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Review

Mitochondrial DNA nucleoid structure ☆

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ABSTRACT

Eukaryotic cells are characterized by their content of intracellular membrane-bound organelles, including mitochondria as well as nuclei. These two DNA-containing compartments employ two distinct strategies for storage and readout of genetic information. The diploid nuclei of human cells contain about 6 billion base pairs encoding about 25,000 protein-encoding genes, averaging 120 kB/gene, packaged in chromatin arranged as a regular nucleosomal array. In contrast, human cells contain hundreds to thousands of copies of a ca.16 kB mtDNA genome tightly packed with 13 protein-coding genes along with rRNA and tRNA genes required for their expression. The mtDNAs are dispersed throughout the mitochondrial network as histone-free nucleoids containing single copies or small clusters of genomes. This review will summarize recent advances in understanding the microscopic structure and molecular composition of mtDNA nucleoids in higher eukaryotes. This article is part of a Special Issue entitled: Mitochondrial Gene Expression.

1. Introduction

45 years ago the Nass [1] and Borst [2] labs independently used the newly-developed Kleinschmidt electron microscopic (EM) spreading technique to document the presence of 5 μm circular DNA genomes in mitochondria isolated from mouse and chick cells. The circularity of the DNA and the lack of apparent abundant DNA binding proteins as visualized with this method reinforced the concept that mitochondria evolved from a prokaryotic ancestor. Later, similar EM observations of the folded chromosome of *E. coli* [3] also revealed a lack of the sort of chromatin structure characteristic of eukaryotic nuclear DNA. While both bacterial DNA and mtDNA lack histones responsible for the regular packaging of nuclear DNA into nucleosomes [4], these genomes are in fact extensively coated with proteins that package the DNA in compact nucleoids, as will be discussed below.

We now know that mtDNA in higher eukaryotes encodes a highly conserved subset of 13 of the ~80 structural subunits of the electron transport chain. These polypeptides are all intrinsic membrane proteins that help to anchor the complexes involved in proton translocation through the mitochondrial inner membrane [5]. The mtDNA encodes none of the polypeptides required for replication, transcription and repair of mtDNA or for processing and translation of the mtRNA transcripts. One can make a back-of-the-envelope estimate that around 200–300 nuclear gene products must be conserved to replicate and express the 13 proteins encoded in mtDNA. This represents close to 20% of the total number of nuclear genes encoding proteins that function in mitochondria, a substantial investment to maintain only 13 genes within mitochondria. The jobs of all of these mtDNA-maintenance genes could be made redundant if only these 13 genes could be moved to the nucleus. This is only one side of the cost-benefit analysis of the mitochondrial ledger. Since these mtDNA-encoded genes have been retained through evolution, it is reasonable to assume that this bi-genomic organization offers substantial compensating benefits, probably in the form of greatly enhanced energy production, as described in a recent review [6]. Elucidating the overall organization of mtDNA is a prerequisite to developing a comprehensive understanding of the coordinate expression of mtDNA- and nuclear-encoded genes.

In addition to this basic research interest in mtDNA organization, this issue has clinical implications. The way that mtDNA is packaged is widely considered to affect the incidence and severity of mtDNA-related diseases [7]. Recent deep sequencing studies have revealed that tissues in normal individuals harbor a measureable incidence of mutant mtDNA genomes [8]. We still know little of the processes that govern fluctuation in the frequency of mutant mtDNA genomes to permit initially rare mutations to predominate within the mtDNA population. Due to the high copy number of mtDNA, mutations in this genome do not exert a great influence on phenotype until the proportion of mutant mtDNA genomes exceeds a critical threshold, often estimated as approximately 75% of total molecules [9]. This threshold reflects the ability of the remaining wild-type mtDNAs to complement the genetic defect in mutant molecules. The degree to which this complementation may depend on the organization of mtDNA molecules as clusters within nucleoids remains uncertain. Clinicians have frequently observed that a mtDNA mutation in an affected proband is often represented at a lower frequency in maternal tissues [10]. There is general agreement that there is a severe reduction in the number of heritable units of mtDNA during oocyte

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development, the so-called bottleneck effect, resulting in wide fluctuation in mutation frequency. Whether the unit of mtDNA inheritance is a single mtDNA molecule or a cluster of mtDNAs in a single nucleoid remains an important unresolved question [11, 12].

