Human Mitochondrial DNA Nucleoids Are Linked to Protein Folding Machinery and Metabolic Enzymes at the Mitochondrial Inner Membrane

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Mitochondrial DNA (mtDNA) is packaged into bacterial nucleoid-like structures, each containing several mtDNA molecules. The distribution of nucleoids during mitochondrial fission and fusion events and during cytokinesis is important to the segregation of mitochondrial genomes in heteroplasmic cells bearing a mixture of wild-type and mutant mtDNA molecules. We report fractionation of HeLa cell mtDNA nucleoids into two subsets of complexes that differ in their sedimentation velocity and their association with cytoskeletal proteins. Pulse labeling studies indicated that newly replicated mtDNA molecules are evenly represented in the rapidly and slowly sedimenting fractions. Slowly sedimenting nucleoids were immunoaffinity purified using antibodies to either of two abundant mtDNA-binding proteins, TFAM or mtSSB. These two different immunoaffinity procedures yielded very similar sets of proteins, with 21 proteins in common, including most of the proteins previously shown to play roles in mtDNA replication and transcription. In addition to previously identified mitochondrial proteins, multiple peptides were observed for one novel DNA metabolic protein, the DEAH-box helicase DHX30. Antibodies raised against a recombinant fragment of this protein confirmed the mitochondrial localization of a specific isofrom of DHX30.

Mammalian cells contain thousands of copies of mitochondrial DNA (mtDNA) organized in several hundred nucleoids (1, 2). These nucleoid structures are dynamic and redistribute actively within mitochondria undergoing fission and fusion (3). It is widely accepted that the segregation of nucleoids may control the inheritance of mutant mtDNAs, thus influencing the development of mitochondrial disorders. The packaging of multiple mtDNA molecules into a single nucleoid helps to explain the classical observation that the segregation of mtDNA mutants is faster than would be expected given the large number of mtDNA genomes in a cell. Although mtDNA-binding proteins are thought to play critical roles in mtDNA maintenance and segregation (4), the composition of mtDNA nucleoids is poorly understood, especially in higher organisms. The organization and inheritance of mtDNA nucleoids has very recently been reviewed (5, 6).

Studies of mtDNA nucleoids in the yeast, Saccharomyces cerevisiae, have progressed more rapidly than experiments in higher eukaryotes due to the simplicity of the yeast nuclear genome and the fact that bakers' yeast is a facultative anaerobe that can survive without mtDNA. Formaldehyde cross-linking studies have revealed several classes of proteins in association with S. cerevisiae mtDNA, including metabolic proteins such as aconitase and Ilv5 (7). Other yeast proteins such as Mmm1p (8), Mdm10p, and Mdm12p (9) may mediate contacts between nucleoids and cytoskeletal components important for mtDNA inheritance. Mmm1p is localized in close proximity to Mgm101p, which has also been characterized as a nucleoid protein (10, 11). Other proteins such as Mmm2p (12), Mdm31, and Mdm32 (13) genetically influence mtDNA nucleoid maintenance but are not as closely associated with mtDNA.

There are two major reasons why yeast mtDNA nucleoids appear to differ from those in higher eukaryotes. First, individual yeast nucleoids contain only one to two mtDNA genomes, perhaps equivalent to one genome that may or may not be replicating, whereas vertebrate mtDNA nucleoids typically contain 5–7 entire genomes (1, 2). Second, many of the yeast nucleoid proteins noted above lack homologs in higher eukaryotes. Biochemical studies of mtDNA nucleoids in higher eukaryotes have reported their association with the inner membrane (3, 14, 15) and cytoskeletal structures (16). However, nucleoid proteins identified to date in mammals are largely confined to a few well characterized DNA-binding proteins. The high mobility group family (HMG) family protein TFAM, related to bacterial HU protein, is a major mtDNA packaging protein conserved in yeast, frogs, and mammals (17–21). MtSSB, a functional and structural relative of bacterial SSB, is a single-stranded DNA-binding protein associated with mtDNA in all eukaryotes (22–24). Twinkle, a mitochondrial DNA helicase, has been shown to co-localize with TFAM and mtSSB (3). MtDNA molecules packaged in nucleoids are engaged in a variety of dynamic processes, including replication and transcription. Thus, the pro-
tein composition of nucleoids may be expected to vary dynamically as well.

Our study of *Xenopus* oocyte mtDNA nucleoids (25) revealed several novel mitochondrial nucleoid proteins, including adenine nucleotide translocator (ANT), prohibitin, and the E2 subunits of two large dehydrogenase complexes, pyruvate dehydrogenase and branched chain ketoacid dehydrogenase. The association of mtDNA nucleoids with proteins known to reside in the mitochondrial inner membrane, such as ANT and prohibitin, is a first step in understanding the classical observation that mtDNA is membrane-associated.

In this study, we report purification of mtDNA nucleoids from cultured human HeLa cells and identification of associated proteins. We found that human mitochondrial nucleoids are quite heterogeneous in nature, comprising two major subsets, both associated with TFAM. 3H]Thymidine pulse labeling indicates that newly replicated DNA is distributed in both nucleoid subsets. The more slowly sedimenting form is closely associated with cytoskeletal proteins, reminiscent of the extensive literature on yeast nucleoids summarized above. The more slowly sedimenting form lacks extensive interactions with cytoskeletal elements. We used this fraction as the starting material for immunoaffinity purification of nucleoids using antibodies directed against either of two abundant mtDNA-binding proteins, TFAM or mtSSB. A set of ~20 proteins was detected with both of these immunoaffinity approaches, including several proteins known to be involved in mtDNA maintenance. A novel member of this set is the DEAH helicase, DHX30, which we characterize for the first time as a mitochondrial mtDNA maintenance. In addition to these DNA metabolic proteins, DHX30, which we characterize for the first time as a mitochondrial protein. We found that human mitochondrial nucleoids are associated with cytoskeletal proteins, reminiscent of the extensive literature on yeast nucleoids summarized above. The more slowly sedimenting form lacks extensive interactions with cytoskeletal elements.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Suspension HeLa cells were grown at 37 °C in minimum essential medium with Earle’s salts, with 1% l-glutamine, 5% bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen).

