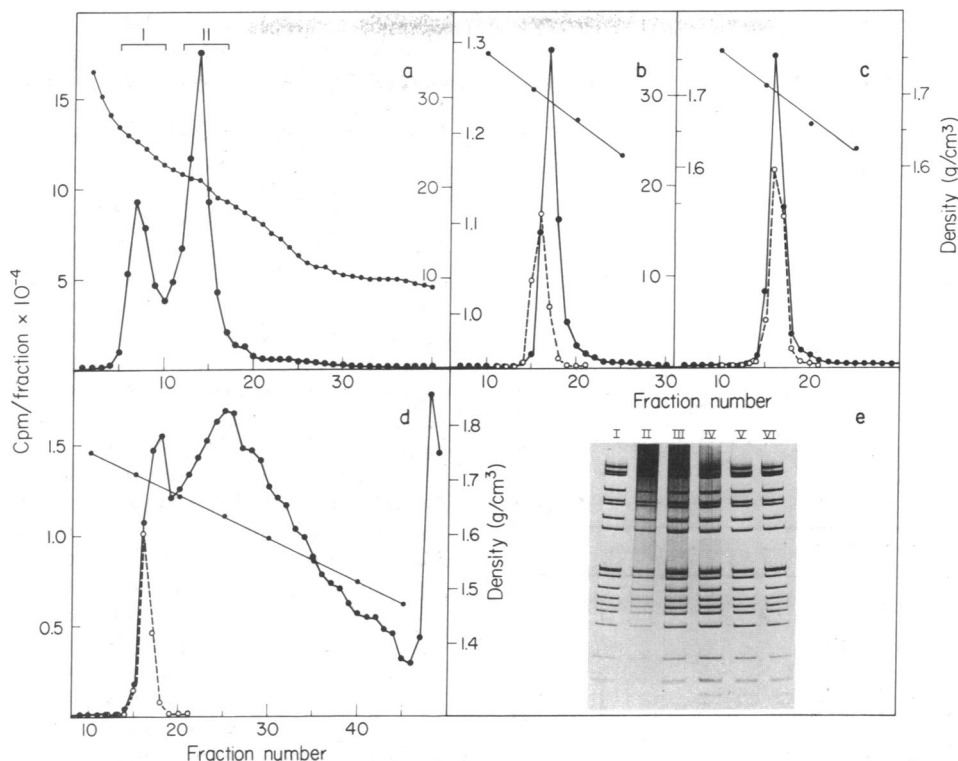


FIG. 1. Purification of mtDNA-protein complexes from a HeLa cell mitochondrial Triton X-100 lysate. (a) Density distribution profile of [³H]thymidine-labeled DNA after centrifugation of the cleared mitochondrial lysate through a preformed 10–40% Metrizamide gradient. (b) Density distribution in a CsCl gradient of the material from peak II in a; ³²P-labeled ϕ X174 RF I DNA was used as an internal marker. (c) Density distribution in a CsCl gradient of the material from peak II in a after treatment with proteinase K. (d) Density distribution in a CsCl gradient of [³H]thymidine-labeled mtDNA-protein complexes isolated from a mitochondrial Triton X-100 lysate after crosslinking with formaldehyde and glutaraldehyde. (e) Polyacrylamide gel electrophoretic profiles of *Hpa*II restriction endonuclease digests of DNA from different samples during the purification of mtDNA-protein complexes. Slots I and VI: mtDNA isolated from NaDodSO₄-lysed mitochondria; slot II: DNA from a crude mitochondrial Triton X-100 lysate; slot III: DNA from a cleared mitochondrial lysate; slot IV: material from peak II in a; slot V: material from the peak in b. Gels were stained with EtBr and photographed under short wavelength UV illumination. See text for details. ●—●, ³H; ○---○, ³²P.

DNA of bacteriophage ϕ X174 (gift of R. L. Sinsheimer and A. Szalay) was used as an internal standard.