2. Microscopic observations of mtDNA nucleoids

The small size of the mtDNA genome and the dynamic mobility and fission–fusion cycles of mitochondria have complicated observations of nucleoid size and dynamics in cells. Satoh and Kuriowa [13] provided one of the earliest characterizations of nucleoids in mammalian cells using DAPI as a fluorescent DNA-binding compound. These workers found that prolonged incubation of human A2780 cells with DAPI permitted visualization of multiple independent nucleoids in some organelles, with an average of 3.2 nucleoids per mitochondrion and a calculated average of 4.6 mtDNA molecules per organelle. In this study, as in several subsequent papers, the apparent mtDNA copy number within an individual nucleoid was calculated using the assumption that fluorescence intensity should increase in a quantal manner in proportion to the discrete number of mtDNA genomes. Bereiter-Hahn and Voth [14] applied this principle in their studies of picogreen stained nucleoids in a variety of cell types. They observed a distribution in fluorescence intensity consistent with a content of 1–6 mtDNA molecules per nucleoid in mouse 3T3 cells. However, this quantitative analysis was undoubtedly confounded by the overall weak staining of nucleoids and the inability of confocal microscopy to eliminate weak signals originating above or below the focal plane. Thus, the observed nucleoid fluorescence intensity was more of a continuous distribution with only a suggestion of peaks representing integral numbers of mtDNA molecules in each nucleoid. They also reported that a substantial fraction, around 30%, of mitochondria lacked nucleoids. Since an organelle that lacks mtDNA for a prolonged period of time will be unable to generate new respiratory complexes, they suggested that mitochondrial fusion events that they carefully documented could provide RNA or protein gene products by complementation. Other reports of nucleoid mtDNA content have circumvented the problem inherent in measurements of fluorescence intensity by calculating the average mtDNA copy number by dividing the total cell mtDNA content by the number of nucleoids [15–17]. The resulting apparent mtDNA content calculated with this method has ranged from about 2.4 to 7.8 per nucleoid, depending on the cell type and the detailed methods employed, as shown in Table 1. These more recent studies have often relied on use of antibodies directed against DNA or the abundant mtDNA binding protein TFAM to detect nucleoids in preference to DNA-binding fluorescent dyes. Table 1 also illustrates the great variability in mtDNA content in different reports, which introduces considerable uncertainty into estimates of nucleoid mtDNA copy number.

Determining the number of mtDNA nucleoids per cell is also surprisingly difficult when the method relies on detection of nucleoids using a wide-field or confocal microscope. These instruments have a resolution within the focal plane that is limited to about 250 nm, and a considerably poorer axial resolution of about 600 nm. Thus, closely spaced nucleoids can be mistaken for single, larger particles. In addition, small nucleoids are more easily overlooked than larger ones. A recent publication using the super-resolution method of STED (stimulated emission depletion) microscopy found that this higher-resolution method revealed 1.6 to 1.9 times as many nucleoids as confocal microscopy [18]. In part, this reflects the ability of this imaging method to resolve larger nucleoid structures into small clusters containing a few distinguishable DNA centers. Kukat et al. [18] calculated that primary human fibroblasts contained 2721 ± 156 mtDNA molecules packaged in 1883 ± 106 nucleoids, for an average mtDNA copy number of 1.45/nucleoid. Interestingly, the average nucleoid mtDNA copy number reported by Kukat et al. [18] was essentially identical to the estimate made by Satoh and Kuriowa [13] 20 years earlier, although this older study reported a much lower mtDNA copy number of only 500 molecules per cell. An independent super-resolution study of nucleoids in mouse 3T3 cells using photoactivated light microscopy (PALM) estimated an average of 3 mtDNA molecules per nucleoid [17]. Both the STED and PALM imaging studies reported that nucleoids had a somewhat ovoid shape with an average diameter of about 100 nm, approximately one-third of the diameter of a mitochondrial tubule. This involves a severe compaction of DNA, as this diameter is equivalent to the length of a 300 bp DNA segment. Brown et al. [17] calculated that nucleoids in 3T3 cells occupied an average volume of 830,000 nm^3, so that three mtDNA molecules would occupy about 6.3% of the available space. This represents a packing density similar to that of the DNA in a papillomavirus capsid [19]. As shown in Table 2, mtDNA appears to be more tightly packed than either bacterial nucleoids or nuclear chromatin, where the volume occupied is roughly in proportional to the DNA content. Together these super-resolution imaging studies suggest that the mtDNA content of a typical nucleoid may be smaller than the earlier estimates of Iborra et al. [15] and Legros et al. [16]. It is not clear how much of this apparent discrepancy is due to the use of different methods or is derived from the use of different cell lines. It is also important to consider that additional work is necessary to determine the size and mtDNA contents of cells in tissues in vivo. The possibility remains that there may be important differences in mtDNA nucleoid structure in cells in animal tissues.