**Mitochondrial Preparation**—All steps in purification of mitochondria and mtDNA-protein complexes were conducted at 4 °C without freezing the sample at intermediate steps. A typical preparation employed 3–3.5 × 10⁶ cells. All buffers included a protease inhibitor mixture containing 0.2 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 2 μg/ml leupeptin, and 5 μg/ml leupeptin. Cells were harvested by centrifugation at 1,000 × g for 5 min and then washed in isonicotic buffer (0.02 M HEPES, pH 8, 5 mM KCl, 1.5 mM MgCl₂, 0.2 mM sucrose, 2 mM DTT). Cells were centrifuged at 900 × g for 5 min and then resuspended in hypotonic buffer (0.02 M HEPES, pH 8, 5 mM KCl, 1.5 mM MgCl₂, 2 mM DTT) and recentrifuged. In some experiments these buffers were supplemented with 100 μg/ml colchicine and 10 μg/ml cytochalasin B in an effort to reduce contamination of nucleoids with cytoskeletal proteins. Pellets were then resuspended in hypotonic buffer and homogenized using a tight-fitting Dounce homogenizer. Two ml of 2.5× MSH was added for every 3 ml of the homogenate to adjust the solution to 1× MSH (210 mM mannitol, 70 mM sucrose, 20 mM HEPES, pH 8.0, 2 mM EDTA). Nuclei were removed by three successive centrifugations for 5 min each at 1,600 × g to generate the postnuclear supernatant, which was layered onto preformed step gradients consisting of three layers with 1× MSH buffer containing different density media. Gradients were formed by layering 8 ml of 14.5% Nycodenz (Histodenz; Sigma) over 5 ml of 29% Nycodenz and then adding a top layer of 10 ml of 10% Percoll (Amersham Biosciences). Gradients were centrifuged for 25 min at 25,000 rpm (92,600 × g) in a Beckman SW32 rotor. The crude mitochondria were collected from the 14.5/ 29% Nycodenz interface, diluted with 1× MSH, and pelleted at 20,000 × g for 15 min.

**Nuclease Treatment of Crude Mitochondria and Purification of Mitoplasts**—Crude mitochondria prepared from 4 liters of suspension culture were resuspended in 5 ml of buffer containing 1 mM ADP, 5 mM sodium pyruvate, 1 mM Na₂-malate, 1 mg/ml bovine serum albumin, 1× MSH, 60 mM KCl, 10 mM MgCl₂, 1 mM K₂HPO₄), 100 units/ml RNase-free DNase I (Sigma Type II) and 50 units/ml Benzonase nuclease HC (Novagen) were incubated with mitochondria at 37 °C for 15 min. 0.5 ml of 250 mM EDTA was added to chelate Mg²⁺ to stop nuclease digestion. The mitochondria were layered over 0.8 M sucrose, 20 mM HEPES, 2 mM EDTA, 2 mM DTT and spun at 12,000 rpm (20,000 × g) in a Sorvall HB-8 rotor for 15 min to remove the digested nuclear DNA fragments and to sediment the pure mitochondria. Mitochondrial pellets were resuspended in MSH buffer and recentrifuged through the 0.8 M sucrose buffer to yield highly purified mitochondria. The purified mitochondria were resuspended in MSH buffer, mixed with 0.12 mg of digitonin/mg of mitochondrial protein, and incubated at 4 °C for 15 min with periodic mixing. The mitochondria were washed in MSH buffer twice by centrifugation at 13,000 × g for 15 min to generate the mitoplast fraction.

**Velocity Sedimentation of Mitochondrial Nucleoids**—Mitoplasts were resuspended at ~7 mg/ml protein in 1.25× lysis buffer (30 mM HEPES, pH 8, 5% glycerol, 2 mM DTT, 1 mM EDTA). One-fourth volume of 6% Triton X-100 was added for a final concentration of 1.2% detergent. The lysate was mixed on ice for 5 min and centrifuged at 3,000 × g for 5 min. The supernatant was loaded on top of a step gradient prepared by layering 4.5 ml of 17% glycerol over 5 ml of 45% glycerol above a pad of 0.5 ml of 30% Nycodenz and 30% glycerol. All gradient layers contained 0.5% Triton X-100, 30 mM HEPES, pH 8, 2 mM EDTA, 2 mM DTT, 0.5% phenylmethylsulfonyl fluoride, and 70 mM NaCl. Fractions were collected following centrifugation at 186,000 × g in an SW41 rotor for 1.5 h.