RESULTS

Isolation of mtDNA-Protein Complexes. Samples of the preparation at various stages of purification were digested with the restriction endonuclease *Hpa* II, known to produce 21 fragments from HeLa cell mtDNA (ref. 13; D. Ojala and G. Attardi, unpublished) (Fig. 1e, slots I and VI). The cleavage pattern obtained from a crude mitochondrial lysate (Fig. 1e, slot II) revealed the presence of mtDNA with a heavy contamination by nuclear DNA (heterogeneous material in the upper part of the gel). The bulk of this contamination was removed by low-speed centrifugation (Fig. 1e, slot III); about three times as much material was run in this slot as in slot II. When the cleared lysate was banded in a preformed Metrizamide gradient, the [³H]thymidine-labeled DNA was found to form two distinct peaks at densities of about 1.2 and 1.14 g/cm³ (Fig. 1a). DNA from the 1.14 g/cm³ peak revealed a typical *Hpa* II mtDNA pattern with only a slight contamination by nuclear DNA (Fig. 1e, slot IV). The DNA from the heavier peak produced, on the contrary, after *Hpa* II cleavage, heterogeneous material spread in the upper portion of the gel (not shown), and appeared therefore to consist almost exclusively of nuclear DNA.

The mtDNA from the 1.14 g/cm³ peak in Fig. 1a was banded to equilibrium in a saturated CsCl solution in the presence of 0.02 or 0.2% Sarkosyl, where it formed a fairly sharp

peak at a density of 1.68 g/cm³ (Fig. 1b). The *Hpa* II restriction pattern of the material from this peak (Fig. 1e, slot V) revealed that it consisted of mtDNA almost completely free of contaminating nuclear DNA. In the gradient shown in Fig. 1b, the mtDNA appeared to band at a slightly lower density relative to the internal marker (phenol/NaDodSO₄-extracted ϕ X174 RF I DNA). The density difference between mtDNA and the marker DNA was no longer detected after treatment of the mtDNA with proteinase K (Merck Co., Darmstadt, West Germany) (100 μ g/ml) at 37° for 30 min in the presence of 1% NaDodSO₄ (Fig. 1c), suggesting the prior presence of protein on the mtDNA.

To verify to what extent mtDNA was complexed with protein in the original lysate, the mitochondrial Triton X-100 lysate was subjected to crosslinking with formaldehyde and glutaraldehyde before the purification procedure. After such fixation, the DNA distribution in the Metrizamide gradients was essentially unchanged, but the material from the lighter peak showed a broad density distribution, ranging from 1.48 to 1.70 g/cm³, when rebanded in CsCl (Fig. 1d). These observations suggested that the crosslinking stabilized DNA-protein associations sensitive to high salt (4–7 M CsCl) and/or to Sarkosyl.

The heaviest DNA fractions in the CsCl profile of crosslinked material gave essentially the same *Hpa* II restriction pattern as observed for noncrosslinked purified mtDNA-protein complexes. On the other hand, the DNA banding at lower densities showed a pronounced reduction in the yield of frag-

