Mitochondrial DNA (mtDNA) occurs in cells in nucleoids containing several copies of the genome. Previous studies have identified proteins associated with these large DNA structures when they are biochemically purified by sedimentation and immunoaffinity chromatography. In this study, formaldehyde cross-linking was performed to determine which nucleoid proteins are in close contact with the mtDNA. A set of core nucleoid proteins is found in both native and cross-linked nucleoids, including 13 proteins with known roles in mtDNA transactions. Several other metabolic proteins and chaperones identified in native nucleoids, including ATAD3, were not observed to cross-link to mtDNA. Additional immunofluorescence and protease susceptibility studies showed that an N-terminal domain of ATAD3 previously proposed to bind to the mtDNA D-loop is directed away from the mitochondrial matrix, so it is unlikely to interact with mtDNA in vivo. These results are discussed in relation to a model for a layered structure of mtDNA nucleoids in which replication and transcription occur in the central core, whereas translation and complex assembly may occur in the peripheral region.

Eukaryotic cells typically contain thousands of mtDNA genomes assembled into hundreds of aggregates known as nucleoids (1–3). These nucleoids show considerable dynamic stability, raising the possibility that this higher order organization is very important to the inheritance and segregation of mtDNAs. Nucleoid organization is thought to contribute to the rapid segregation of mtDNA mutations during oocyte maturation, the so-called “bottleneck” (4, 5), since early primordial germ cells appear to have only a small number of segregation units (6). The degree of mtDNA heteroplasmy varies considerably during embryonic development of somatic tissues as well, reflecting an uneven distribution of mtDNA genotypes that may be influenced by the clustered organization of mtDNA. A high rate of mitochondrial fusion appears to be essential for the maintenance of mtDNA in nucleoids (7, 8). One of the consequences of active cycling of mitochondrial fusion and fission may be to permit an individual nucleoid to have access to a larger pool of diffusible proteins required for replication or transcription than it would if it were trapped within a single mitochondrion.

Quantitative analysis of the size and mtDNA content of nucleoids in cultured mammalian cells suggests that an average nucleoid may contain 5–7 mtDNA genomes packed in a space with a diameter of only 70 nm (9). A human mtDNA nucleoid with seven 16.6-kb mtDNA genomes is a large structure containing 70 million daltons of DNA and a comparable mass of protein. These nucleoids resemble their counterparts in eubacteria, which employ abundant basic DNA binding proteins to package their genomes in a restricted volume. The packing density of bacterial nucleoids is impressive, since 4700 kb of E. coli DNA is folded into a volume of 0.08–0.24 μm³ (10). If 5–7 mtDNA molecules are packed in a sphere with a diameter of 70 nm, the calculated packing density of mtDNA nucleoids would be similar to that of a bacterial nucleoid. The parallel between bacterial and mitochondrial nucleoids is extended by the recognition that both are tethered to membrane components. For the case of the mtDNA genome, the concept that the DNA may be physically associated with the inner membrane was raised shortly after the discovery of mtDNA (11). The identity of a possible membrane anchor has been an open question since the pioneering work from the Attardi laboratory (12) implicated a specific interaction with an unknown protein.

The packaging of animal mtDNA in nucleoids is accomplished with the aid of DNA-binding proteins, including two particularly abundant proteins, TFAM and mtSSB. Although a recent study has suggested that the properties of TFAM may be especially important for the compaction of mtDNA in nucleoids (13), there is some controversy regarding the number of TFAM molecules per mtDNA. Some studies have suggested that there is sufficient TFAM to literally coat the mtDNA genome (14), whereas others have reported much lower levels (15, 16). The ratio of TFAM per mtDNA is developmentally regulated in Xenopus oocytes (17). Immature oocytes that are actively replicating and transcribing mtDNA have a lower ratio of TFAM to mtDNA. TFAM accumulates to high levels in mature oocytes as mtDNA replication and transcription become repressed. Somatic cell types generally require active transcription of mtDNA to provide subunits of respiratory...
Formaldehyde-cross-linked Human mtDNA Nucleoids

complexes and frequent replication to replace mtDNA genomes lost to turnover.

We previously characterized a set of proteins associated with human mtDNA nucleoids using gentle lysis of mitochondria with nonionic detergents followed by sedimentation and immunoaffinity purification using polyclonal antibodies directed against TFAM and mtSSB (18). We found that the use of two distinct antibody reagents identified a common set of protein components, including many of the proteins previously known to function in mtDNA replication and transcription. However, this procedure also identified several metabolic proteins and chaperones as well as evidence of association of large nucleoid structures with cytoskeletal elements. The fact that this gentle approach to purify native nucleoids used non-denaturing conditions raised the possibility that some of the proteins recovered were not actually intrinsic nucleoid proteins but might simply represent abundant or “sticky” proteins that contaminated the preparation.

In the present study, we adopted a different procedure for biochemical isolation of nucleoids that is intended to identify the subset of nucleoid proteins that are in close contact with mtDNA. We prepared nucleoprotein complexes from formaldehyde-treated mitochondria under conditions that exposed the complexes to harsh ionic detergents and high salt to strip away all proteins not covalently bound to the DNA. Formaldehyde cross-linking has been used in previous studies of the protein composition of yeast mtDNA nucleoids (19) that identified a number of metabolic proteins in contact with mtDNA. Formaldehyde has desirable properties for such cross-linking studies, since it has a very short range of action and is reversible at high temperatures. This accounts for the popularity of this method as a probe of DNA-protein interactions in chromatin immunoprecipitation experiments (20).