Other methods that do not rely on microscopy have also provided estimates of the mtDNA content of nucleoids. Cavelier et al. [20] used flow cytometry of isolated mitochondrial particles to measure the mtDNA of individual mitochondrial particles, resulting in a mean content of 2.0 molecules per nucleoid (ranges 1–11). A particularly interesting aspect of this study was that an additional analysis of particles from heteroplasmic cells revealed that individual particles did not commonly contain both wild-type and mutant mtDNA genomes. A conceptually similar particle analysis using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) also revealed an average content of 6.2 mtDNA molecules per particle [21, 22] although particles with a single mtDNA molecule were the most numerous. These two studies share the limitation that it may be technically difficult to assure that the isolated mitochondrial particles contained single nucleoids with no artifactual aggregation.

Microscopy has also been used to study the kinetics of replication of mtDNA in nucleoids by following the incorporation of the thymidine analog bromodeoxyuridine (BrdU) into mtDNA using indirect immunofluorescence with anti-BrdU antibodies. Biochemical pulse labeling experiments using ^{3}H-thymidine in mouse L and human HeLa cells did not detect significant incorporation of the precursor

Table 1
Measurements of the absolute mtDNA copy number per cell and per nucleoid.

MtDNA/cell	Species	Cell	MtDNA/nucleoid	Reference
8800	Human	HeLa		[91]
800	Human	Fibro		[92]
500	Human	A2780	1.4	[13]
7200	Human	HeLa		[93]
7200	Human	KB		[93]
2933	Human	Fibroblast		[93]
5200	Human	fibroblast		[93]
1632	Human	Male Fibro	2.3	[16]
1961	Human	Fem Fibro	2.4	[16]
2637	Human	HeLa	5.7	[16]
4126	Human	143B	7.5	[16]
3500	Human	ECV304	7.3	[15]
2721	Human	Fibroblast	1.4	[18]
12,900	Human	Fibroblast		[94]
821	Mouse	3T3	3	[17]
1100	Mouse	L		[91]
720	mouse	LA9		[92]

Table 2
Comparison of DNA packaging densities.

Source	Genome size, bp	Vol, nm^3	ρ_{PACK}	Notes
mtDNA nucleoid	$3\times16{,}295$	8.3×10^5	0.063	[17][a]
Papilloma virus	7.9×10^3	1.13×10^5	0.07	[19][b]
E. coli nucleoid	4.7×10^6	5×10^8	0.01	[95][c]
Human nucleus	6×10^9	6×10^{11}	0.01	[96][d]

[a] Assuming 3 mtDNA molecules/nucleoid.
[b] ρ_{PACK} for DNA cylinder with 1 nm radius $= 0.34\pi N_{bp}$/volume.
[c] Nucleoid volume estimated as 0.5 μm^3.
[d] Assuming a lower-range estimate of nuclear volume of 600 μm^3.