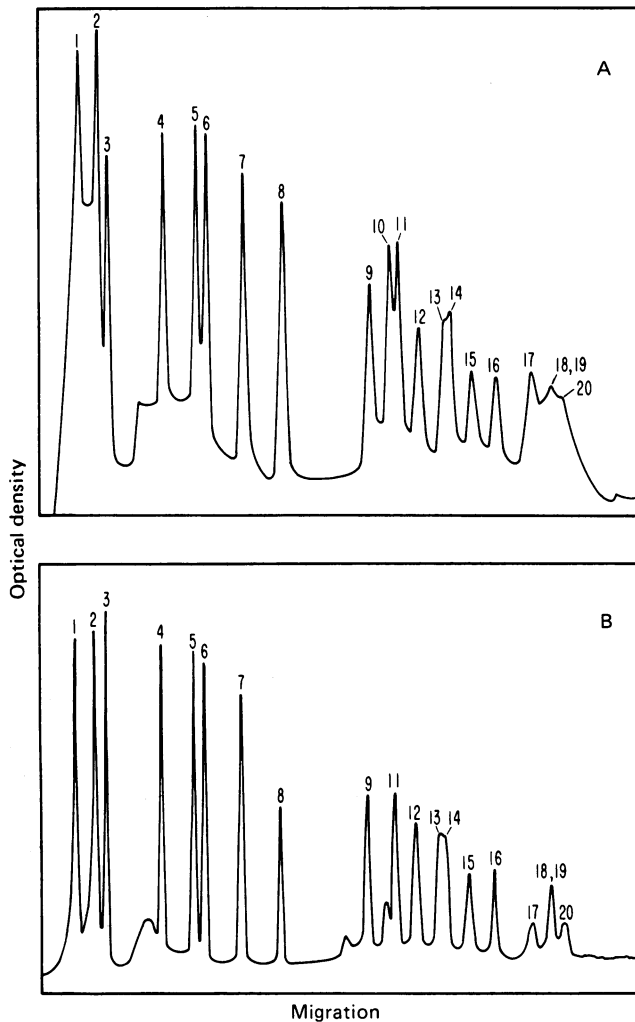


FIG. 2. Microdensitometer tracings of polyacrylamide gel electrophoretic profiles: (A) *Hpa* II restriction endonuclease digest of the HeLa cell NaDodSO₄-extracted mtDNA, and (B) mtDNA-protein complexes crosslinked with formaldehyde and glutaraldehyde. The origin of the gel is at the left.

ments 10 and 17 and a slighter reduction in that of fragment 8 (Fig. 2); this was presumably due to the association of these fragments with protein, which either decreased their electrophoretic mobility or protected some restriction site(s) from digestion. It has been recently shown (ref. 13; D. Ojala and G. Attardi, unpublished) that fragment 17 includes or is very near to the origin of replication, and is flanked on the physical map on one side by fragment 10, which contains the major portion of the D-loop sequences, and on the other side by fragment 8.

Visualization of a Membrane-Like Structure or Protein Complex Bound to mtDNA. EM techniques designed to visualize complexes of mtDNA with protein or membrane were applied to examine the noncrosslinked mtDNA from the light density peak in the Metrizamide gradient (Fig. 1a). This material appeared as typical 5 μ m mtDNA circles, except that a prominent structure was found at one or more sites on the DNA. An analysis of 200 DNA circles showed 95% with one such structure, 1% with two or more, and 4% with none. These structures varied in size from about 0.01 to 0.5 μ m. The smaller structures had the appearance of large protein complexes, while the larger ones appeared like membrane patches, as visualized by the EM procedures employed here (Fig. 3A). When this

material was rebanded in CsCl, the overall frequency of mtDNA molecules with associated protein structures was not reduced, but the large structures were now absent, and the smaller complexes were the only ones observed (Fig. 3B). In contrast, when the material was crosslinked prior to banding in Metrizamide and CsCl, the larger mtDNA-associated structures were predominant.

After isolation of mtDNA by NaDodSO₄ lysis of mitochondria and banding in a CsCl/EtdBr gradient, about 30% of the open circular DNA molecules examined by EM showed a single small (5–10 nm) knob (Fig. 3C); the remainder had none. After Pronase digestion, the frequency of DNA molecules exhibiting such a knob was reduced to a background level (about 5%), suggesting a protein nature for this structure. An NaDodSO₄-resistant structure was also observed with similar frequency in closed circular mtDNA molecules.

Location of the Membrane-Like Structure or Protein Complex on the mtDNA Map. In order to decide whether the membrane-like structures or protein complexes are at a specific location on the circular mtDNA molecules, mtDNA-protein complexes (Fig. 1b) were cleaved with *Hind*III endonuclease to yield fragments corresponding to about 5%, 33%, and 62% of the total contour length (8). The 62% fragment contains the origin of mtDNA replication at about 60% fractional distance from the end adjacent to the 33% *Hind*III fragment, while the D-loop corresponds to the region at 60–66% fractional distance from the same end (8). Upon examination of the cleaved complexes, the protein structures were found only on linear DNA fragments 50–65% the length of the intact circle (Fig. 3D). Often the DNA appeared to be tangled around the membrane-like structure. Those molecules in which the length of the DNA fragment was within 10% of the full 62% length were selected for analysis. The histogram in Fig. 4A shows that the protein structure is at about 62% fractional distance from one of the two ends of the *Hind*III fragment. In order to establish which end this was, relative to the *Hind*III map, mtDNA-protein complexes were digested with *Eco*RI. This enzyme cleaves mtDNA into three fragments of about 6.8%, 45%, and 49% fractional lengths (8). The origin of mtDNA replication is nearly equidistant from the two ends of the 49% *Eco*RI fragment. Following digestion with *Eco*RI and preparation for EM by the method of Koller *et al.* (11), the protein structure was found on DNA fragments of about 50% the total contour length (Fig. 3E). As shown in Fig. 4B, the structure is very close to the middle of these fragments. The above-described results localize the position of the protein structure to the region of the mtDNA containing the origin of replication and the D-loop sequences.