**[3H]Thymidine Labeling of Cultured Cells to Detect mtDNA Replication**—HeLa cells grown in suspension were incubated with 1.2 μCi/ml of [3H]thymidine (MP Biomedical) for 45 or 90 min before harvesting. Nucleoids from the labeled cells were prepared as described above. MtDNAs were then deproteinized by phenol–CHCl₃ extraction, ethanol precipitated, and digested with HindIII. Restriction fragments were separated by electrophoresis on a 0.7% agarose gel. The gel was soaked first in three changes of 100% methanol for 30 min each, then 3% diphenoxy-
Human Mitochondrial DNA Nucleoid Proteins

Mitochondrial Nucleoids Can Be Separated into Rapidly and Slowly Sedimenting Fractions—The mtDNA nucleoid purification conditions reported here reflect extensive experimentation to optimize this preparation. In early experiments, we found that mitochondria prepared by standard gradient purification procedures were contaminated significantly with fragments of nuclear DNA. This was not observed in our previous work with *Xenopus* oocyte mtDNA nucleoids because these cells contain considerably more mtDNA than nuclear DNA. We found that this contamination could be minimized by treating mitochondria purified through Percoll/Nycodenz gradients with DNase I and Benzonase using a procedure modified from that of Higuchi and Linn (29). This nuclease treatment was conducted in a buffer supplemented with metabolic substrates and Benzonase to retain activity of the isolated mitochondria. Whereas nuclease treatment reduced contamination by histones to a level below the limit of detection of mass spectrometry, we routinely observed some contamination of the mtDNA with short DNA fragments presumably derived from nuclear DNA.

Further Purification of Nucleoids and Identification of Associated Proteins—Immunofractionation purification using antibodies directed against either TFAM or mtSSB was performed essentially as described (21, 25), along with control experiments using non-immune immunoglobulins. Immunoglobulins were prepared and antigen affinity purified from the sera of rabbits immunized with recombinant human TFAM or mtSSB purified in our laboratory. Immunoglobulins for the nonspecific control column were prepared by affinity chromatography on Protein A-agarose using a standard protocol. The antibodies were coupled to magnetic tosyl-activated M-280 Dynabeads (Dynal) in 0.1 M sodium phosphate, pH 7.5, under conditions recommended by the manufacturer. Approximately 5 × 10^8 antibody-coated beads were incubated with 0.6 ml of a glycerol gradient fraction enriched in mtDNA nucleoids in glycerol gradient buffer containing 70 mM NaCl and 0.5% Triton X-100. Following a 90-min incubation with mixing, the beads were collected on a magnet, and the unbound proteins were removed as a supernatant fraction. The column was then washed three times by resuspension of the beads in buffer containing 20 mM HEPES, pH 7.5, 70 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.5% Triton X-100. Bound proteins were eluted with 0.5% SDS. Proteins were separated by SDS-PAGE and fragmented by in-gel digestion with trypsin using standard methods (25). Peptides were identified by Nano LC-MS/MS on a QSTAR Pulsar i mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA) integrated with a Nano-LC system (LC Packings, San Francisco, CA). The mass spectral data were analyzed by Analyst QS with integrated PROID and Pro-Group software (Applied Biosystems) with reference to an interrogator data base prepared from the current GenBank non-redundant protein data base.

Immunoblotting—Samples of gradient fractions mixed with sample loading solution were subjected to electrophoresis on 12% acrylamide gels in Tris glycine buffer (26). Gels were either stained with silver (27), or electrophoretically transferred to polyvinylidifluoride membrane (Immobilon PVDF, Waters) to permit detection of proteins by immunoblotting with specific primary antiserum followed by the appropriate secondary antibody conjugated to alkaline phosphatase and colorimetric detection. The antibodies used included: homemade polyclonal rabbit sera directed against human TFAM, polymerase γ(A), polymerase γ(B), mtSSB, and DHX30 (see below); ANT, HSP60, kinectin 1, and Tom20 (Santa Cruz); voltage-dependent anion channel (Calbiochem); prohibitin (Neomarkers); vimentin and actin (Sigma). Antibodies directed against components of respiratory complex I (MS111), complex II (MS204), complex III (MS303), and complex IV (COX1) were obtained from MitoSciences. Polyclonal antibodies directed against aconitase were a gift from Dr. Luke Szweda; both polyclonal and monoclonal antibodies against mitofilin (IMMT (28)) were kindly provided by Drs. Paul Odgren and Reid Gilmore.

Antibodies to human DHX30 were raised against a 28-kDa domain of DHX30 expressed in bacteria. A cDNA clone containing isoform 2 of DHX30 (accession number NM_014966) obtained from Origene was used as template for PCR amplification using primers 5'-ACACATATGGGAAGAGCCCTCGGATC and 5'-CAATGCAGGCGCTTATTTTCT to amplify a 747-bp fragment with unique NdeI and NotI sites. These two enzymes were used to transfer the fragment to pET22b + to permit expression of a 28.3-kDa polypeptide in BL21 cells. This polypeptide was insoluble upon expression in *Escherichia coli*, but was solubilized in buffer containing 8 M urea and purified by His tag affinity chromatography followed by ion exchange chromatography in the presence of 8 M urea. A portion of this polypeptide was used to inoculate a rabbit (Cocalico Biologicals) to prepare a polyclonal antibody and a second portion was coupled to Affi-Gel resin (Bio-Rad) to permit affinity purification of antibodies. The affinity purified antibodies were used for Western blots as described above and for immunofluorescence.

RESULTS

Mitochondrial Nucleoids Can Be Separated into Rapidly and Slowly Sedimenting Fractions—The mtDNA nucleoid preparation was further purified by affinity chromatography using antibodies that had been affinity purified from rabbit sera directed against human TFAM, polymerase γ(A), polymerase γ(B), mtSSB, and DHX30 (see below); ANT, HSP60, kinectin 1, and Tom20 (Santa Cruz); voltage-dependent anion channel (Calbiochem); prohibitin (Neomarkers); vimentin and actin (Sigma). Antibodies directed against components of respiratory complex I (MS111), complex II (MS204), complex III (MS303), and complex IV (COX1) were obtained from MitoSciences. Polyclonal antibodies directed against aconitase were a gift from Dr. Luke Szweda; both polyclonal and monoclonal antibodies against mitofilin (IMMT (28)) were kindly provided by Drs. Paul Odgren and Reid Gilmore.

