The Rat Liver Mitochondrial DNA-Protein Complex: Displaced Single Strands of Replicative Intermediates Are Protein Coated

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ABSTRACT Mitochondrial DNA (mtDNA)-protein complexes were released from the organelles by sodium dodecyl sulfate-lysis and purified by Phenyl-Sepharose CL-4B chromatography. The mitochondrial DNA-binding protein P16 was the only detectable protein in the complex. Treatment of the complex with proteinase K, or subtilisin, revealed the presence of a protease-insensitive, submolecular domain ($M_r \cong 6{,}000$) that retained the capacity to bind tenaciously to the DNA. Analysis of chemically fixed complexes by CsCl isopycnic gradient centrifugation showed that P16 was bound to a large subpopulation of mtDNA enriched in displacement loops (D-loops). Based upon the effective buoyant density of the complex in CsCl gradients and the molecular weights of P16 and mtDNA, it was estimated that a mean of 49 P16 molecules were bound per mtDNA. For this measurement, the variation in hydration of protein and DNA at different CsCl concentrations was ignored. Analysis of restriction endonuclease-digested complexes by glass fiber filters that bind only protein-associated DNA resulted in the retention of a single fragment regardless of the enzyme, or enzymes, used. In each case, the retained fragment was the D-loop-containing fragment. With direct electron microscopy, the protein was readily visualized on the displaced single strand portions of D-loops and expanding D-loops. The nucleoprotein fibers were ~12 nm in diameter without correcting for the thickness of tungsten coating and roughly ⅓ the length of the double strand segment of the corresponding D-loop structure. In addition, occasional molecules with the characteristics of gapped circles were seen exhibiting a nucleoprotein fibril, presumably containing the single strand gap segment, linking the ends of double strand DNA. P16 was not seen on the double strand portions in any of the complexes.

Evidence from a number of laboratories suggests that the region of the origin of replication of animal mtDNA is associated with a proteinaceous component. For example, an mtDNA-protein complex was isolated from Triton X-100-lysates of HeLa cell mitochondria (1). Electron microscopic examination of restriction endonuclease fragments containing this structure revealed the presence of a membrane-like patch in the vicinity of the displacement loop (D-loop)[1]. A fraction of these complexes retained a smaller proteinaceous structure (5–10 nm) after treatment with SDS. In *Drosophila melanogaster* (2) the mtDNA was shown to have several regions near the replication origin that were precluded from cross-linking with photoactivated trimethylpsoralen. The protection against cross-linking was interpreted to result from the association of proteins at the non–cross-linked sites. In studies with *Xenopus laevis* mitochondria (3), a polymeric protein with a subunit molecular weight of 12,500 was isolated by DNA-cellulose affinity chromatography. In reconstitution experiments this protein appeared to exhibit an affinity for the single strand of the D-loop structure as judged from electron microscopy of the reconstituted complex. In a subsequent report (4), a protein fraction from *Xenopus laevis* mitochondria appeared to have specific affinity for supercoiled molecules containing the D-loop region of mtDNA.

[1] *Abbreviations used in this paper:* D-loop, displacement loop; H- and L-strand, heavy and light strand of mtDNA, respectively; mtDNA, mitochondrial DNA; TE, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4.

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In our previous study (5), an mtDNA-protein complex was released from rat liver mitochondria by SDS lysis and purified by hydroxyapatite column chromatography. The only detectable polypeptide in the complex was a low molecular weight species P16 ($M_r \simeq 16{,}000$) having a pI of 7.6–7.8. It was shown that the nascent 7S D-loop fragment in the complex, in contrast to completely deproteinized mtDNA, was protected against branch migrational loss upon restriction endonuclease-cleavage of the covalently closed parental strands. These results were interpreted as evidence that the presence of P16, presumably in the region of the D-loop, played a critical role in maintaining the integrity of these triplex replication loops.

The work presented here further characterizes the rat liver mtDNA-protein complex. P16 was found associated with the population of mtDNA molecules enriched in replicative intermediates. Evidence is provided to suggest that the specific binding site of P16 is the displaced single strand of the replicative intermediates. The relationship of P16-binding to the asymmetrical mode of mtDNA replication is discussed.

MATERIALS AND METHODS

Materials: Male Sprague-Dawley rats weighing 150–250 g were purchased from Flow Laboratories, Inc. (Dublin, VA). [Methyl-^{3}H]dThd was obtained from ICN Chemical and Radioisotope Division (Irvine, CA). The restriction endonucleases *HindIII*, *EcoRI*, *HhaI*, and *BamHI*, and the protein molecular weight standards were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, MD). Phenyl-Sepharose CL-4B was obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). Ultrapure optical grade CsCl was a product of Harshaw Chemical Co. (Solon, OH). "Highly polymerized" calf thymus DNA and ultrapure *Micrococcus lysodeikticus* DNA were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrofluor was obtained from National Diagnostics (Somerville, NJ).