The small protein found on the DNA after NaDodSO₄ extraction was similarly mapped. When the *Eco*RI digest of NaDodSO₄ extracted mtDNA was spread by the technique described by Koller *et al.* (11), many of the 49% *Eco*RI fragments had a V shape (possibly due to flow forces during the adsorption of DNA on the mica sheet), and about 50% of them had a clearly recognizable "knob" at the vertex (Fig. 3F). Measurements on 90 of these V-shaped molecules with a "knob" showed that this structure was also approximately equidistant from the two ends (Fig. 4D). In a *Hind*III digest of NaDodSO₄-extracted mtDNA, the V-shaped molecules were exclusively found in the 62% size class; in these, a "knob" could not be clearly recognized in general, possibly due to the low contrast. Measurements of 29 molecules showed that the bending point was at about 60% fractional distance from the farther end (Fig. 4C), again placing it close to the origin of mtDNA replication.

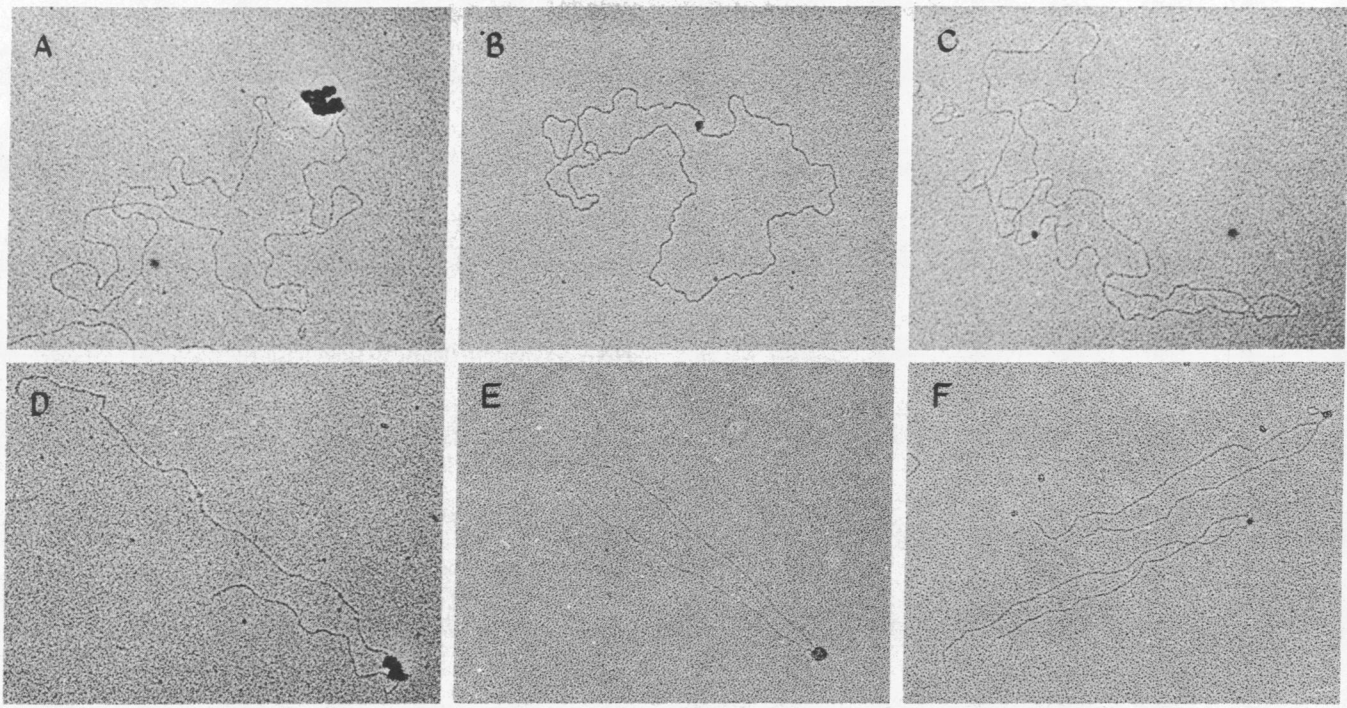


FIG. 3. Visualization of HeLa cell mtDNA-protein complexes released by Triton X-100 or NaDodSO₄ lysis of mitochondria. (A) Triton X-100-released complex banded in Metrizamide. (B) Triton X-100-released complex banded in Metrizamide and CsCl. (C) Complex isolated by NaDodSO₄ lysis of mitochondria and CsCl/EtdBr banding. (D, E) Fragments, with associated protein structure, from a *Hind*III (D) or *Eco*RI (E) digest of material as in A. (F) Fragments with "knob" from an *Eco*RI digest of material as in C. Uncut mtDNA-protein complexes were relaxed by x-irradiation prior to examination. A-D were prepared by spermidine-facilitated mounting and tungsten shadowed (10). E and F were prepared by the procedure of Koller *et al.* (11) and Pt-Pd shadowed. Bar equals 1 μ m.

Evidence against an Artfactual Nature of the mtDNA-Protein Complexes. To exclude the possibility that these complexes artifactually formed during lysis of mitochondria (possibly due to sticking of proteins or membrane fragments to the single-strand DNA portion of the D-loop), ³²P-labeled closed-circular mtDNA [which contains the majority of the D-loop DNA (14, 15)] was mixed with lysis buffer before addition to a [³H]thymidine-labeled mitochondrial suspension; one half of the lysate was treated with formaldehyde and glutaraldehyde, the other half was left untreated, and mtDNA-protein complexes were isolated from the two portions of the lysate. As shown in Fig. 5, in both samples, there was very