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Immunofluorescence—Monolayer HeLa cells were grown in glass slide tissue culture chambers in Dulbecco’s modified Eagle’s medium with 5% bovine serum, 5% fetal calf serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO2 in air. Cells were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature, and permeabilized in 0.25% Triton X-100 for 15 min. Cells were then rinsed 3 times in PBS for 5 min each, and blocked with either 0.5% milk or 5% normal goat serum in PBS at 37 °C for 1 h. The cells were incubated with primary antibodies in 0.25% Triton X-100/PBS at 4 °C for 24 h. In some experiments 3% normal goat serum was included in the antibody incubation. Cells were rinsed in PBS for 10 min and incubated in fluorescent secondary antibody in PBS for 45–60 min. After rinsing with PBS for 10 min, the cells were mounted with Vectashield (Vector Labs). Fluorescence was visualized using a Leica TCS SP2 or a Zeiss LSM 510 Meta confocal microscope. The primary anti-protein antibodies used for immunofluorescence were from the sources described above; the mouse monoclonal IgM anti-DNA antibody was AC-30-10 from Progen.
DNA. The overall scheme for mitochondrial nucleoid purification is shown in Fig. 1.

MtDNA nucleoids were prepared as described under “Experimental Procedures” beginning with velocity sedimentation of mitochondrial lysates in glycerol gradients. Nucleoids sediment very heterogeneously in these gradients and can be divided on the basis of a rapid fluorometric stain for DNA into rapidly and slowly sedimenting fractions as shown in Fig. 2A. A portion of each fraction was saved for later analysis that confirmed that the DNA has the correct restriction pattern for mtDNA (Fig. 2B). The remainder of peak fractions was immediately processed further as described below. Immunoblots show that TFAM and mtSSB, well characterized mtDNA-binding proteins, are associated with both slowly and rapidly sedimenting nucleoid fractions. On the other hand, the cytoskeletal proteins vimentin and actin are detected readily only in the rapidly sedimenting nucleoid fraction (Fig. 2C). In one attempt to sequence proteins in the rapidly sedimenting fraction we observed extensive contamination with cytoskeletal proteins including tubulin, spectrin, and other proteins. We tried to reduce the content of cytoskeletal proteins in nucleoid preparations by including cytochalasin B and colchicine in the culture medium just prior to cell harvesting or in the cell homogenization buffer, but found that these changes had little effect (data not shown). Therefore, the proteomic analyses reported below used only the slowly sedimenting nucleoid fraction.

Do the Two Forms of mtDNA Nucleoids Have Different Replication Status?—One hypothesis to account for the existence of two classes of mtDNA nucleoids is that the association of nucleoids with a structure attached to the cytoskeleton may be correlated either positively or negatively with mtDNA replication. In yeast, Meeusen and Nunnari (11) have shown that only a subset of nucleoids is engaged in replication at any instant and that replication occurs in association with a large two-mem-

FIGURE 1. Experimental scheme for mitochondrial nucleoid purification. Nucleoids purified through the glycerol gradient step are analyzed either by isopycnic centrifugation in Nycodenz gradients or by immunoaffinity purification with antibodies directed against either human TFAM or mtSSB. TX-100, Triton X-100.

FIGURE 2. Velocity sedimentation of HeLa mitochondrial nucleoids. Glycerol gradients of mitochondria lysed with Triton X-100 were performed as described under “Experimental Procedures,” with the direction of sedimentation from right to left in this figure. 1-ml fractions were collected and numbered from the tube bottom. Panel A, DNA analysis. 30 μl of each glycerol gradient fraction was mixed with 150 μl of TE containing a 1:300 dilution of Picogreen stain (Invitrogen) and 30 μg/ml RNase A in a 96-well plate. Samples were incubated at 37 °C for 30 min and DNA was detected using a Fluorimeter 595. DNA peaks representing rapidly and slowly sedimenting nucleoids are in fractions 3 and 9, respectively. Panel B, 150 μl of glycerol gradient fractions were deproteinized by extraction with phenol-CHCl₃, ethanol precipitated, and digested with HindIII. Restriction fragments of 10.2, 5.5, and 0.9 kb were separated by electrophoresis on a 0.7% agarose gel, stained with Vistra Green (Amersham Biosciences), and imaged using a Fluorimeter 595. Panel C, immunoblots of proteins in glycerol gradient fractions. Samples of glycerol gradient fractions were subjected to SDS-PAGE, blotted to polyvinylidene difluoride membranes, and detected with the indicated antisera and appropriate secondary antibodies.
brane spanning complex. In vertebrate cells in culture, Iborra et al. (2) concluded that the entire mtDNA nucleoids do not replicate as a unit, but that single mtDNA molecules within a larger nucleoid structure replicate individually. It is conceivable that mtDNA molecules may need to be released from larger nucleoid structures when the DNA is replicated. Thus, we sought to determine whether replicating and newly replicated mtDNA molecules might be selectively enriched in either the rapidly or slowly sedimenting fraction. We pulse-labeled HeLa cell cultures with [3H]thymidine for 45 or 90 min immediately prior to isolation of nucleoids. Because approximately 1 h is required for replication of a single mtDNA molecule (30), and because several minutes are required for equilibration of [3H]thymidine in the precursor pool, these pulse labeling intervals should provide selective incorporation of [3H] into mtDNA replication intermediates and newly replicated molecules. Mitochondrial lysates were fractionated by glycerol gradient sedimentation and mtDNA was recovered by phenol-CHCl₃ extraction and ethanol precipitation. MtDNA was digested with HindIII and analyzed by agarose gel electrophoresis and fluorography. A comparison of the distribution of total mtDNA and pulse-labeled mtDNA in gradient fractions (Fig. 3) showed no preferential labeling of either rapidly or slowly sedimenting nucleoids. Quantification of DNA and radioactivity revealed no significant difference in the specific activity of the two peaks (data not shown). We conclude that neither nucleoid fraction is enriched in replicating or newly replicated molecules.