We found that only a subset of the nucleoid proteins found in our native nucleoid preparations were cross-linked to mtDNA, including TFAM and mtSSB, along with DNA metabolic proteins, such as DNA polymerase γ and mtRNA polymerase, and a small number of other proteins that we consider to be components of the nucleoid core. An important advantage of this cross-linking study is that it distinguished a class of proteins found in association with native nucleoids that were not represented in the formaldehyde-cross-linked set, including a number of chaperone proteins. We studied one of these proteins, ATAD3, in greater detail, since this protein has recently been claimed to have the ability to bind mtDNA D-loop structures directly (21). Immunofluorescence indicated that it is not confined to nucleoids, which is consistent with our inability to cross-link it to mtDNA. Limited proteolysis of mitochondria showed that the putative DNA-binding domain of ATAD3 reported by He et al. (21) is not directed toward the mitochondrial matrix, so that it is not properly positioned to bind D-loop structures. Our results are discussed with reference to a layered model of nucleoid structure in which replication and transcription occur within a central core, and RNA processing, translation, and respiratory complex assembly may occur in a peripheral zone.

EXPERIMENTAL PROCEDURES

Formaldehyde Cross-linking and Preparation of Nucleoids—Mitochondria were prepared from 4 liters of suspension-cultured HeLa cells as described (18), including a nuclease treatment step, but omitting treatment with digitonin to produce mitoplasts. Following the final pelleting, mitochondria were resuspended in 0.9 ml of MSH buffer (210 mM mannitol, 70 mM sucrose, 20 mM HEPES, pH 8, 2 mM EDTA, 2 mM dithiothreitol supplemented with 0.2 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin, and 5 μg/ml leupeptin), mixed with 0.1 ml of 10% formaldehyde in MSH, and incubated on a rotator at 4 °C for 30 min. 125 μl of 2 mM glycine, pH 7.3, was added for an additional 5 min before mitochondria were lysed by the addition of one-tenth volume of 20% SDS. The lysate was layered onto two preformed composite gradients in Beckman SW41 polylamellar tubes. The top gradient buffer contained 15% glycerol, and the bottom contained 30% glycerol plus 30% Nycodenz (Histodenz; Sigma). Gradients contained 20 mM Hepes, pH 8, 0.5% Sarkosyl (sodium sarcosinate; Sigma), 100 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin, and 5 μg/ml leupeptin throughout and were generated by layering over a pad of 0.6 ml of 5.7 M CsCl. Gradients were centrifuged for 4.5 h at 38,000 rpm (247,000 × g) at 4 °C. Fractions of ~0.8 ml were collected and assayed for DNA either by staining with Picogreen with detection by fluorometry (22) or by digestion with restriction endonuclease HindIII and agarose gel analysis to identify mtDNA fragments. When restriction analysis was performed, as in Fig. 2, 50 μl of each fraction was treated as described below to reverse cross-links and deproteinized by extraction with phenol–CHCl₃. DNA was precipitated with ethanol, treated with HindIII restriction endonuclease, and analyzed by agarose gel electrophoresis using standard methods.

Fractions containing mtDNA were diluted to 10 ml with gradient buffer lacking either glycerol or Nycodenz and layered over a pad of 5.7 M CsCl in an SW41 tube. Samples were centrifuged for 6–8 h at 38,000 rpm and 4 °C to sediment nucleoids to the CsCl shelf. The upper buffer was withdrawn, and the interface was collected and mixed with 7 M CsCl to adjust the solution density to 1.5–1.55 g/ml as determined by refractometry. Gradients were centrifuged to equilibrium for 40–44 h at 18 °C and 40,000 rpm (215,000 × g) in a Beckman SW60Ti rotor. Fractions were collected and analyzed for DNA content using Picogreen fluorescence as described above. Fractions containing the peak of DNA fluorescence at a density of 1.5–1.6 g/ml were pooled and rebanded. Following measurement of density and DNA content, fractions from the second gradient surrounding the peak of DNA fluorescence were diluted with TE buffer to 0.5 ml and precipitated with two volumes of ethanol. If a dense white CsCl precipitate formed, the samples were resuspended in 0.3 ml of 0.3 M sodium acetate, and ethanol precipitation was repeated. Following a rinse with 70% ethanol, the samples were dried briefly and redissolved in 75 μl of SDS sample loading buffer (10% glycerol, 20 mM dithiothreitol, 62.5 mM Tris, pH 6.8, 2% SDS). Samples were heated at 80 °C for 3 h to reverse formaldehyde cross-links.
Protein Analysis and Sequencing—Following reversal of cross-links, protein samples from gradient fractions were analyzed by SDS-PAGE on gels containing 8–12% polyacrylamide, depending on the molecular weight range desired. Proteins were detected by silver staining (23) or were transferred to polyvinylidene difluoride membranes that were probed with various antibodies. Polyclonal antisera directed against TFAM, mtSSB, polymerase γA, SHMT, and DHX30 were prepared in the Bogenhagen laboratory and were used at a dilution of 1:1000–1:5000. Antibodies directed against the ATAD3 N-terminal sequence PAPKDKWSNFDP (residues 51–62) and the C-terminal sequence LKAEGPGRGDEPS (residues 620–634) were prepared in the Rousseau laboratory. Additional antipeptide antibodies directed against ATAD3 sequences were obtained from Dr. Olivier Gires (University of Munich). Antibodies directed against mTERF (from Dr. Giuseppe Attardi, Caltech), LRPPRC (from Dr. Serafin Pinol-Roma, CUNY), PDIP38 (from Dr. Marietta Lee, New York Medical College), and Lon and Tid1 (from Dr. Carolyn Suzuki, New Jersey Medical School) were used as directed by the suppliers. Other antibodies obtained from commercial sources directed against prohibitin (Neomarkers), Tom20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and DNA (Fitzgerald Industries) were used as recommended by the suppliers. Following incubation of blots with primary antibodies, membranes were washed with several changes of 0.5% Tween 80 in PBS prior to incubation with secondary anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase. Complexes were incubated with reagents from the Pierce Supersignal kit to develop a chemiluminescent signal detected using an Alpha Innotech CCD imager. Antibodies were also used for immunofluorescence microscopy as described (18). For protein sequencing, the gel lanes of interest were divided into 10–16 zones, as shown in Fig. 4. Proteins were digested to peptides with sequencing grade trypsin and analyzed by liquid chromatography-tandem mass spectrometry as described (18).