into parental DNA strands as might be expected if repair of endogenous DNA damage made a substantial contribution to precursor incorporation [23, 24]. Thus, labeling with BrdU may be expected to indicate mainly semi-conservative DNA replication, with a potential minor contribution from turnover of the 7S DNA strand. Bogenhagen and Clayton [25] found that a three-hour labeling with BrdU resulted in replication of 10–12% of pre-labeled mtDNA molecules in mouse L cells, consistent with random selection of mtDNA molecules for replication. Iborra et al. [15] performed a similar BrdU labeling experiment to determine the fraction of nucleoids labeled with BrdU using immunofluorescence to detect incorporation into individual nucleoids. They reported that incubation for 1 h with BrdU resulted in labeling of 50% of nucleoids, while incubation for 3 h or longer labeled 100% of nucleoids. Assuming that only a full round of replication would lead to sufficient BrdU incorporation to be observed with this assay, such a high labeling index could only be observed if individual nucleoids contain multiple mtDNA molecules. They concluded that this result ruled out the model that certain nucleoids were preferentially engaged in replication, because such a model would require labeling of only a subset of nucleoids. The observation in their experiments that such a high percentage of nucleoids contained replicating molecules after such a short pulse interval is consistent with a high copy number of mtDNA molecules per nucleoid, which they reported to be 7.3 mtDNA molecules per nucleoid in the ECV304 cells used in their study. BrdU labeling was also employed in the super-resolution imaging study by Kukat et al. [18] using cells reported to have only one mtDNA molecule in most nucleoids. However, these workers did not report the fraction of nucleoids labeled during a one-hour incubation with BrdU, although the known kinetics of mtDNA replication would suggest that only a minor subset of nucleoids should be labeled.

BrdU can also be used to monitor mtDNA replication in nucleoids in vivo by injection of the analog, as has been done routinely to monitor nuclear DNA replication in tissues. When Wai et al. [26] performed this sort of BrdU labeling to follow incorporation of the analog into replicating mtDNA in developing oocytes, they found that a 2 h or 24 h incubation resulted in labeling of only a subset of mtDNA nucleoids. They interpreted this result as evidence of a preferential replication of a subset of mtDNA molecules. However, if most nucleoids contain only a single mtDNA molecule, as suggested by Kukat et al. [18], the results obtained by Wai et al. [26] are entirely consistent with random selection of molecules for replication. This limited series of BrdU labeling experiments illustrates the great potential of this technique to explore localized, nucleoid-based mtDNA replication kinetics in cells and tissues.

3. Does nucleoid size matter?

As detailed above, a number of publications have gone to great lengths to quantify the copy number of mtDNA in nucleoids. But is this really an important parameter? The reason this has become a focus of attention is due to the interest in improving our understanding of the inheritance of mutant mtDNA genomes in human disease. The high polyploidy of mtDNA provides protection against deleterious mutations as one remaining wild-type mtDNA genome may complement a genetic defect in neighboring genomes. If several mtDNAs are clustered in a nucleoid, it is easy to see how this complementation may occur. However, if each mtDNA is often packaged individually, genetic complementation must depend on a combination of diffusion of gene products within one mitochondrion or fusion of mitochondria to permit exchange of their contents. The ability of mitochondrial gene products to diffuse within the organelle has received little attention. Previous studies have shown using fluorescence recovery after photobleaching (FRAP) that soluble GFP-tagged proteins in the mitochondrial matrix are relatively free to diffuse [27], but other studies have emphasized that the cristae structure of mitochondria provides a considerable barrier to free diffusion of matrix proteins [28, 29]. Diffusion of the membrane-bound mtDNA-encoded subunits of large respiratory complexes is likely to be greatly hindered by the high density of proteins in the mitochondrial inner membrane. Partikian et al. [27] showed that a GFP-tagged membrane protein did not diffuse freely in the mitochondrial inner membrane. These considerations suggest that effective genetic complementation of mitochondrial gene defects will depend more on diffusion of RNAs in the matrix, rather than of proteins within the membrane. RNA binding proteins, including LRPPRC and SLIRP [30] and PPR-domain proteins [31, 32] may stabilize mtRNAs to enhance diffusion. The exchange of such RNA-protein complexes between mitochondria may be an important consequence of the frequent mitochondrial fusion events observed in cells. The importance of fusion events for mitochondrial maintenance has been demonstrated with direct genetic analysis [33, 34] as well as mathematical modeling studies [35, 36].