little evidence of aggregation of the ³²P-labeled DNA with protein components causing its displacement to the density range typical of the mtDNA-protein complexes. Similar results were obtained in a similar reconstruction experiment utilizing ³²P-labeled open circular mtDNA.

DISCUSSION

The EM observations reported above have clearly indicated the association of Triton X-100-released HeLa cell mtDNA molecules with a protein complex or membrane fragment of variable size, at a specific site corresponding to the DNA region normally occupied by the D-loop, and probably near the origin of replication of the L strand (14, 15). The observation that *Hpa*II fragment 17, which contains or is very near to the origin of mtDNA replication, and the adjacent fragments in the *Hpa*II map, 10 and 8 (ref. 13; D. Ojala and G. Attardi, unpublished), showed a decreased yield on polyacrylamide gel analysis, when the Triton X-100-released mtDNA was formaldehyde- and glutaraldehyde-treated, is consistent with their being associated with a protein-containing structure, which either decreased their mobility or protected some restriction sites. Quite possibly, extensive crosslinking of the protein complexes or membrane fragments (some of which are very large) to the DNA, as well as inter- or intraprotein crosslinking, would protect these structures during the various experimental manipulations, in particular during the CsCl density gradient centrifugation in the presence of Sarkosyl.

The uniqueness and site specificity of the association of the protein complex or membrane fragment with mtDNA would tend to exclude a random adsorption of such structures to the DNA during or after the Triton X-100 lysis of the organelles. Also the possibility of a protein complex or membrane fragment

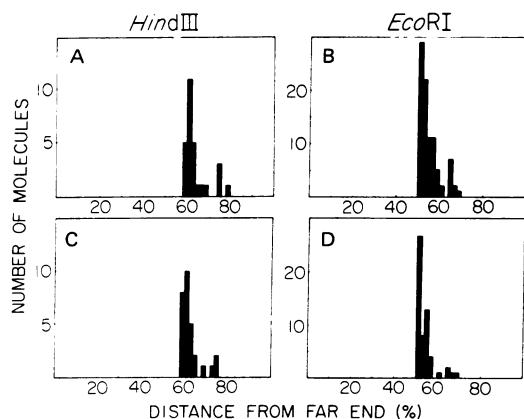


FIG. 4. Histograms of the positions of the protein complex or membrane structure on the largest fragments produced by *Eco*RI (A) or *Hind*III (B) cleavage of Triton X-100-released mtDNA. Histograms of the positions of the NaDodSO₄-resistant protein on the largest fragments produced by *Eco*RI (C) or *Hind*III (D) cleavage of NaDodSO₄-extracted mtDNA.