RESULTS

Preparation of Cross-linked Nucleoids—The protocol employed to prepare formaldehyde-cross-linked nucleoids from HeLa cells (Fig. 1) began with purification of mitochondria as described in our previous work on native nucleoids (18). In the modified protocol, the mitochondria in mannotol-sucrose buffer were incubated with 1% formaldehyde for 30 min before glycine was added to react with excess formaldehyde. Mitochondria were then lysed with 2% SDS, and DNA-protein complexes were separated from free protein by sedimentation through a composite glycerol/Nycodenz gradient containing 0.5% Sarkosyl. Following sedimentation, mtDNA was found in a broad peak in the center of the gradient in association with a significant fraction of the TFAM and mtSSB. TFAM and mtSSB molecules not cross-linked to mtDNA were released and remained at the top of the gradient with the vast majority of mitochondrial protein (Fig. 2). In the experiment shown in Fig. 2, 40–45% of both TFAM and mtSSB was contained in cross-linked complexes, although the extent of cross-linking varied somewhat from one preparation to another. Fragments of nuclear DNA contaminating the preparation were also enriched at the top of the gradient (data not shown). The fractions containing mtDNA were identified and pooled. To concentrate the cross-linked complexes, they were first diluted with gradient buffer lacking glycerol or Nycodenz to reduce the density of the solution. The complexes were then concentrated by sedimentation onto a pad of 5.7 M CsCl. This pad was collected and adjusted to a CsCl density of 1.5–1.55 g/ml and centrifuged to equilibrium in an SW60Ti rotor. For a typical preparation beginning with 4 liters of HeLa cells in suspension culture, two CsCl gradients were used for the first round of isopycnic centrifugation. Since early pilot experiments showed that a considerable amount of free protein was contained at the top of these initial gradients, the mtDNA peaks from two CsCl gradients were pooled and rebanded. mtDNA-protein com-
plexes were found at a density of 1.5–1.57 g/ml in the second CsCl gradient (Fig. 3). Following reversal of the formaldehyde cross-links with heat, a sample of each fraction was digested with restriction endonuclease HindIII to confirm that the DNA in the gradients has a digestion pattern characteristic of mtDNA (Fig. 3). Another fraction was subjected to SDS-PAGE to detect proteins. This revealed that the DNA complexes that banded at lighter density contained a larger amount of cross-linked protein than those on the dense side of the peak, as expected (compare the protein/DNA ratios of fractions 3 and 5 in Fig. 3, B and C). No significant amount of free protein was detected at the top of the gradient when the complexes were purified using two rounds of isopycnic centrifugation.

**Proteins Identified in Cross-linked Nucleoids**—Proteins from the peak fractions of two independent nucleoid preparations were fractionated by SDS-PAGE and detected by silver staining (Fig. 4). This revealed a very reproducible pattern of polypeptides. Each gel lane was divided into a series of slices containing proteins of decreasing molecular mass, and each slice was processed by in situ trypsin digestion to generate peptides. The peptides from the two lanes shown in Fig. 4 were analyzed using a total of 27 liquid chromatography-tandem mass spectrometry runs as described under “Experimental Procedures.” Proteins identified by at least two high confidence peptide hits are listed in supplemental Table 1. In a few cases, such as for PDIP38 and DNAJ3 (Hsp40, TID1), proteins identified by a single high confidence peptide hit were included when the protein was already characterized as a nucleoid component. Occasional peptide hits representing keratins and histones were omitted from this list, since keratins were considered to be introduced by sample handling, and the histones appeared to result from trace contamination with nuclear DNA fragments. In addition to the list of proteins and peptides in supplemental Table 1, we observed numerous ribosomal proteins and other proteins involved in translation. Interestingly, these represent both mitochondrial and nonmitochondrial ribosomes. These cytoplasmic ribosomes may be contaminants, since it is estimated that a HeLa cell has about 160 times as many cytoplasmic ribosomes as mitochondrial ribosomes (24). Still, their presence may be significant in light of the model of Iborra et al. (9),
suggestion that nucleoids are centers for mitochondrial translation and occur near sites where proteins synthesized on cytoplasmic ribosomes docked to the outer membrane may be co-translationally imported. However, since we could not rule out the possibility that these peptide hits represent proteins cross-linked to rRNA, we did not include these in supplemental Table 1 but listed them separately in supplemental Table 2. Although the model for co-translational import and respiratory complex assembly is of great interest, a comprehensive test of this is beyond the scope of the present paper.