4. mtDNA nucleoid protein composition

MtDNA is not packaged in a regular nucleosomal array characteristic of nuclear DNA. Nuclear chromatin consists of two distinct protein fractions, a salt-resistant histone octamer core in which the 8 histones wrap about 140 base pairs of DNA in a repeating structure that has a roughly equal mass of protein and DNA [4], and a much more diverse set of non-histone nuclear proteins. This latter class of proteins is generally dissociated from chromatin by washing with moderately high salt concentrations, such as the 0.42 M salt extraction used by Dignam et al. [37] to generate a soluble fraction of proteins enriched in factors involved in replication, transcription and RNA processing. The only protein known to bind mtDNA in a salt-resistant manner is mtSSB. mtSSB has a primary sequence and structure [38, 39] similar to bacterial SSB and has been shown to coat the extensive ssDNA regions of mtDNA replication intermediates [40]. In an early study, Albring et al. [41] noted the tight binding of a large complex to the control region of mtDNA, but the identity of proteins in this complex remains unknown.

The best-characterized and most abundant protein binding duplex mtDNA is the HMG-box protein TFAM [42]. TFAM binds and wraps the duplex mtDNA [43, 44] altering the writhe of the DNA helix, and is probably the major factor responsible for the remarkably tight packaging of mtDNA within nucleoids as noted above [45]. TFAM binds mtDNA with some specificity allowing the interaction to be detected by DNase I footprinting [43, 44] in the vicinity of transcription promoters, where it markedly stimulates transcription initiation. Whether the protein has other similarly tight binding sites elsewhere in the mtDNA genome has not been determined. A recent study of the accessibility of mtDNA to nucleases found evidence of extensive protein binding in vivo [46], although mtDNA sequences were generally accessible to the nuclease probe. The highest binding site occupancy observed in this study appeared to be the binding site for mTERF1. TFAM is related to the yeast HMG-box protein abf2p, which does not stimulate transcription but has been reported to be sufficiently abundant to coat the mtDNA [47]. A number of laboratories have

quantified the stoichiometry of TFAM binding to mtDNA, as reported in Table 3. The Kang and Larsson laboratories reported a very high TFAM copy number of about 1000 proteins/mtDNA genome or higher. Association of such a large number of TFAM molecules with mtDNA would require one protein bound per 16 bp of DNA with a total TFAM protein mass 2.4-fold greater than the mass of mtDNA. This very dense TFAM binding has the potential to block access of other DNA binding proteins, including transcription factors. Exactly how many TFAM molecules are necessary to saturate the mtDNA genome is not known. The first crystal structures of TFAM bound to a specific mtDNA sequence have shown that a single copy of TFAM occupies 22 bp of the heavy-strand mtDNA promoter [48, 49]. Thus, a stoichiometry of 1000 or more TFAM molecules per mtDNA may be excessive, unless TFAM binds with an altered mode to different sequences. Cotney et al. [50] found a vastly lower TFAM copy number of only 50 molecules of TFAM/mtDNA in HeLa cells. The basis for this 20-fold discrepancy is unknown and should be resolved. Our laboratory has not studied the quantity of TFAM in human mitochondria but did explore this topic in *Xenopus* oocytes, where mtDNA replication and transcription are known to be under strict developmental control. We found that immature stages I–II oocytes that are still actively engaged in mtDNA replication and transcription contain approximately 400–600 molecules of TFAM per mtDNA genome. Mature stages V–VI oocytes contain well over 1000 molecules of TFAM per mtDNA, potentially a sufficient amount to contribute to the developmental down-regulation of mitochondrial transcription in mature oocytes.