Purification of mtDNA-Protein Complexes: Unless otherwise noted, all procedures were carried out at 0–4°C. Mitochondria were generally isolated from 20 to 30 g of rat liver by differential centrifugation and labeled with [methyl-^{3}H]dThd in vitro as previously described (6). The labeled mitochondria were lysed with SDS, and the released [^{3}H]mtDNA-protein complexes were isolated by sucrose density gradient centrifugation (5). The fractions containing the [^{3}H]mtDNA-protein complex were pooled and pelleted by centrifugation through a 5-ml shelf of 50% (wt/vol) sucrose, 0.5 M NaCl, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0 in a Beckman 50.2 Ti rotor (Beckman Instruments, Palo Alto, CA) for 12–16 h at 40,000 rpm. The pellets were resuspended with gentle shaking in a total of 0.6 ml or less of 0.5 M NaCl, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0 (0.5 M NaCl; TE). The suspension was layered onto a 0.9 × 6 cm Phenyl-Sepharose CL-4B column previously equilibrated with 0.5 M NaCl, TE, and the [^{3}H]mtDNA-protein complex was eluted with the same buffer. The column was monitored by absorbance at 260 and 280 nm and by scintillation counting of 10-μl samples of each 0.6 ml fraction in Hydrofluor.

Preparation of Deproteinized mtDNA: Isolated mtDNA-protein complex was made 1% (wt/vol) with SDS and extracted twice with cold phenol as previously described (6). The deproteinized preparation was then passed through a Phenyl-Sepharose CL-4B column as described above to remove any remaining phenol and SDS.

SDS PAGE of Protein Contained in the mtDNA-Protein Complex: Samples to be analyzed by gel electrophoresis were concentrated either by centrifugation overnight at 45,000 rpm in a Beckman 65 rotor (Beckman Instruments) or by evaporation to dryness in a Savant Speed Vac Concentrator (Savant Instruments, Inc., Hicksville, NY). The pellets were dissolved in 50–100 μl of SDS-sample buffer described by O'Farrell (7) and electrophoresed on exponential gradient gels consisting of 5–20% polyacrylamide prepared as described previously (5). The gels were run under constant voltage at 200 V until the tracking dye Pyronin Y migrated to the bottom of the gel. The staining procedure was the same as that previously described for isoelectric focusing gels (5). The molecular weight standards used were ovalbumin, 43,000; α-chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; lysozyme, 14,300; cytochrome *c*, 12,300; and bovine trypsin inhibitor, 6,200. With these conditions, lysozyme and cytochrome *c* migrated as a single band.

Isopycnic CsCl Gradient Analysis of [^{3}H]mtDNA-Protein Complexes: Purified [^{3}H]mtDNA-protein complexes were dialyzed overnight against 0.1 M NaCl, 20 mM sodium phosphate, pH 7.7, and fixed with formaldehyde and glutaraldehyde as previously described (5). The fixed samples were diluted to 3 ml with 0.1 M NaCl, 20 mM sodium phosphate, pH 7.7 and solid CsCl was added until the solution gave a refractive index reading of 1.3960 at room temperature. The solutions were centrifuged for 60 h at 22°C at 33,000 rpm in a Beckman SW 50.1 rotor (Beckman Instruments). *M. lysodeikticus* DNA (1.731 g/cm^3) and calf thymus DNA (1.700 g/cm^3) were used as buoyant density markers in identical, parallel gradients. Under these conditions, completely deproteinized [^{3}H]mtDNA bands at a position corresponding to the peak of marker calf thymus DNA with a density of 1.700 g/cm^3. Gradients were fractionated dropwise and monitored by absorbance at 260 nm and scintillation counting of small aliquots in Hydrofluor. The density of the experimental gradient fractions was determined by correlation of refractive indices of these fractions with those of the gradients containing the buoyant density markers.