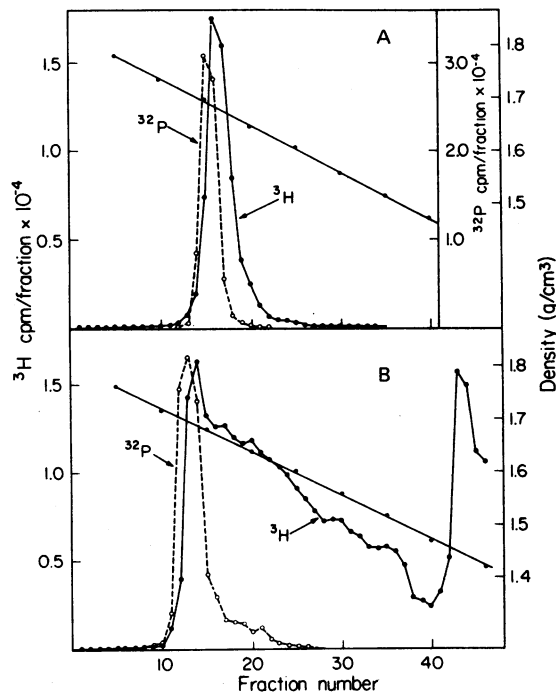


FIG. 5. Equilibrium banding in CsCl gradients of ^{32}P -labeled purified closed circular mtDNA which had been added to a suspension of mitochondria from ^3H -labeled cells together with the Triton X-100 lysis buffer, and subjected to the usual procedure for purification of the mtDNA-protein complexes, as described in *Materials and Methods*. (A) Untreated mitochondrial lysate. (B) Mitochondrial lysate treated with formaldehyde and glutaraldehyde. ●—●, ^3H ; ○—○, ^{32}P .

sticking to the single-stranded DNA portion of the D-loop during mtDNA isolation seems unlikely. In fact, the frequency of D-loops in HeLa cell mtDNA is normally low (16). Furthermore, the association with mtDNA of the protein structure appears to withstand exposure to both high ionic strength (7 M CsCl) and low ionic strength (as used for the *Hind*III digestion), as well as to 0.2% Sarkosyl. Finally, the reconstruction experiment described in the *Results* section failed to show any evidence of a significant aggregation of protein complexes or membrane fragments with mtDNA leading to a decrease in its density in a CsCl gradient.

A plausible interpretation of the evidence presented above, and of the previous EM observations by Nass (1), is that HeLa cell mtDNA is attached *in vivo* to the inner mitochondrial membrane at or near the origin of replication, and that a fragment of variable size of this membrane remains associated with the DNA during the isolation under the conditions used here. However, the mitochondrial membrane derivation of the protein structure detected here remains to be proven.

When HeLa cell mtDNA was isolated after NaDodSO_4 lysis of mitochondria, only a small (5–10 nm) Pronase-sensitive “knob” was observed on a portion of the DNA molecules. The results of restriction enzyme cleavage of this NaDodSO_4 released mtDNA are consistent with the location of this “knob”

at or close to the site of the origin of mtDNA replication. It seems possible that the NaDodSO_4 -resistant protein is a residue of the membrane fragment which withstood NaDodSO_4 treatment. However, further experiments are required to establish this point. A NaDodSO_4 -resistant protein associated with mtDNA has also been observed in L cells (H. Kasamatsu and M. Wu, personal communication). A salt-stable protein complex has been recently described at the site of the origin of simian virus 40 DNA replication (17), and a portion of it has been reported to be NaDodSO_4 -resistant and may be covalently bound to the DNA (18).

An association with membrane at the site of the origin of DNA replication has been shown for the bacterial chromosome (19) and for the plasmid ColE1 DNA (20). As hypothesized for these systems, the membrane association of mtDNA may have an important role in the mechanism and regulation of mtDNA replication and/or in the segregation of daughter molecules.

We wish to thank Ms. D. Ojala and Dr. A. Szalay for advice in the polyacrylamide gel electrophoretic analysis. The help of Ms. A. Drew and Ms. G. Engel is gratefully acknowledged. This work has been supported by a research grant from the U.S. Public Health Service (GM-11726) and a fellowship of the Deutsche Forschungsgemeinschaft to M.A.

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