The ability of TFAM to wrap DNA resembles that of the histone-like proteins HU and INT in bacteria. Expression of yeast abf2 in *E. coli* can even compensate for a deficiency in HU protein [51]. Given this functional conservation, it is interesting to compare the simple protein composition of mtDNA nucleoids with the more complex and variable composition of their bacterial counterparts. Of course, the bacterial nucleoid is much larger, occupying a substantial fraction of the volume of the bacterial cell, but it is interesting to note that it is compartmentalized into independent ~10 kbp supercoiled domains of a size comparable to a mtDNA genome [52]. A recent super-resolution imaging study has shown that HU is widely distributed throughout the nucleoid, while other proteins, such as H-NS have a more confined localization [53]. Purification of nucleoids from *E. coli* has revealed a set of 12 major DNA binding proteins, many of which vary in abundance in logarithmically growing and stationary-phase cells [52, 54, 55]. Fig. 1 illustrates how some of the most abundant DNA binding proteins in logarithmically growing cells (HU, INT, Fis and Hfq) are effectively replaced by CpbA, Dfq and other proteins as cells enter stationary phase. During this adaptation, the total number of binding proteins per cell appears to remain relatively steady, with about 300,000 copies of proteins complexed with the 4.6 megabase *E. coli* genome. Interestingly, this translates to one protein bound per 15 base pairs, similar to the density of TFAM binding mtDNA, although other sources cite a significantly lower protein content for bacterial nucleoids and point out that very high protein contents may be deleterious [56]. One striking difference between mtDNA and bacterial nucleoids is that, to our knowledge, higher eukaryotic cells employ TFAM as the single protein that substantially coats the mtDNA. This arrangement may reflect the smaller size of the mtDNA genome and limited genetic rearrangements to which it is exposed. It may also reflect the rather consistent environment of mtDNA in higher eukaryotic cells provided by normal physiological regulations. Interestingly, in the yeast *S. cerevisiae*, two other proteins have been found to be capable of complementing a deficiency of abf2p. These are Ilv5p [57] and Aco1p [58]. These proteins have not been found to date in mammalian nucleoids. Indeed, mammals cannot synthesize isoleucine de novo and lack an Ilv5 gene. It may be the case that these two yeast proteins play roles in binding and regulating mtDNA under relatively extreme conditions of nutritional adaptation or sporulation in yeast. To date, no proteins have been shown to be capable of compensating for a deficiency in TFAM in mammals. Knockout of the mouse TFAM gene results in embryonic lethality [59] and tissue-specific knockouts of the TFAM gene have been used to generate several models of tissue-specific cell dysfunction [60–62].

The problem inherent in coating mtDNA with TFAM is that this binding should not preclude access of transcription, replication and repair factors to the mtDNA. Our laboratory has conducted experiments to identify proteins associated with nucleoids in HeLa cells using two very different methods. In one study [63], we cross-linked proteins to mtDNA in freshly isolated mitochondria using formaldehyde, then quenched the aldehyde with excess glycine, denatured proteins with SDS and purified the DNA protein complexes through sedimentation followed by equilibrium centrifugation in buoyant CsCl gradients. All proteins not covalently cross-linked to mtDNA should be stripped off by this harsh processing. In another approach [64], we lysed freshly isolated mitochondria with the non-ionic detergent Triton X-100, sedimented the large protein–DNA complexes away from excess free proteins by glycerol gradient centrifugation in the presence of TX-100 and then purified complexes by immunoaffinity purification using antibodies directed against TFAM or mtSSB. Together these two approaches identified a series of proteins previously known to be engaged in mtDNA transactions, including DNA pol γ, mtRNA polymerase, TFB1M, TFB2M, mTERF1, topoisomerase I and the replicative DNA helicase C10orf2 (Twinkle) as well as TFAM and mtSSB, which were the "baits" in the affinity purification procedure. TFAM was also recovered in nucleoids purified by affinity of the mtDNA for immobilized HU protein [65], although the less abundant DNA binding proteins were not detected in this study. Other proteins known to influence mtDNA transcription, such as mTERF2 and mTERF3 have been detected bound to mtDNA in separate studies [66, 67]. Another protein found very consistently in nucleoid preparations is serine hydroxymethyltransferase (SHMT2), which we identified as a protein with high affinity for ssDNA (Whitford and Bogenhagen, unpublished). This is a very abundant

Table 3
Measurements of the TFAM/mtDNA copy number in mammalian cells.

Species	Cell	mtDNA/cell	TFAM/mtDNA	Reference
Human	HeLa	1000	1700[a]	[97][a]
	HeLa	5000	50.3	[50][b]
	Fibroblast	2721	1000	[18]
Mouse	Kidney	nd	977	[98]
	Liver	nd	1480[c]	[67]

[a] Also reported 3000 +/− 1000 mtSSB/mtDNA.
[b] Also reported 6 mtRNA Pol/mtDNA, 2.7 TFB1M and 8.6 TFB2M/mtDNA.
[c] Calculated from reported one TFAM/10–12 bp; also noted one TERF2/260 bp, about 63/mtDNA.