Glass Fiber Filter Assays: Purified [^{3}H]mtDNA-protein complexes and SDS/phenol-deproteinized [^{3}H]mtDNA prepared as described previously were applied to Whatman GF/C glass fiber filters (Whatman Chemical Separation, Inc., Clifton, NJ) and serially washed essentially as described by Thomas et al. (8). Serial washing was carried out at room temperature as follows: a double layer of 6-mm Whatman GF/C glass fiber filter disks was centered onto a 2.4-cm Whatman GF/A glass fiber filter and washed by slowly pipetting 100 μl of 0.5 M NaCl, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4 onto its surface. The GF/C disks were then transferred to a second underlying GF/A filter and a 100-μl sample in 0.5 M NaCl, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4 was applied in the same manner. Subsequent transfer and wash cycles served to wash unbound mtDNA through the GF/C disks onto a series of underlying GF/A filters. To release filter-bound mtDNA, the washing cycle was interrupted to incubate the GF/C disks in 25 μl of 1% (wt/vol) SDS for 30 min at 37°C before continuing the washing series. The mtDNA present on upper or underlying filters was determined by scintillation counting of the dried filters in a toluene-based cocktail. The amount of [^{3}H]mtDNA retained by the GF/C disks following each wash was calculated by subtracting the amount of radioactivity released by each wash from the total amount of radioactivity recovered on all filters in the series. To recover filter-bound restriction fragments for electrophoretic analysis, mtDNA-protein complexes were cleaved with various restriction endonucleases for 1 h at 37°C in the buffer system recommended by the supplier. After adjusting to 0.5 M NaCl, the restriction endonuclease-digested mtDNA-protein complexes were applied to prewashed GF/C disks and serially washed as described above. The GF/C disks were then placed in the recessed cap of a 1.5-ml Eppendorf tube and subjected to the 30-min incubation with 1% (wt/vol) SDS at 37°C. The restriction fragments released by the SDS treatment and several successive washes with distilled water were collected through pinholes in the cap as described by Thomas et al. (8). Filtrates were concentrated in a Savant Speed Vac Concentrator and electrophoresed in 0.8% (wt/vol) agarose as previously described (5).

Electron Microscopy: MtDNA-protein complexes were prepared for direct visualization using techniques described elsewhere without change (9, 10). This included fixation with formaldehyde and glutaraldehyde, mounting onto glow-charged carbon films, washing, drying, and shadowcasting with tungsten. DNA lengths were measured using a computer-coupled tracing system.

RESULTS

Purification of the [^{3}H]mtDNA-P16 Complex

The radioactively labeled complex obtained from preparative sucrose density gradients was contaminated with loosely bound proteins and large proteinaceous aggregates. The bulk of this contaminating material was removed by pelleting the pooled complex through a 50% sucrose cushion in a fixed-angle rotor. The pelleted complex was then subjected to Phenyl-Sepharose CL-4B column chromatography to remove the last traces of loosely bound material. The mtDNA-protein complex eluted in the void volume and only the tightly bound protein, P16, remained associated with the mtDNA under these conditions (Fig. 1, lane *A*). The high salt included in the elution buffer is apparently sufficient to effect the retention of all other proteinaceous components by the column. The yields of labeled mtDNA from the column often exceeded 90%.

It should be noted also that comparable amounts of P16 were found associated with mtDNA isolated from mitochon-

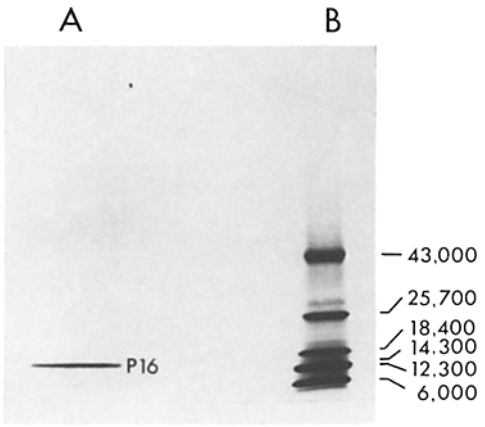


FIGURE 1 SDS polyacrylamide gel showing that P16 is the only detectable polypeptide present in a typical preparation of mtDNA-protein complex. The methods for preparation of complexes through the Phenyl-Sepharose CL-4B chromatography step are given under Materials and Methods. The complex eluting in the void volume was pelleted by centrifugation overnight at 45,000 rpm in a Beckman 65 rotor at 4°C. The pellet was resuspended in SDS-sample buffer, heated in a boiling water bath for 5 min, and electrophoresed on a 5–20% polyacrylamide gradient gel as described under Materials and Methods. (*A*) Protein from the mtDNA-protein complex; (*B*) molecular weight standards.

dria that were first subjected to a digitonin washing procedure as described previously (6). This procedure removes the outer mitochondrial membrane and any spuriously bound extramitochondrial components that may be associated with the outer membrane (data not shown).