**Immunopurification of Nucleoids Using TFAM- or MtSSB-specific Antibodies**—In early experiments, we used equilibrium centrifugation in isopycnic, non-ionic Nycodenz gradients for further purification of nucleoids. These experiments showed that the rapidly sedimenting fraction formed a very sharp band, characteristic of high molecular weight complexes, whereas the slowly sedimenting fraction had a less compact banding pattern (supplemental materials Fig. S1). Significantly, nuclease treatment of the rapidly sedimenting complex prior to the buoyant density gradient resulted in dispersal of the proteins to a broad region of the density gradient (supplemental materials Fig. S2), providing evidence that the proteins were initially associated with mtDNA, as previously shown for Xenopus mtDNA nucleoids (25). Because the density of nucleoids in Nycodenz gradients overlaps with that of some free proteins, we used affinity purification with antibodies directed against either TFAM or mtSSB to immunopurify nucleoids as described under “Experimental Procedures.” Fig. 4 shows that mtSSB was recovered in complexes immunopurified with anti-TFAM antibodies, and vice versa. Both reagents precipitated complexes containing Hsp60, prohibitin, and kinectin. A control column made with non-immune IgG failed to precipitate any of these proteins, indicating that proteins were not recovered based on nonspecific adsorption to antibodies coupled to the magnetic beads. We concluded that immunopurification with either anti-TFAM or anti-mtSSB antibodies is a highly selective purification step. Additional Western blotting experiments presented below support this conclusion. During the process of developing the two-step sedimentation and immunopurification procedure reported here, we experimented extensively with use of alternative detergents and variations in the ionic strength and gradient conditions. In general, variations in the protocol such as substitution of Triton X-100 with octyl glucoside and variation of the salt concentration resulted in little change in the recovery of nucleoid proteins, as shown in supplemental materials Fig. S3.

**Identification of Nucleoid Proteins**—We used mass spectrometry to identify the proteins in several independent preparations of slowly sedimenting nucleoids generated by
Human Mitochondrial DNA Nucleoid Proteins

Table 1: Proteins found in slowly sedimenting HeLa mtDNA nucleoids

| Protein          | Gene   | Accession No. | Peptide hits | Homolog observed |
|------------------|--------|---------------|--------------|------------------|
|                  |        |               | Anti-TFAM    | Anti-mtSSB       | Frog          | Yeast         |
| I. DNA binding and metabolism |        |               |              |                 |               |               |
| Tlam             | TFAM   | NP_003192     | 4            | 5                | +             | +             |
| mtSSB            | SSBP1  | NP_003134     | 5            | 5                | +             | +             |
| mtDNA polymerase | POLRMT |               |              |                 |               |               |
| DEAH box polypeptide 30-2 | DHX30  | NP_055781     | 4            | 3                |               |               |
| Hydroxacyl dehydrogenase A | HADHA  | NP_000173     | 6            | 16               |               |               |
| Serine hydroxymethyltransferase | SHMT2  | NP_005403     | 4            | 5                |               |               |
| Other proteins with known roles in mtDNA replication and transcription |        |               |              |                 |               |               |
| MtDNA polymerase γ A | POLG   | NP_002684     | 3            | 0                | +             |               |
| MtDNA polymerase γ B | POLG2  | NP_009146     | 1            | 0                |               |               |
| TFB2M            | TFB2M  | NP_001761     | 0            | 3                |               |               |
| DNA helase, twinkle | PE01   | NP_068602     | 3            | 0                |               |               |
| SUV3-like helase | SLIP3L1 | NP_003162     | 3            | 0                |               |               |
| II. Chaperones    |        |               |              |                 |               |               |
| HSP 70           | HSPA1  | NP_004125     | 9            | 14               | +             |               |
| HSP 60           | HSPD1  | NP_055472     | 9            | 6                | +             |               |
| Prohibitin 1     | PHB1   | NP_002625     | 7            | 4                | +             |               |
| Prohibitin 2     | PHB2   | NP_009204     | 7            | 6                | +             |               |
| LRP130           | LRPPRC | NP_573566     | 8            | 27               |               |               |
| AAA-ATPase       | ATAD3A | NP_060658     | 10           | 6                |               |               |
| III. Other proteins |        |               |              |                 |               |               |
| ANT2             | SLC25A5 | NP_001143     | 9            | 6                | +             |               |
| Carnitine palmitoyltransferase 1A | CPT1A | NP_001867 | 3 | 9         |               |               |
| NADH dehydrogenase S1 | NDHFS1 | NP_004997 | 4 | 4         |               |               |
| NADH dehydrogenase 1α 9 | NDHFA9 | NP_004993 | 3 | 4         |               |               |
| NADH dehydrogenase FeS 30K | NDHFS3 | NP_004542 | 0 | 4         |               |               |
| Carbamoyl phosphate synthetase I | CPS1 | NP_001866 | 5 | 21        |               |               |
| Bcl-XL-binding protein v68 | MGC5352 | NP_612642 | 3 | 4         |               |               |
| Mitofilin        | IMMT   | NP_006830     | 3            | 7                |               |               |
| Kinectin 1       | KTN1   | NP_001866     | 10           | 23               |               |               |
| Ribophorin        | RPNI   | NP_002941     | 6            | 6                |               |               |

* Bogenhagen et al. (25) and unpublished observations (D. Bogenhagen).
* Chen et al. (5).
* Extramitochondrial annotations suggest this is likely a contaminant.