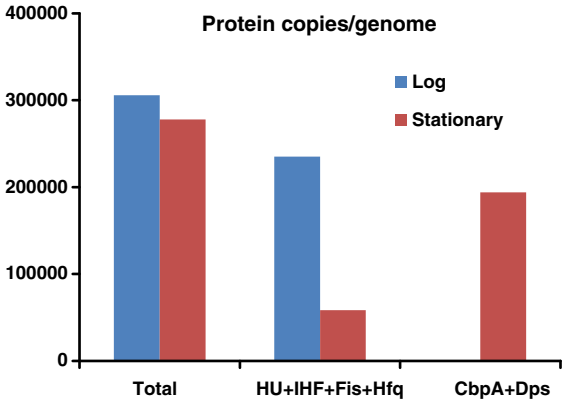

Fig. 1. Variation in bacterial nucleoid proteins with culture stage. Data are taken from [54] and are considered approximate due to interpolation from graphs.

metabolic protein and is not confined to nucleoids. The presence of this and other abundant proteins associated with nucleoids may have compromised our ability to detect the presence of lower-abundance proteins known to function in mtDNA maintenance and repair, including DNA ligase III, RNaseH, and several DNA damage-specific glycosylases, among others. A number of repair proteins have been reported to reside in close association with the mtDNA membranes [68], and these proteins must have access to mtDNA following DNA damage.

Proteomic analysis of native and cross-linked nucleoids identified a number of nucleoid associated proteins not previously considered to play direct roles in mtDNA transactions. Many of these are abundant proteins that may represent contaminants in nucleoid preparations. However, several of them represent classes of proteins that might be enriched in the vicinity of nucleoids for important functional reasons. These groups are discussed below.

4.1. Helicases and RNA binding proteins

Nucleoids were found to contain the Suv3L1 helicase and its binding partner polynucleotide phosphorylase, which have been implicated in mitochondrial RNA stability [69, 70]. In addition, we detected two other RNA helicases, DDX28 and DHX30, the latter of which had not previously been shown to be mitochondrial. Proteins that bind mitochondrial mRNAs and tRNAs, such as LRPPRC and EFTu, were also found in nucleoid preparations, although they have a much broader distribution in the mitochondrial matrix. Interestingly, Sondheimer et al. [71] have suggested that LRPPRC has a direct role in modulating mitochondrial transcription. These helicases and binding proteins probably reflect RNA processing events that must occur in the vicinity of the mtDNA.

4.2. Chaperones and quality control proteases

A diverse set of chaperones and proteases involved in binding and degrading unfolded proteins was identified in nucleoid preparations [63], including HSP70, HSP60, and DNAJ3 (HSP40). HSP60 was previously reported as a component of the yeast mtDNA nucleoid due to its DNA binding ability [72], and a DNAJ homolog has been reported to influence mtDNA stability in yeast [73]. However, we were quite surprised to find prohibitin associated with nucleoids given that this chaperone resides in the intermembrane space [74]. Prohibitin exerts a broad range of effects on mitochondrial structure and function as it influences cristae morphology and interacts with Opa1, mutation of which can lead to mtDNA deletions [75–77]. Prohibitin has been reported to affect stability of nucleoids [78], although this effect may be indirect. In addition to chaperones, the Lon and ClpX proteases, both of which are involved in mitochondrial quality control and the mitochondrial unfolded protein response, were also found associated with nucleoids. The observation of Lon protease in nucleoids reflects its reported ability to bind mtDNA [79] and to participate in mtDNA copy number control through its ability to degrade TFAM [80].

4.3. Mitochondrial ribosomal proteins

Numerous mitochondrial ribosomal proteins were found associated with nucleoids [63, 64]. Interestingly, Rorbach et al. [81] found a variety of nucleoid proteins associated with immunoprecipitated mitochondrial ribosome recycling factor, providing additional evidence for this physical association. The fact that mitochondria have a relatively low content of ribosomes [82] suggests that this association is significant. We have previously noted the possibility that nucleoids may be held near the inner membrane by a coupled transcription–translation process analogous to bacterial transertion [63, 83]. If such a coupling does occur in mitochondria, the mechanism would be rather different than in bacteria, since mitochondrial transcripts are not thought to be translated as prokaryote-like long polycistronic arrays, and even lack ribosome binding sites. Another explanation for the association of ribosomal proteins with nucleoids is that these proteins may bind nascent mitochondrial rRNA sequences that have not yet been released from primary transcripts. Alternatively, translation by fully assembled ribosomes may occur in the vicinity of nucleoids.