Determination of the DNA-to-Protein Mass Ratio of the Complex

Isolated [^{3}H]mtDNA-protein complexes were fixed by formaldehyde and glutaraldehyde treatment and analyzed in isopycnic CsCl gradients as shown in Fig. 2. Two major peaks were observed. A portion of the mtDNA (A_{260} profile) banded at a density of 1.700 g/cm^3, the buoyant density of "naked" rat liver mtDNA (11). In the experiment shown, the major portion of the total sample, as judged from A_{260} measurement, banded at a lower density of 1.677 g/cm^3 reflecting the presence of the bound protein. This large subpopulation also contained the bulk of the radioactively labeled species. Because it is known from previous studies (5) that our in vitro labeling conditions result in the incorporation of label primarily into 7S D-loop fragments, it follows that the protein-bound molecules banding at the lower density are highly enriched in D-loop-containing species. Using the formula of Brutlag et al. (12), the DNA-to-protein mass ratio of the fixed complex was calculated from the measured effective buoyant density of the more buoyant subpopulation of molecules. For this calculation, we have chosen a value of 0.73 cm^3/g (13) for the partial specific volume of the DNA-bound protein. The DNA-to-protein mass ratio for the lower density peak obtained from four different preparations was 14.4 ± 0.36. Using values of 10.8×10^6 and 15,200 (14) for the M_r of rat liver mtDNA and P16, respectively, we have calculated that a mean of 49 P16 molecules are bound per mtDNA in the lower density population of molecules.[2]

[2] This is an approximate value in that this formula neglects the variation in hydration of protein and DNA at different CsCl concentrations and ignores the difference between partial specific volume and the reciprocal of effective buoyant density (15, 16). The molecular weight assigned for rat mtDNA is consistent with other mammalian mtDNA species for which the complete sequences have been published (17–19).

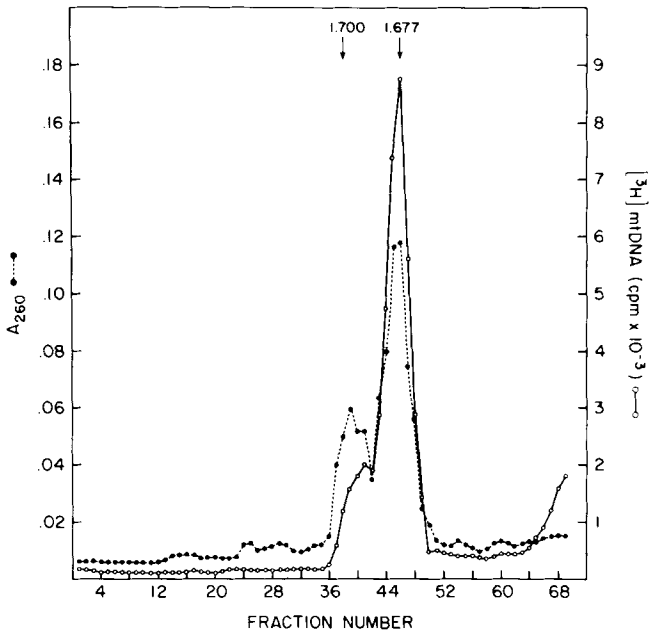


FIGURE 2 Isopycnic CsCl gradient analysis of fixed ^{3}H-mtDNA-protein complexes. The methods used are described under Materials and Methods. The densities shown with arrows were determined from buoyant density markers run in a parallel gradient and using refractive indices to correlate densities of gradient fractions.

Localization of the P16-Binding Site to the Vicinity of the D-Loop

To examine whether the bound P16 molecules were distributed throughout the length of the circular mtDNA or localized to a selected region of the genome, complexes were digested with a series of restriction endonucleases for which the rat mtDNA restriction maps were known (20–22). For these experiments, litter mate rats having only A type mtDNA were used to avoid the confusion of having both A and B types in the preparation (22). The P16-bound restriction fragments were retrieved on glass fiber filters previously shown to retain only protein-associated DNA (23). The filter-bound fragments were eluted with SDS and analyzed on agarose gels alongside the corresponding, unfiltered digests. As shown in Fig. 3, lanes labeled *B*, only a single fragment was retained by the filters regardless of the enzyme, or enzymes, used. Furthermore, the retained fragments in all cases were those known to encompass the D-loop structure (20, 21; personal communication, Dr. G. Brown, Department of Biology, McGill University). These results suggest that the bound P16 is localized in the vicinity of the D-loop structure.