As expected from the complexity of the silver-stained gel patterns in Fig. 4, peptide analysis led to the identification of a large number of peptides listed in supplemental Table 1. The proteins identified are listed in Table 1 along with a comparison with the set of proteins we found in native nucleoids (18). The proteins in Table 1 are divided into three classes. Class I includes 31 proteins observed in both native and cross-linked nucleoid preparations. Class II includes 10 proteins observed in native nucleoids but not found cross-linked to mtDNA. These appear to represent peripheral proteins that might have been retained in native preparations on the basis of their interactions with other nucleoid components. We cannot rule out the possibility that some of these peripheral nucleoid proteins are merely contaminants, although nucleoid-associated roles for some of them, such as Hsp60 and prohibitin, are supported by other data. The third class of proteins listed in Table 1 is a set of 16 proteins identified in cross-linked nucleoids but not in native nucleoids. In most cases, these were identified in only one of the two experiments. It is most likely that these proteins were adventitiously cross-linked to core nucleoid proteins, and they are not considered further.

We performed a series of immunoblotting experiments to validate the identification of a number of the nucleoid proteins and to test for the presence of certain other proteins of interest. In these experiments, two independent cross-linked nucleoid preparations were generated, and proteins released by heat treatment of the nucleoid peak fraction and flanking fractions were separated by SDS-PAGE, blotted to polyvinylidene difluoride membranes, and probed for reactivity with a series of antisera. In most cases, a positive control lane was included, containing a portion of the mitochondrial protein preparation before cross-linking. Fig. 5A shows the DNA distribution for one of these final CsCl gradients, displaying a peak in fraction 7. Immunoblots of proteins from fractions 5–9 of this gradient are shown in Fig. 5B, whereas Fig. 5C shows the immunoblots from a similar analysis of samples from an independent nucleoid preparation. Results in Fig. 5 confirm that TFAM, mtSSB, polymerase γA, SUV3L, SHMT, and DHX30 are contained in CsCl gradient fractions containing the nucleoid peak. We note that for mtSSB and DHX30, gradient fractions on the lighter side of the DNA peak showed greater reactivity with antibodies. This suggests that these proteins are found in complexes with a higher protein/DNA ratio. Lon protease, which has been reported to be a nucleoid component and to have the ability to bind mtDNA (25), was identified by immunoblotting and by several peptide hits in one of our preparations. TID1, which interacts with Lon, was identified by a single peptide in our sequence analysis but was not observed in cross-linked complexes by immunoblotting, although this may reflect a limited sensitivity of the antibody. Fig. 5 also shows that LRPPRC was only very weakly detected in gradient fractions and that ATAD3 was not seen. Another protein reported to associate with nucleoids, PDIP38 (26), was identified by only one peptide hit in one cross-linked preparation and was not detected by immunoblotting. PDIP38 was first identified as a protein associating with DNA polymerase δ in whole cell detergent lysates (27) but was later found to be a rather abundant mitochondrial protein (28). The fact that PDIP38 interacts with a nonmitochondrial DNA polymerase has been taken as a suggestion that it might interact with the mitochondrial DNA polymerase, DNA polymerase γ. However, our efforts to detect any interaction between these proteins or any effect of PDIP38 on polymerase γ activity have been unsuccessful (data not shown). The precise role of this protein in mitochondria is still unknown, although a recent publication reported that its down-regulation resulted in mitochondrial fragmentation (29).

Further Analysis of the Peripheral Nucleoid Protein ATAD3—The ATAD3 locus is tandemly duplicated in the human genome, generating two paralogs, ATAD3A and ATAD3B, both of which were represented in our native HeLa cell nucleoid preparations (18). Shortly after we reported the observation of
They also reported that an N-terminal domain of the protein
had the ability to bind specifically to the mtDNA D-loop,
although they did not document that the intact protein retained
this ability. Interestingly, ATAD3 (also known as TOB3) has

ATAD3 in native nucleoids, another group also reported this
protein among a short list of candidate nucleoid proteins (21).
They also reported that an N-terminal domain of the protein

\* LRRC59 is a protein found in nucleoids that has not yet been shown to be a mitochondrial component.
been reported to be an inner membrane protein (30) and to be up-regulated in certain tumors (31). ATAD3 is an AAA-domain protein, a class of proteins that is known to have important roles in DNA and RNA transactions in other systems (32, 33). Although we failed to observe ATAD3 in cross-linked nucleoids, these facts suggested that it is still of particular interest as a potential nucleoid protein. To determine the degree to which ATAD3 is localized to nucleoids, we performed immunofluorescence using an antipeptide antibody directed against a sequence within the C-terminal AAA domain of the protein in combination with anti-DNA antibodies. For this analysis, we used SAOS2 cells, which have very elongated mitochondria and with nucleoids studded along the mitochondrial profiles. The epitope recognized by the antipeptide antibody was progressively degraded by proteinase K for increasing amounts of time, up to 20 min, the intact protein recognized by the C-terminal antibody was progressively lost and replaced by a fragment of ~42 kDa that represents a C-terminal domain of the protein protected by the mitochondrial membranes. The epitope recognized by the N-terminal antibody was progressively degraded by proteinase K without generating a stable product. Controls showed that a known outer membrane protein, Tom20, is quickly degraded by proteinase K, whereas a protein localized to the intermembrane space, prohibitin, is considerably more stable. Proteinase K may have some accessibility to the intermembrane space of purified mitochondria under our treatment conditions to account for the slower rate of degradation observed for prohibitin and ATAD3 in comparison with Tom20. These results are consistent with the likelihood that both ATAD3A and -B are
Formaldehyde-cross-linked Human mtDNA Nucleoids

embedded via their single transmembrane segments in the mitochondrial inner membrane with a topology leaving the C-terminal AAA domain directed toward the matrix. The proteinase K susceptibility of the N-terminal domain suggests that it would not have the opportunity to bind mtDNA D-loops.