4.4. Lipid metabolic enzymes

Both subunits of the trifunctional protein involved in beta-oxidation of fatty acids, HADHA and HADHB, were found to be associated with native nucleoids and to cross-link to mtDNA, while a lower frequency of peptide hits was obtained for CPT1A, ACADVL, ACAA2, ACAT1, ECHS1 and ETFB. These are all abundant proteins involved in lipid metabolism. We have found HADHA immunofluorescence is not at all confined to nucleoids (D.F.B, unpublished observation). In addition, a AAA ATPase, ATAD3A, that was little-studied at the time, was also observed in native nucleoids [64] as well in the short list of proteins identified by He et al. [65]. This is an abundant mitochondrial protein that appears to have an unusual topology spanning the mitochondrial IM and OM and to function in cholesterol metabolism [84]. ATAD3A is necessary for maintenance of mitochondrial morphology and, like prohibitin, may indicate that nucleoids reside in contact with an atypical membrane environment. This specialized membrane environment involves association of nucleoids with contact point proteins, such as ANT and VDAC isoforms, and may bear a relationship to the mitochondrial-ER associated membrane fraction [85]. Such protein associations are currently quite speculative, but provide a fertile ground for future research.

4.5. Cytoskeletal attachment

A large literature too expansive to cite here has established that mitochondria can be transported within cells through numerous interactions with molecular motors and cytoskeletal elements (reviewed in [86, 87]). Whether such cytoskeletal elements are docked to mitochondria preferentially in the vicinity of nucleoids is not well established. Over the years there have been a number of reports of association of mtDNA with matrix components [88] and vimentin [89], for example. We reported that mtDNA nucleoids from HeLa cells sedimented heterogeneously in glycerol gradients, with the more rapidly sedimenting fraction associated with actin and vimentin [64]. We found a large number of other cytoskeletal-associated proteins in this fraction (Y. Wang, unpublished observation) but did not pursue these proteins further due to concern that the association might be artifactual. The sort of Triton X-100 lysis procedure we employed is similar to a classical cytoskeletal protein purification protocol and is known to leave large aggregates of cytoskeletal proteins intact in rapidly sedimenting complexes. More recently, Reyes et al. [90] reported the observation of non-muscle myosin heavy chain and β-actin in nucleoids prepared from rat liver by a high salt lysis method. They further suggested that these cytoskeletal proteins enter the mitochondrial matrix to form direct contacts with nucleoids. Defining the molecular nature of these putative attachment sites remains an interesting subject for future research.

5. Summary points

1. The clustering of mtDNA molecules in nucleoids appears to be a significant factor influencing both the genetic complementation among mutant and wild-type mtDNA genomes and the inheritance of mutant mtDNAs.
2. A review of the literature indicates there is a remarkable lack of consensus regarding the number of mtDNA copies that may be

localized within single nucleoids or clusters of nucleoids in cultured cells. We know even less concerning nucleoid structure in animal tissues.

3. While mtDNA may be considered as a minimal-size genome derived from a prokaryotic ancestor, the diversity of nucleoid associated proteins observed in contemporary prokaryotes has not been preserved in mammalian mitochondria, where a single HMG-box protein, TFAM, appears to account for the compaction of mtDNA within nucleoids. Published measurements of the number of TFAM molecules per mtDNA vary considerably.
4. Initial publications using super-resolution light microscopy methods have had a major impact on our appreciation of the fine structure of nucleoids and hold great promise for further advances in visualizing key steps in mitochondrial biogenesis at unprecedented resolution.
5. The array of proteins found associated with nucleoids includes RNA helicases, mitochondrial ribosomal proteins, chaperones and quality control proteases and lipid metabolic enzymes. Together, these classes of proteins imply that the immediate nucleoid neighborhood is devoted to key steps in mitochondrial biogenesis.

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