There was the possibility, however, that filter retention of the observed fragments was not due solely to the presence of bound P16, but that the filter-binding may be a function of the presence of the D-loop structure itself on these fragments. To provide evidence against such a possibility, the filter-binding efficiency of mtDNA-P16 complexes was compared with that of completely deproteinized mtDNA labeled in the nascent D-loop fragment in vitro (5). As shown in Fig. 4, the

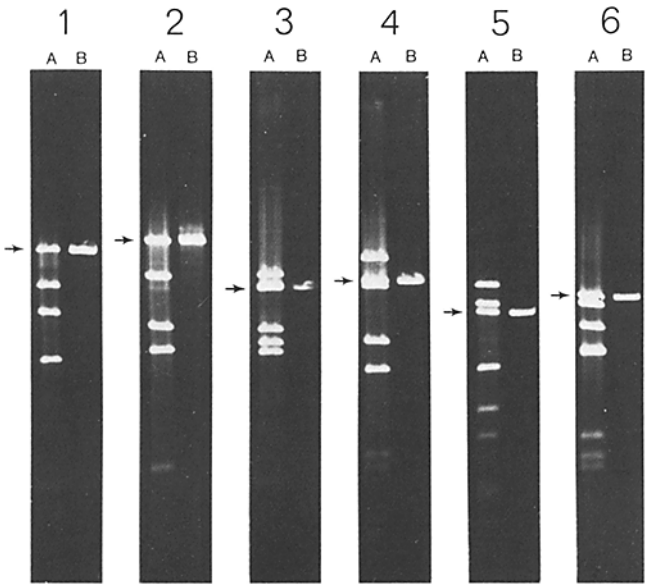


FIGURE 3 Agarose gel electrophoresis of restriction fragments retained by GF/C filter disks. mtDNA-protein complexes were digested with various restriction endonucleases and two thirds of each sample was applied to GF/C filter disks and serially washed to remove unbound fragments. Restriction fragments retained by the filter were released by treatment with SDS, recovered by filtrate collection, and electrophoresed in 0.8% (wt/vol) agarose. The remaining one third of each sample was incubated in the presence of 1% (wt/vol) SDS for 30 min at 37°C and served as a control restriction digest of the mtDNA-protein complex. The restriction digests shown are (*1*) *EcoR*I; (*2*) *Hind*III; (*3*) *EcoR*I/*BamH*I; (*4*) *Hind*III/*BamH*I; (*5*) *EcoR*I/*Hha*I; (*6*) *Hind*III/*Hha*I. For each set of digests: lane *A*, control, unfiltered restriction digest of the mtDNA-protein complex; Lane *B*, restriction fragment retained by the GF/C disk. Arrows indicate restriction fragments known to encompass the D-loop structure. This figure is a composite of two agarose gels: restriction digests shown in *1*, *2*, *5*, and *6* are from a different gel than digests shown in *3* and *4*.

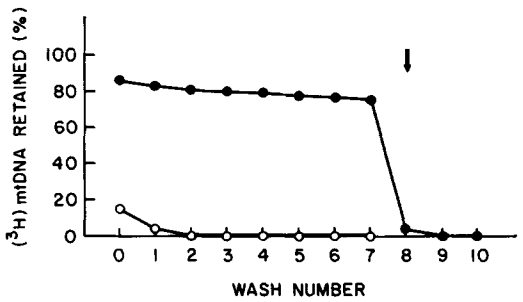


FIGURE 4 The retention of intact ^{3}H-mtDNA-protein complexes and of SDS/phenol-deproteinized ^{3}H-mtDNA by Whatman GF/C glass fiber filters. Samples were loaded onto GF/C filter disks (designated wash number zero) and serially washed. At the wash indicated by the arrow, the GF/C filter disks containing intact mtDNA-protein complexes were treated with SDS prior to continuation of the serial washing. The methods used are described under Materials and Methods. ●; intact mtDNA-protein complex; O; SDS/phenol-deproteinized mtDNA.