DISCUSSION

Formaldehyde Cross-linking as a Probe of DNA-Protein Interactions in Nucleoids—This paper provides a direct extension of our earlier work identifying a set of proteins associated with mtDNA nucleoids in cultured HeLa cells (18). The gentle conditions used to prepare native nucleoids did not permit us to determine which proteins in these complexes were in direct contact with the mtDNA and which of them might be peripheral components. We used reversible cross-linking with formaldehyde to help resolve this issue. This cross-linking agent has been used extensively in chromatin immunoprecipitation experiments in which cross-linked chromatin is sonicated and the protein of interest is immunoprecipitated along with any DNA to which it may be cross-linked, permitting the DNA to be identified by PCR (20). Our biochemical isolation procedure for mtDNA nucleoids is the inverse of a typical chromatin immunoprecipitation approach in which we take advantage of the large size and high density of DNA to analyze proteins that co-precipitate with the DNA rather than to analyze the DNA that co-precipitates with the protein. At the end of the gradient purification procedure, the cross-links are reversed by heat treatment, permitting separate analysis of the largely intact mtDNA and the liberated proteins.

It is important to recognize certain limitations of the sensitivity and specificity of the formaldehyde-cross-linking approach. Our ability to capture rather rare mitochondrial DNA metabolic enzymes and DNA-binding proteins indicates that the method is reasonably sensitive. The two transcription factors TFBM1 and -2 and mtRNA polymerase have recently been shown to occur with abundances ranging from 2.5 to 8.5 molecules per mtDNA genome (16). These proteins act by equilibrium binding of the free protein to the mtDNA. In each case, the equilibrium distribution between free and bound pools is not known. Since only a fraction of each protein may be bound to mtDNA at any time, the fact that we were able to document the presence of these relatively rare proteins in nucleoids shows that the method is relatively sensitive. However, we note that we did not detect some other proteins known to act in mtDNA metabolism, such as MTERF3 (34), the mitochondrial isoforms of DNA ligase III (35, 36), and RNase H1 (37).

It is tempting to think that additional rare nucleoid proteins could be documented by scaling up the preparation or by increasing the sensitivity of peptide analysis. However, such efforts are likely to encounter problems with the specificity of formaldehyde cross-linking, since very abundant proteins may be recovered in either native or cross-linked nucleoid preparations due largely to their high copy number relative to mtDNA rather than to a specific role in nucleoid structure or function. For example, it is possible to calculate that rat liver contains 2500–7500 copies of ANT per copy of mtDNA, since a single rat liver mitochondrion has an average content of 5.5 copies of mtDNA (11), 0.23 pg of protein (38), and 0.1–0.3 nmol of ANT/mg of protein (39). Although HeLa cell mitochondria are not nearly as rich in metabolic proteins as rat liver, a very low level of contamination or artifactual cross-linking to major proteins might provide a more robust proteomic signal than a rare authentic nucleoid protein. For this reason, we cannot conclude that ANT, for example, has a significant functional role in nucleoids. As a general rule, the likelihood that a protein found in a nucleoid preparation is an authentic nucleoid component is increased if the protein has a very low abundance.

A second mechanism that limits the specificity of formaldehyde cross-linking is that this agent is much more active as a protein-protein cross-linking reagent than it is as a DNA-protein cross-linker. Formaldehyde reacts mainly with exocyclic amino groups on A, C, and G that are engaged in base pairing in the grooves of the DNA helix and, in doing so, generates a very short arm cross-linking agent. Many DNA-interacting proteins may not bind in a mode that permits facile reaction, as reported in other systems (40, 41). The relative ease of protein-protein cross-linking with formaldehyde implies that it is quite possible that proteins identified only by this procedure might be cross-linked to other proteins that are, in turn, cross-linked to the DNA. This is a well recognized problem with the chromatin immunoprecipitation method (42). Among our results, the recovery of VDAC (porin) isoforms in cross-linked nucleoids (Table 1) probably reflects such piggy-backed cross-linking resulting from the known ability of these proteins to bind to ANT isoforms (43). This may be a common issue for many of the Class III proteins listed in Table 1. These considerations indicate that it will be difficult to document many additional novel nucleoid proteins using biochemical purification and protein sequencing as the major approach. Genetic methods will continue to be very useful for identification of additional nucleoid proteins.

A Model for the Layered Structure of the Nucleoid—With the combined results of this study and Wang and Bogenhagen (18), we have used two very different methods to study the protein composition of nucleoids. Given these methodological differences, it is remarkable that 24 of the 31 core nucleoid proteins listed in Table 1 were identified in both native and cross-linked preparations. A few proteins, such as DNA polymerase γB, TFBM1 and -2, MTERF, and mitochondrial topoisomerase I, were not documented very convincingly only on the basis of our peptide identifications. They are included in the list largely on the basis of their established roles in mtDNA transactions. Another protein that has been shown to bind mtDNA, Lon protease (25, 44, 45), was detected in cross-linked nucleoids in only one preparation, but its presence was confirmed by immunoblotting (Fig. 5).