* sedimentation and immunofluorination purification used either anti-TFAM or anti-mtSSB antibodies. Proteins eluted from the magnetic beads with SDS were separated by SDS-PAGE. The entire gel lane was sliced into 12 to 15 zones selected to separate major polypeptide species. Proteins were fragmented by in vitro trypsin digestion and peptides were analyzed by LC-MS/MS. Protein identifications were accepted only when proteins were found at the appropriate size in SDS-PAGE and when at least three peptides were identified with greater than 90% confidence by ProID software. We routinely observed a rather large number of polypeptides, typically 25–40, when either anti-TFAM or anti-mtSSB antibodies were used in these purifications. Of 28 proteins purified using anti-TFAM antibodies and 35 purified using anti-mtSSB antibodies, the majority, 21 proteins, were observed with both approaches. We consider proteins to be tightly associated with nucleoids when they were identified using both anti-TFAM and anti-mtSSB antibodies independently. These proteins are listed in Table 1, along with a number of proteins known to bind mtDNA that were observed with only one of the two antibody reagents. Lists of all peptide hits with confidence >90% supporting these protein identifications are included in supplemental materials Table 1.

Nucleoid proteins reported in Table 1 are divided into several major functional groups. First, as Group I we identified the majority of proteins documented to date as participants in the replication and transcription of mtDNA. This includes abundant DNA-binding proteins TFAM, mtSSB, as well as mtRNA polymerase. Some relatively rare proteins, including both subunits of DNA polymerase γ, the DNA helicase twinkle (31), and the transcription factor TFB2M (32) were observed in some, but not all preparations. These are included in Table 1 because previous studies have documented their roles in mtDNA transcription. We have confirmed the presence of both subunits of DNA polymerase γ in nucleoid preparations using Western blotting (see below). Two other proteins with helicase motifs were observed and are discussed below.

Group II in Table 1 lists a remarkably large number of chaperone proteins found in nucleoids. Their potential significance is discussed below. We also detected a series of proteins involved in intermediary metabolism, membrane transport, and the cytoskeleton, listed as Group III in Table 1. Metabolic proteins have been recovered in mtDNA nucleoid preparations from Xenopus and yeast as well. It is particularly noteworthy that we did not observe aconitase in the human mtDNA nucleoids, despite the elegant work by Chen et al. (7) documenting the role of this protein in maintenance of yeast mtDNA in the absence of TFAM.

To validate our protein identification results, we also conducted an extensive series of Western blotting experiments using 17 distinct antibodies. To search for proteins that may be included preferentially in either fast or slowly sedimenting nucleoids, we performed these experiments on both glycerol gradient fractions. We included antibodies directed against 11 proteins identified by mass spectrometry as well as several other proteins that we failed to detect, such as aconitase. The
immunoblotting experiments shown in Fig. 5 permit several conclusions. First, as a technical note, we did not observe quantitative binding of proteins to the antibody-coated beads in these experiments, because we used a large quantity of starting material to increase the sensitivity of detection. Thus, many proteins found in the eluate (E) are also contained in the unbound supernatant fraction (S). Second, TFAM, mtsSB, polymerase γ(A), ANT, prohibitin (PHB), HSP60, vimentin, actin, kinectin, mitofilin, and NDUFA9 (CI) antigens were selected for immunoblotting in an effort to confirm the protein identification results, whereas other antigens, including aconitase, voltage-dependent anion channel (VDAC), CII (SDHA), CIII (UQCRC1), CIV (COX1), and Tom20 were selected as controls not detected by mass spectrometry.

**FIGURE 5.** Confirmation of the presence and absence of proteins in mitochondrial nucleoids using immunoblotting. Fractions from rapidly and slowly sedimenting nucleoids were used for immunofinity purification using antibodies directed against TFAM. Proteins were fractionated by SDS-PAGE and detected by silver staining or immunoblotting as in Fig. 4. M, protein markers with selected masses in kilodaltons indicated on the left; L, load; S, unbound supernatant; W1 and W2, successive washes; E, eluted with 0.5% SDS. The upper panel shows a silver-stained gel of proteins. The lower panels show immunoblots with antibodies directed against the indicated antigens. TFAM, mtsSB, polymerase γ(A), ANT, prohibitin (PHB), HSP60, vimentin, actin, kinectin, mitofilin, and NDUFA9 (CI) antigens were selected for immunoblotting in an effort to confirm the protein identification results, whereas other antigens, including aconitase, voltage-dependent anion channel (VDAC), CII (SDHA), CIII (UQCRC1), CIV (COX1), and Tom20 were selected as controls not detected by mass spectrometry.
that DHX30 has a clear physical relationship with mtDNA nucleoids. We conclude that the DHX30 gene is another example of a eukaryotic gene with alternate isoforms expressed in mitochondria and other cellular compartments. Further studies will be required to test whether the protein isoform shown in Fig. 6B explains this mitochondrial localization or if other isoforms with less obvious mitochondrial localization signals provide the mitochondrial variant.