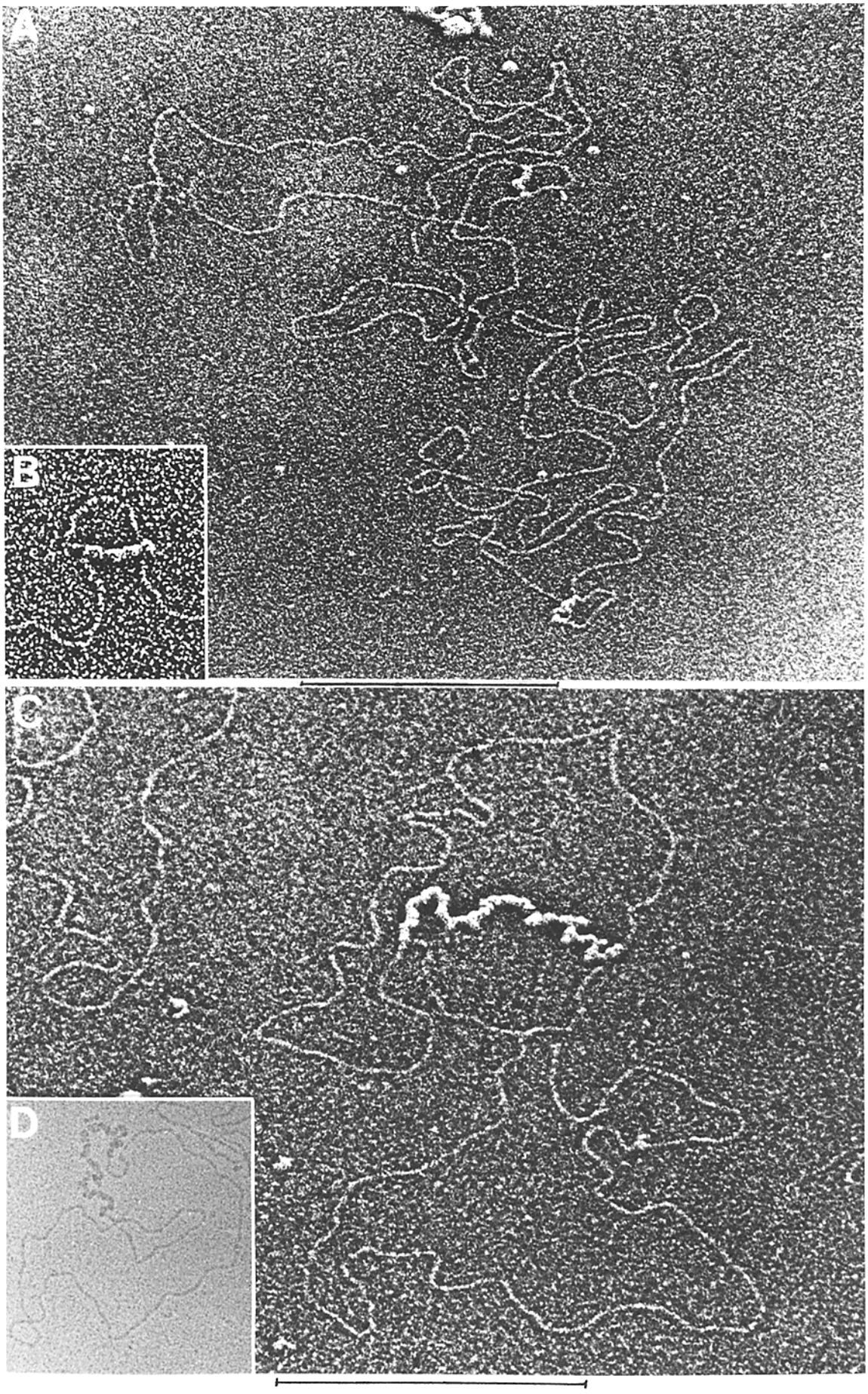
mtDNA-P16 complexes were retained with high efficiency and were able to be removed quantitatively with SDS. The deproteinized, D-loop mtDNA was not retained indicating that the triplex D-loop structure per se does not cause filter retention. It is, therefore, reasonable to conclude from the restriction endonuclease analyses above that in the bulk of the mtDNA-P16 Complexes, the P16 molecules are bound near, or on, the D-loop.

Visualization of mtDNA-Protein Complexes by Direct Electron Microscopy

Purified complexes were fixed by treatment with formaldehyde and glutaraldehyde, mounted directly onto thin carbon films, washed, dried, and shadow-cast with tungsten. Samples prepared in this manner revealed circular mtDNA molecules, many of which contained distinct D-loop structures. The DNA appeared to be free of protein along its length except where a short, thick nucleoprotein fiber bridged the two ends of the duplex part of the D-loop (Fig. 5, *A* and *B*). This fiber appeared typical of complexes formed between single stranded DNA and known single strand DNA-binding proteins such as *Escherichia coli* single strand binding protein (24), or recA (10). When 65 molecules from a single sample were counted sequentially, 20% showed no D-loops or bound protein, 70% had a single D-loop-bridge structure as described, and 10% appeared to be expanded D-loop molecules. Without correcting for the thickness of tungsten coating, the nucleoprotein bridge segment of nonexpanded D-loops was measured to be roughly 50 nm long and 12 nm wide, whereas the length of the corresponding duplex portion was 150 ± 20 nm (28 molecules measured). Thus, if the bridge does indeed consist of the single stranded portion of the D-loop bound by protein, its length is compacted threefold relative to the length of the duplex DNA. The extended nucleoprotein segment of expanded D-loops (Fig. 5 *C*) had the same general appearance and diameter characteristic of the normal size D-loops. This nucleoprotein segment also exhibited the threefold reduction in length relative to the duplex portion of the loop. When single stranded DNA alone is prepared by these techniques, it appears as a highly collapsed bush of nucleic acid and could not provide the mass seen here. Efforts to visualize the D-loop complexes following deproteinization were not successful, very possibly because the single strand portion was either broken or extended indiscernibly along the duplex segment.