Additional Class I proteins listed in Table 1 have roles in intermediary metabolism unrelated to DNA or RNA transactions. The identification of subunits of hydroxyacyl dehydrogenase in nucleoids has been documented previously by us and others (18, 21). Both SHMT (73) and HADHB (46) have the ability to bind polynucleotides in vitro, but neither protein has as yet been shown to influence mitochondrial DNA or RNA metabolism. The E2 subunit of branched chain ketoacid dehydrogenase is another metabolic protein that has been observed
repeatedly in native nucleoid preparations from Xenopus oocytes (22) and cultured human cells (18). Further work is necessary to determine whether any of these may be bifunctional proteins with a role in nucleoid maintenance in addition to their metabolic activities. This criterion has been met for yeast aconitase (47, 48), but this abundant protein has not been observed in HeLa nucleoids with either native or cross-linking approaches. A second yeast metabolic protein with a role in mtDNA maintenance, with a role in mtDNA maintenance, Ilv5 (49, 50), does not have a recognized mammalian ortholog.

The nucleoid core is clearly a biosynthetic center for mtDNA replication and transcription (Fig. 8). An important question regarding mtDNA inheritance is whether a selective subset of nucleoids might engage in high rates of replication, whereas other nucleoids may be relatively inactive. In an extreme model, mtDNA heteroplasmy might shift rapidly if replication of mtDNA were restricted to only a few nucleoids. An increased replication rate for a subset of mtDNA genomes has been proposed for some cell types (51) but has not been supported by studies in other cells (52). Iborra et al. (9) addressed this question by determining the fraction of nucleoids that were pulse-labeled with the DNA precursor bromodeoxyuridine. In a 3-h labeling interval in cultured cells, they found that essentially all nucleoids contained replicating mtDNA molecules, consistent with the random selection of molecules for replication throughout the cell cycle (53). Similar experiments using bromouracil to label nascent transcripts and to permit detection with anti-bromouracil RNA antibodies were interpreted as showing that newly synthesized mtRNA remained in the vicinity of nucleoids for ~40 min. This is a surprisingly long time, considering that most newly synthesized mtRNAs have half lives of only 1–2 h (54, 55) and that a substantial fraction of newly synthesized mtRNA is rRNA. The time-dependent loss of nucleoid-associated mtRNA may in part reflect RNA degradation rather than transport away from the nucleoid. Other mitochondrial matrix components, such as mitochondrially targeted GFP, have been found to move rapidly within mitochondria at close to diffusion-limited rates (56). Therefore, the diffusion of free macromolecules is not strictly impeded within the mitochondrial matrix, so that the persistence of newly synthesized RNA in the vicinity of nucleoids may indicate that it is not free to diffuse.

The relationship of nucleoids to the mitochondrial translation apparatus is especially interesting. It is possible that either ribosome assembly or translation may be more active in the immediate vicinity of nucleoids. Recently, mitochondrial ribosomal protein L12 has been reported to bind mtRNA polymerase and to stimulate transcription in a partially purified in vitro system (57). It has not yet been determined whether a pool of free MRPL12 exists independently of mitochondrial ribosomes. Interestingly, we did not obtain peptide hits on MRPL12, although we did find peptides from 15 other mitochondrial ribosomal proteins as well as EFTU, EFG, MRRF, and four mitochondrial tRNA synthetases (supplemental Table 2). We cannot rule out the possibility that MRPL12 was missed in our experiments for technical reasons.

As nascent polycistronic RNAs are threaded away from the nucleoid core, these RNAs may be processed and translated in the peripheral zone surrounding the core, as noted above and in Fig. 8. Some chaperones, such as Hsp70 and LRPPRC, were found in cross-linked nucleoids. LRPPRC is thought to function as a chaperone involved in the assembly of complex IV (58, 59), but it has also been shown to have nucleic acid binding ability (60). Only a small fraction of LRPPRC is cross-linked to mtDNA (Fig. 5). It is remarkable that several chaperone and AAA proteins represented in native nucleoids were not observed to cross-link to mtDNA, including Hsp60 and the complex of prohibitin 1 and 2. We consider that the recovery of these Class II proteins in native nucleoids is an indication that they may be associated with other nucleoid components as nearest neighbors.

ATAD3 is an example of a protein found in association with nucleoids but not in direct contact with mtDNA. We studied ATAD3 in greater detail than other Class II proteins, since Holt’s laboratory (21, 61) suggested that ATAD3 interacts directly with the mtDNA through its N-terminal domain. Their model is ruled out by our demonstration that this domain is exposed to protease outside the mitochondrial inner membrane. It will be quite interesting to determine whether ATAD3 does employ its AAA domain in an essential role to support mtDNA maintenance within nucleoids.