**Physical Relationship of Mitofilin to MtDNA Nucleoids**—Mitofilin (IMMT) is a recently discovered mitochondrial protein that is important for preservation of cristae morphology (33). Mitofilin contains a coiled-coil domain as well as a motif characteristic of motor proteins. Residues thought to be required for motor protein activity have diverged within mitofilin, so that it is not considered to be an active motor protein. Mitofilin is anchored to the inner mitochondrial membrane with the bulk of its coiled-coil structure exposed to the intermembrane space. Mitofilin persists in foci within mitochondria following extraction with non-ionic detergents (28). Because mitofilin was reproducibly found in our human nucleoid preparations, we used immunofluorescence to determine whether it is colocalized with TFAM as a nucleoid marker. Fig. 8 reveals punctate staining of mitofilin within mitochondria with some foci adjacent to but not co-incident with TFAM-stained nucleoids. This is reminiscent of the spatial relationship described previously for Mmm2p and yeast mtDNA nucleoids (12).

**DISCUSSION**

**Definition of the MtDNA Nucleoid**—The mtDNA nucleoid is a dynamic structure containing several mtDNA genomes, only some of which may be actively involved in replication or transcription at any instant. Hence, the protein composition of mtDNA nucleoids can be expected to vary under diverse physiological conditions. Our biochemical purification of nucleoids provides an averaged snapshot of protein-DNA and protein-protein contacts that survive our fractionation procedures. This work has identified a larger fraction of nucleoid proteins known to function in mtDNA transactions than any previous study. However, it is likely that more proteins remain to be identified because we have not reproducibly observed significant peptide
hits for some proteins that have been shown in other studies to bind to mtDNA or within nucleoids, such as mTERF (34), Lon (35), and PDIP38 (36).

We have found that a substantial fraction of nucleoids adhere to cytoskeletal proteins, such as actin and vimentin. The association with vimentin is particularly interesting because vimentin can be cross-linked to mtDNA in higher eukaryotic cells (37), although it is possible that this cross-linking is mediated by other primary DNA-binding proteins that have not been identified. Actin has been identified in human mitochondrial nucleoid preparations by Kanki et al. (38) as well as in our work. It is noteworthy that we did not observe any significant association of cytoskeletal proteins with Xenopus oocyte mitochondrial nucleoids in our previous studies (25).

Our proteomic investigation focused on the slowly sedimenting nucleoid fraction not tightly associated with cytoskeletal proteins. At this time, we cannot discern whether these nucleoids are loosely associated with cytoskeletal proteins in vivo or whether they are released during our handling procedures. To obtain the most robust list of tightly associated proteins, we used two complementary immunoaffinity reagents to further purify these nucleoids. This identified a set of 21 proteins recovered by immunopurification with antibodies directed against either TFAM or mtSSB. An equally large set of proteins was identified by only one of the two immunoprecipitation approaches. These may be less tightly bound to mtDNA or other nucleoid components. The protein set that was recovered with both reagents includes the best established mtDNA-binding proteins, TFAM and mtSSB, as well as other proteins detected either due to their abundance or to their affinity for mtDNA and other protein components of nucleoids. This discussion will focus on the two broad classes of nucleoid-associated proteins, those proteins that appear to be involved directly in mtDNA metabolism and those that do not. In general, we consider that the nucleoid proteins that are required for their function to bind mtDNA constitute the core of a nucleoid structure, whereas other proteins identified in nucleoids are likely to have a more peripheral interaction. The presence of these peripheral proteins may depend on protein-protein interactions with other nucleoid components.

Nucleoid Proteins Involved in MtDNA Metabolism—Table 1 indicates that our nucleoid preparations included several proteins previously characterized as factors involved in replication and transcription of mtDNA, including mtRNA polymerase, TFAM, and mtSSB. Other proteins, including DNA polymerase\(\gamma\), twinkle helicase, and the transcription factor TFBM2 were observed in some preparations, but not others. Interestingly, although the work of Garrido et al. (3) has established twinkle helicase as a signature component of
nucleoids, we identified two additional helicases in the nucleoid fraction, the Suv3-like helicase and DEAH helicase 30, DHX30. The Suv3-like helicase has recently been documented as a mitochondrial protein with DNA helicase activity (39), although its potential role in mtDNA replication has not been tested. In contrast, our observation of multiple peptides derived from DHX30 in nucleoids represents the first evidence that this protein can be imported into mitochondria. Our inspection of the genetic loci encoding DHX30 in humans and mice led to a clear suggestion for a conserved exon structure for a mitochondrial isoform of DHX30 in mammals (Fig. 6). To confirm the presence of DHX30 in mitochondria, we raised a polyclonal antibody to the protein and showed that an isoform of the protein copurifies with mitochondria and is detectable by immunofluorescence in the vicinity of mtDNA nucleoids. Further work is required to define the role of DHX30 in mtDNA replication and/or transcription.

Two additional proteins previously shown to have nucleic acid binding ability were reproducibly observed in nucleoids. One of these, hydroxacyl dehydrogenase has been shown to bind RNA through its β subunit (40). The second, serine hydroxymethyltransferase, is the human homolog of a Xenopus mitochondrial protein shown to bind single-stranded DNA (61). These two proteins are representative of other metabolic proteins with nucleic acid binding ability, a group that includes aconitase (41).