Partial digestion of the complexes with the restriction en-

FIGURE 5 Visualization of mtDNA-protein complexes by direct electron microscopy. The mtDNA-protein complexes were eluted from Phenyl-Sepharose CL-4B and fixed for electron microscopy as described under Materials and Methods. Following fixation the samples were mounted onto thin carbon supports, washed, air dried, and tungsten shadow-cast with rotation. *A* shows two examples. The single stranded portion of the D-loop structure is shortened and thickened by the bound protein. A higher magnification from another such molecule is shown in *B*. *C* is an example of an expanded D-loop molecule in which the single stranded segment has the same shortened, thickened appearance but is much longer. The DNA strands appear to cross over each other close to the middle of the molecule. An example of a gapped circle is shown in *D*. Bar, 0.5 μm (*A*); 0.25 μm (*B*); 0.5 μm (C); and 0.89 μm (*D*). The electron microscopy of *A*–*C* were done by Dr. Jack Griffith at the University of North Carolina using a Philips EM 400. The gapped circle visualized in *D* was obtained from a separate preparation of mtDNA-P16 complex and photographed using a Hitachi HU-12 electron microscope.


A
B
C
D

donuclease *EcoRI* for 10 min at 37°C produced many full length linear molecules containing the nucleoprotein-bridged loop. The loop was most often located 0.43 ± 0.05 μm from one end of the 4.9 μm DNA (25 molecules measured). This distance corresponds very closely to the known distance between the well-characterized D-loop and the nearest *EcoRI* restriction site (20) indicating that the nucleoprotein-bridged loops observed in our micrographs were in fact D-loops. In some preparations, molecules exhibiting characteristics of gapped circles were occasionally observed. One of these is shown in Fig. 5*D*. The double stranded portion of this molecule is about ⅔ the length of an intact mtDNA suggesting that the remaining ⅓ is present within the nucleoprotein segment as compacted single strand DNA. These characteristics are consistent with previous descriptions of gapped, β-daughter molecules prior to completion of nascent heavy strand (H-strand) synthesis (25, 26).

Thus, based upon the electron photomicrographs and the physicochemical data presented above, it appears that P16 is selectively bound to the displaced single strand of D-loops and to the extended single strand portions of advanced replicative intermediates that arise as a result of the asymmetrical mode of mtDNA replication.

DNA-bound P16 Contains a Protease-insensitive Domain That Retains the DNA-binding Capacity

Treatment of isolated mtDNA-protein complexes with nonspecific proteases such as Proteinase K or subtilisin resulted in the digestion of the bound P16 to a protease-insensitive remnant ($M_r \simeq 6{,}000$) that remained associated with the mtDNA through subsequent Phenyl-Sepharose CL-4B chromatography (Fig. 6, lanes *C* and *D*). It is concluded from this observation that the peptide bonds in at least one region of the DNA-bound P16 are accessible to these proteases thus releasing a large portion of each protein from the complex. In addition, there is also an inaccessible segment of P16 that resists proteolytic digestion and retains that portion of the protein crucial to the DNA-binding function.

Treatment of the intact complex with trypsin yielded apparently intact P16 as judged from migration rate in the SDS polyacrylamide gel (Fig. 6, lane *E*). This result suggests that the region of P16 that is accessible to the nonspecific proteases may contain few, or no, basic amino acid residues, and that arginines or lysines present elsewhere in the protein are protected either by the tight conformation of the polypeptide in these regions or as a result of the interaction of the protein with the DNA molecule.

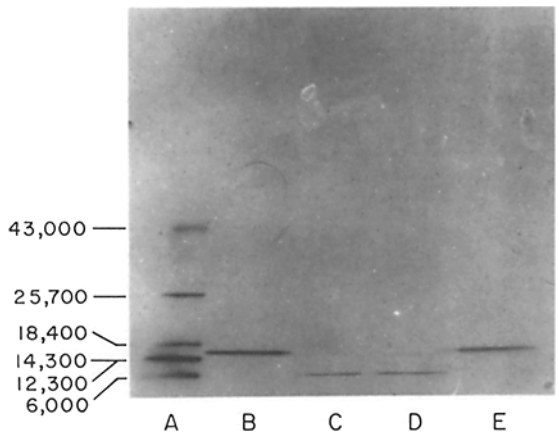


FIGURE 6 SDS PAGE of polypeptides remaining bound to mtDNA following protease-digestion of complexes. Purified ^{3}H-mtDNA-protein complexes were incubated for 1 h at 37°C in 0.5 M NaCl, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0 with 20 μg/ml of either proteinase K, subtilisin BPN', or trypsin. The control sample was incubated similarly except that no protease was added. Each sample was then passed through a 0.7 × 7-cm column of Phenyl-Sepharose CL-4B in 0.3 M NH_4HCO_3, 1 mM disodium EDTA, pH 7.8. The mtDNA and any polypeptides remaining bound to the DNA eluted in the void volume. The peak fractions were concentrated to dryness in a Savant Speed Vac Concentrator. The residue was dissolved in SDS sample buffer and electrophoresed in 5–20% polyacrylamide gradient gels as described under Materials and Methods. (*A*) Molecular weight standards; (*B*) undigested control; (*C*) proteinase K-digested complex; (*D*) subtilisin-digested complex; (*E*) trypsin-digested complex.