Do Peripheral Nucleoid Proteins Participate in Coupled Transcription, Translation, and Complex Assembly?—Previous studies have suggested that the mtDNA nucleoid may represent a general center for mitochondrial biogenesis (62). All of the proteins encoded by mtDNA are hydrophobic membrane-associated subunits of respiratory complexes I, III, IV, and V. The association of both mtDNA nucleoids and mitochondrial ribosomes with the inner membrane (63) may reflect coordination between the synthesis of mitochondrially encoded proteins and their assembly into complexes. The notion that respiratory complexes may be assembled on the periphery of nucleoids may...
help explain why subunits of complex I, which contains more mtDNA-encoded subunits than any other complex, are frequently found in this peripheral zone despite the fact that this is the least abundant respiratory complex (38, 64). In bacteria, the coupling between transcription, translation, and membrane insertion of nascent proteins has been given the name translocation (10, 65). Considerable evidence has been accumulated to show that this coupling may occur in yeast mitochondria (66). The recent claim that MRPL12 may be a transcription-translation coupling factor in human mitochondria (57) could represent a feature of this model. As noted above, we did not observe any peptides derived from MRPL12 in our nucleoid fractions, although we did see peptides from several other ribosomal proteins and translation factors, representing both the mitochondrial and cytoplasmic translation apparatus (Supplemental Table 2). The presence of mitochondrial ribosomal proteins in the nucleoid fraction may be significant, since HeLa cells are thought to contain only 34,000 mitochondrial ribosomes, about 4 ribosomes/mtDNA (24). It is tempting to speculate that these observations support the model advocated by Iborra et al. (9), in which nascent mitochondrial proteins synthesized on cytoplasmic ribosomes are imported into mitochondria through import channels located in the vicinity of nucleoids as a means to coordinate respiratory complex assembly. This translocation model, which couples translation to nucleoid structure and the mitochondrial inner membrane, may help explain why it has so far proven impossible to reconstitute an active mitochondrial translation system. Clearly, much more work is necessary to explore the potential role of mtDNA nucleoids and their nearest neighbors as biosynthetic centers within mitochondria and to test the model of a layered nucleoid structure shown in Fig. 8.

Acknowledgments—We thank Dr. Weiping Xie (SUNY Stony Brook Proteomics) for assistance with mass spectrometry. We thank several colleagues mentioned throughout for gifts of antibodies used in this work.