Association of Nucleoids with Chaperones and Metabolic Proteins Characterizes an Intra-mitochondrial Microenvironment—Many proteins found in nucleoid preparations have other established roles in mitochondria, such as ANT and the chaperones noted in Table 1. In many of these cases, only a fraction of the entire mitochondrial content of these proteins may be in contact with nucleoids. Due to the sensitivity of protein identification using mass spectrometry, it is conceivable that some of the abundant mitochondrial proteins reported in Table 1 may be contaminants. Nevertheless, the association of these proteins with nucleoids may still be significant because the identities of these interacting proteins help to define the microenvironment in which the nucleoid resides within mitochondria. Extensive investigation is needed to explore the potential roles of most of these proteins in mtDNA maintenance.

Table 1 reports multiple peptide hits on mitochondrial proteins with chaperone activity, including HSP70, HSP60, LRPPRC, prohibitin, and the mAAA ATPase ATAD3A/B, which is also known as TOB3. The first two of these are not surprising because yeast HSP60 has been reported to have mtDNA binding activity (42) and HSP70 is the eukaryotic homolog of E. coli dnaK protein, which has an established role in replication of bacteriophage and bacterial DNA genomes (43). These and other chaperones use the energy of ATP hydrolysis to assist in loading and unloading proteins at replication forks in bacterial (44) and eukaryotic (45) systems. We also identified peptides derived from LRPPRC, a factor required for cytochrome oxidase assembly (46) that has also been reported to have RNA and single-stranded DNA-binding activity (47).

Prohibitin 1 and prohibitin 2 were identified both in our current study of human mtDNA nucleoids and in our previous characterization of Xenopus mtDNA nucleoids (25). These proteins comprise a large toroidal structure associated with the m-AAA complex in yeast (48–51). We consistently obtained multiple peptide hits on ATAD3A and its closely related paralog ATAD3B, which have recently been identified in the mitochondrial inner membrane in mouse hepatocytes (52). The ATAD3A/B and AFG3L2 AAA-ATPases are closely related to paralgp (53). In S. cerevisiae, Afg3p and Rca1p, contribute to the matrix protease, m-AAA complex, whereas yme1p is a component of the inter-membrane space i-AAA complex. The m-AAA proteases have recently been shown to control ribosome assembly (54), a process that has not been studied extensively in higher eukaryotes. It is conceivable that ribosome assembly may occur in the vicinity of nucleoids as rRNAs are transcribed from the mtDNA template and processed.

In contrast to our results, prohibitin and AAA-ATPases were not reported by Chen et al. (7) in their study of yeast nucleoids cross-linked with formaldehyde. This does not mean that these proteins are irrelevant to the stability of yeast mtDNA. Prohibitin has been linked to mtDNA inheritance in yeast (55) and yme1p was initially named based on the phenotype of mutants at this locus, which showed enhanced yeast mtDNA escape to the nucleus (56). It may be the case that AAA-ATPases and prohibitin are not located in physical contact with mtDNA or with proteins tightly bound to mtDNA in yeast.

Other Group III proteins listed in Table 1 are involved in membrane transport and metabolism. ANT isoforms were identified in this study as well as in our previous characterization of Xenopus nucleoids (25). The conserved presence of ANT in mtDNA nucleoids is intriguing because mutations in ANT have been correlated with multiple mutations in mtDNA (57). The physical basis for the association of ANT with nucleoids is not clear at present.

Metabolic proteins have been found consistently in yeast, Xenopus, and human mtDNA nucleoids, although no individual protein has been reported in all sources. We were surprised to identify multiple peptides from two subunits of NADH dehydrogenase in human nucleoid preparations. This result, validated by Western blotting in Fig. 5, is of interest because complex I is the least abundant of the respiratory complexes (58). The selective association of complex I with the nucleoid fraction without the more abundant respiratory complexes suggests that this association does not simply reflect contamination with abundant proteins. This reinforces the concept that nucleoids reside in a specialized microenvironment adjacent to the mitochondrial inner membrane.

Evolutionary Diversity of MtDNA Nucleoid Composition—This work documents significant differences in the spectrum of proteins associated with human mtDNA nucleoids as compared with their counterparts in Xenopus oocytes and yeast. Certain key DNA-binding proteins like TFAM and mtSSB are conserved, but there is a marked variability in the metabolic proteins associated with mtDNA. One of the most interesting results in this regard is the apparent absence of
Human Mitochondrial DNA Nucleoid Proteins

...aonitase from human mtDNA nucleoids. Chen et al. (7) detected this TCA cycle protein in yeast nucleoids and, using a genetic approach, confirmed that it has an unanticipated ability to compensate for a deficiency in yeast TFAM (for example, Afb2p). It may be that this secondary role for aconitase may reflect selective pressures more important for yeast than for mammalian cells.

We detected a significantly larger number of human nucleoid proteins than we detected in mtDNA nucleoids purified from Xenopus oocytes (25). Both mitochondrial sources provided clear peptide signatures for TFAM, mtSSB, ANT, and prohibitin, but the only other major components of Xenopus oocyte nucleoids were the E2 subunits of pyruvate dehydrogenase and branched chain ketoacid dehydrogenase. The reason for this apparent discrepancy is not clear, although it may reflect the fact that the Xenopus mitochondria used in our previous work were derived mainly from mature oocytes not actively engaged in mtDNA replication or transcription. Another intriguing possibility is raised by recent work showing that oocytes rely on an unusual metabolic flux that depends heavily on amino acid catabolism and the pentose cycle (59). In contrast, cultured HeLa cells use glycolysis and the catabolism of glutamine as major sources of energy (60). Thus, branched chain ketoacid dehydrogenase may be relatively more abundant in Xenopus oocyte mitochondria. This discrepancy provides an interesting hypotheses to guide further research.

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