DISCUSSION

Rat liver mtDNA was released from the organelles by treatment with SDS and purified by Phenyl-Sepharose CL-4B chromatography. Under these conditions, a single protein species designated as "P16" was found associated with the D-loop-enriched population of the mtDNA. As judged from the buoyant density of the fixed complex, P16 was present in multiple copies with a mean of 49 P16 molecules per mtDNA in the protein-bound population. Using a filter-binding analysis of restriction endonuclease-digested complexes, the predominant binding site for P16 was localized to fragments known to contain the site of the D-loop structure. By direct electron microscopy, the specific binding site of P16 was shown to be the displaced single strands of D-loops and expanded D-loops and the single strand portion of gapped circular molecules. The nucleoprotein fibrils appeared more densely compacted than those observed on a similar complex from *Xenopus laevis* mitochondria (3). In our case, the nucleoprotein segments were more similar in appearance to nucleoprotein fibers formed by *E. coli* single strand-binding protein (24) and recA protein (9). These bacterial proteins form a fiber of roughly 12 nm diameter and condense single strand DNA to about ⅓ the equivalent duplex length. Both of these features were characteristic of the mitochondrial P16 as well. Furthermore, in some of the electron micrographs there was a discernible repeating beaded superstructure similar in appearance to that reported for complexes of *E. coli* single strand-binding protein with single strand DNA (24).

Our electron microscopy analysis also revealed that 70% of the molecules from a typical preparation contained D-loops and 10% expanded D-loops. These values are probably representative of the in vivo state of rat mtDNA because branch migrational loss of nascent strands during isolation is unlikely to occur. We previously demonstrated that the presence of P16 in the complex virtually completely inhibits branch migration during parental strand scission in vitro (5). The high percent of D-loop DNA we observed is consistent with values reported for mouse L cells (27) and *Xenopus laevis* oocytes (28).

The novel, asymmetrical mode of replication of animal mtDNA (29) has been shown to occur by displacement expansion of 7S nascent H-strands until the origin of light strand (L-strand) replication is exposed at a distance of about ⅔ genome length from the H-strand origin. As L-strand synthesis in the opposite direction approaches the H-strand origin, synthesis is arrested until the daughter molecules are segregated releasing a nearly completed α-daughter and the β-

gapped circle (25, 26). Thus, at some time during a single round of replication, virtually every segment of parental H-strand exists as displaced single strand DNA. It also follows that most, and perhaps all, L-strand synthesis occurs on the displaced single strand template. In this report, we provide evidence that the displaced single strands of these replicative intermediates do not exist as "naked" DNA, but that they appear to be coated by tightly bound P16 molecules. It is conceivable that the unique mode of mtDNA replication, exhibiting relatively long lived displaced single strand segments has necessitated the existence of a protein capable of forming a protective sheath on exposed single strand DNA. It is likely that its functions are manifold and may include (*a*) protection against nuclease digestion, (*b*) protection against branch migrational loss of nascent strands during nicking-closing cycles, and (*c*) removal of inhibitory secondary structures in the displaced template strand during L-strand synthesis.

The existence of DNA-binding proteins with domains distinguished by limited proteolysis has been widely demonstrated (30). In the mitochondrial system described here, the bound P16 contains an $M_r \simeq 6{,}000$ segment that is resistant to proteolysis by even broad-spectrum proteases. This domain retains the capacity to bind tenaciously to the mtDNA and thus represents the smallest detectable portion of P16 that is involved in the DNA-binding function. The fate of the remainder of the molecule upon proteolysis is not yet known. In isoelectric focusing gels the proteolytic remnant migrated as a single ionic species with a pI of ~8.0 (Manam, S., and G. C. Van Tuyle, unpublished results), i.e., ~0.3–0.5 pH units higher than the intact P16 (4). Thus although the $M_r \simeq 6{,}000$ remnant represents at least a portion of the DNA-binding domain, the lack of extensive clustering of basic amino acid residues in this fragment supports our previous contention that electrostatic interactions may represent only a small contribution to the DNA-binding function, and that other interactions of a stronger nature must be involved to maintain the extraordinary stability of the complex (5).

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