REFERENCES

1. Chen, X. J., and Butow, R. A. (2005) Nat. Rev. Genet. 6, 815–825
2. Malka, F., Lombes, A., and Rojo, M. (2006) Trends Cell Biol. 17, 376–382
3. Kucej, M., and Butow, R. A. (2007) Mol. Cell Proteomics 6, 1205–1216
4. Morrissey, J. (1981) Anal. Biochem. 117, 307–310
5. Attardi, G., and Schatz, G. (1988) Annu. Rev. Cell Biol. 4, 289–333
6. Lu, B., Yadav, S., Shah, P. G., Liu, T., Tian, B., Pusztas, S., Villaluna, N., Kutejova, E., Newlon, C. S., Herzog, H. and Suzuki, C. K. (2007) J. Biol. Chem. 282, 17363–17374
7. Cheng, X., Kanki, T., Fukushima, O., Ohgaki, K., Takeya, R., Aoki, Y., Hamasaki, N., and Kang, D. (2005) J. Biol. Chem. (Tokyo) 138, 673–678
8. Liu, L., Rodriguez-Belmonte, E. M., Mazloum, N., Xie, B., and Lee, M. Y. W. T. (2003) J. Biol. Chem. 278, 10041–10047
9. Xie, B., Li, H., Wang, Q., Xie, S., Rahmei, A., Dai, W., and Lee, M. Y. W. T. (2005) J. Biol. Chem. 280, 22875–22884
10. Arakaki, N., Nishihama, T., Kohna, O., Owaiki, H., Kuramoto, Y., Abe, R., Kita, T., Suena, M., Himeda, T., Kuwajima, M., Shibata, H., and Higuti, T. (2006) Biochim. Biophys. Acta 1760, 1364–1372
11. Da Cruz, S., Xenarios, I., Langridge, J., Vilbois, F., Parone, P. A., and Martinou, J.-C. (2003) J. Biol. Chem. 278, 41566–41571
12. Schaffrik, M., Mack, B., Matthias, C., Rauch, J., and Gires, O. (2006) Mol. Life Sci. 63, 2162–2174
13. Lee, D. G., and Bell, S. P. (2000) Curr. Biol. 12, 280–285
14. Neuwald, A. F. (2005) Nucleic Acids Res. 33, 3614–3628
15. Park, C. B., Asin-Cayuela, J., Camara, Y., Shi, Y., Pellegrini, M., Gaspari, M., Wilbom, R., Hultenby, K., Erdjument-Bromage, H., Tempst, P., Falkenberg, M., Gustafsson, C. M., and Larsson, N.-G. (2007) Cell 130, 273–285
16. Perez-Jannotti, R. M., Klein, S. M., and Bogenhagen, D. F. (2001) J. Biol. Chem. 276, 48978–48987
17. Lakshmipathy, U., and Campbell, C. (1999) Mol. Cell. Biol. 19, 3869–3876
18. Ferri, S. M., Frolova, E. G., Feng, C., Grinberg, A., Love, P. E., and Crouch, R. J. (2003) Mol Cell 11, 807–815
19. Schwerzmann, K., Cruz-Orive, L., Eggman, R., Sanger, A., and Weibel, E. (1986) J. Cell Biol. 102, 97–103
20. Tzagoloff, A. (1982) Mitochondria, p. 211, Plenum Publishing Corp., New York
21. Solomon, M. J., and Varshavsky, A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6470–6474
22. Kurdistani, S. K., and Grunstein, M. (2003) Methods 31, 90–95
23. Wells, J., and Farnham, P. J. (2002) Methods 26, 48–56
24. Crompton, M. (1999) Biochem. J. 341, 233–249
25. Fu, G. K., and Markovitz, D. M. (1998) Biochemistry 37, 1905–1909
26. Liu, T., Lu, B., Lee, L., Onodriovcica, G., Kutejova, E., and Suzuki, C. K. (2004) J. Biol. Chem. 279, 13902–13910
27. Adams, D. J., Beveridge, D. J., van der Weyden, L., Mangs, H., Leedman, P. J., and Morris, B. J. (2003) J. Biol. Chem. 278, 44894–44903
28. Chen, X. J., Wang, X., Kaufman, B. A., and Butow, R. A. (2005) Science 307, 714–717
29. Chen, X. J., Wang, X., Kaufman, B. A., and Butow, R. A. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 13738–13743
30. Zelenaya-Troitskaya, O., Perlman, P. S., and Butow, R. A. (1995) EMBO J. 14, 3268–3276
31. Bateman, J. M., Iacovino, M., Perlman, P. S., and Butow, R. A. (2002) J. Biol. Chem. 277, 47946–47953
32. Davis, A., and Clayton, D. (1996) J. Cell Biol. 135, 883–893
52. Magnusson, J., Orth, M., Lestienne, P., and Taanman, J.-W. (2003) *Exp. Cell Res.* **289**, 133–142
53. Bogenhagen, D., and Clayton, D. (1977) *Cell* **11**, 719–727
54. Gelfand, R., and Attardi, G. (1981) *Mol. Cell Biol.* **1**, 497–511
55. Cantatore, P., Flagella, Z., Fracasso, F., Lezza, A. M. S., Gadaleta, M. N., and de Montalvo, A. (1987) *FEBS Lett.* **213**, 144–148
56. Partikian, A., Olveczky, B., Swaminathan, R., Li, Y., and Verkman, A. S. (1998) *J. Cell Biol.* **140**, 821–829
57. Wang, Z., Cotney, J., and Shadel, G. S. (2007) *J. Biol. Chem.* **282**, 12610–12618
58. Partikian, A., Olveczky, B., Swaminathan, R., Li, Y., and Verkman, A. S. (1998) *J. Cell Biol.* **140**, 821–829
59. Mootha, V. K., Lepage, P., Miller, K., Bunkenborg, J., Hjerrild, M., Delmonte, T., Villeneuve, A., Sladek, R., Xu, F., Mitchell, G. A., Morin, C., Mann, M., Hudson, T. J., Robinson, B., Rioux, J. D., and Lander, E. S. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 605–610
59. Xu, F., Morin, C., Mitchell, G., Ackerley, C., and Robinson, B. H. (2004) *Biochem. J.* **382**, 331–336
60. Mootha, V. K., Lepage, P., Miller, K., Bunkenborg, J., Reich, M., Hjerrild, M., Delmonte, T., Villeneuve, A., Sladek, R., Xu, F., Mitchell, G. A., Morin, C., Mann, M., Hudson, T. J., Robinson, B., Rioux, J. D., and Lander, E. S. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 605–610
61. Holt, I. J., He, J., Mao, C.-C., Kirk-up, J. D. B., Martinsson, P., Sembongi, H., Reyes, A., and Spellbrink, J. N. (2007) *Mitochondrion* **7**, 311–321
62. Capaldi, R. A., Aggeler, R., Gilkerson, R., Hanson, G., Knowles, M., Marcus, A., Marginientu, D., Marusich, M., Murray, I., Oglesbee, D., Remington, S. J., and Rossignol, R. (2002) *Biochim. Biophys. Acta* **1555**, 192–195
63. Liu, M., and Spremulli, L. (2000) *J. Biol. Chem.* **275**, 29400–29406
64. Hatefi, Y. (1985) *Annu. Rev. Biochem.* **54**, 1015–1069
65. Lynch, A. S., and Wang, J. C. (1993) *J. Bacteriol.* **175**, 1645–1655
66. Bryan, A. C., Rodeheffer, M. S., Wearn, C. M., and Shadel, G. S. (2002) *Genetics* **160**, 75–82
67. Falkenberg, M., Gaspari, M., Rantanen, A., Trifunovic, A., Larsson, N.-G., and Gustafsson, C. M. (2002) *Nat. Genet.* **31**, 289–294
68. McCulloch, V., Seidel-Rogol, B. L., and Shadel, G. S. (2002) *Mol. Cell Biol.* **22**, 1116–1125
69. Fernandez-Silva, P., Martinez-Azorin, F., Micol, V., and Attardi, G. (1997) *EMBO J.* **16**, 1066–1079
70. Garrido, N., Griparic, L., Jokitalo, E., Wartiovaara, J., van der Bliek, A. M., and Spellbrink, J. N. (2003) *Mol. Biol. Cell* **14**, 1583–1596
71. Valgardsdottir, R., Brede, G., Eide, L. G., Frengen, E., and Prydz, H. (2001) *J. Biol. Chem.* **276**, 32056–32063
72. Zhang, H., Barcelo, J. M., Lee, B., Kohlhagen, G., Zimonjic, D. B., Popescu, N. C., and Pommier, Y. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 10608–10613
73. Whitford, T. A. (1993) *Isolation and cDNA Cloning of a Xenopus Mitochondrial Serine Hydroxymethyltransferase Which Binds to ssDNA*. Ph.D. thesis, State University of New York at Stony Brook