Characterization of a histone-like protein extracted from yeast mitochondria

(DNA-binding protein/chromatin/evolution)

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ABSTRACT   Analysis of proteins isolated by affinity chromatography on DNA-cellulose from highly purified yeast mitochondria shows that these organelles do not contain histones but have in abundance a DNA-binding protein of 20,000 daltons. The purification yield of this protein, called HM, indicates that mitochondria have at least an equal mass of HM relative to DNA. The amino acid composition and its electrophoretic characterization reveal that HM, rich in lysine, is slightly basic and heat stable. HM appears to be coded by the yeast nucleus, as shown by its presence in several "petite" mutants. We have shown that HM, like histones or histone-like proteins, is able to introduce superhelical turns into circular relaxed DNA in the presence of a nicking-closing activity.

The nucleosome is the primary folding unit of eukaryotic nuclear DNA (1–3). It is of interest to know if non-nuclear DNA is also associated with histones to give a nucleosomal structure. To investigate this question, we selected yeast mitochondrial DNA for several reasons. Yeast nuclear chromatin has a repeating structure of 165 base pairs (bp) per nucleosome and contains the four major histones H3, H4, H2A, and H2B (4). A detailed genetic map and a large number of mutants (5) affecting the mitochondrial structure and its functions are available and represent a precious tool for biochemical studies (6). Also, yeast mitochondrial DNA is a 70,000-bp circular molecule, 5 times larger than mammalian mitochondrial DNA. In stationary phase, about 100 copies are present in the diploid cell, which means that the total amount of mitochondrial DNA per yeast cell (7×10^6 bp) represents 15% of the total amount of nuclear DNA (7). Because the total volume of mitochondria per cell is of the order of 5 μm^3 (8), the problem of DNA packaging is not as great as in yeast nucleus, which contains 7 times more DNA in approximately the same volume (8). In fact, in this respect, the mitochondria can be compared to *Escherichia coli*, in which 4×10^6 bp of DNA is compacted in 1.5 μm^3.

This similarity to *E. coli* raises the possibility that the mitochondrial genome, instead of having a nucleosomal structure characteristic of eukaryotic nuclear DNA, may have an organization resembling that of the bacterial nucleoid. It has recently been shown that the DNA-binding protein HU isolated from *E. coli* (9) introduces, in the presence of a nicking-closing enzyme, up to 16–18 negative superhelical turns into relaxed circular simian virus 40 (SV40) or plasmid DNA (10). In fact, this bacterial protein can compact SV40 DNA into a beaded structure. This low molecular weight protein (M_r 10,000) is particularly well conserved in prokaryotes. HU proteins isolated from two different species of cyanobacteria, from *Salmonella typhimurium* and from *Bacillus subtilis*, show immunoprecipitation bands with the serum prepared against the *E. coli* HU

(ref. 11 and unpublished results). Therefore, it was of interest to see if mitochondria possess either histones or an HU-like protein.

We report here that yeast mitochondria do not contain histones but have in abundance a 20,000-dalton DNA-binding protein which we call HM. HM is present in the same quantity in wild-type cells and in "petite" mutants. This mitochondrial protein introduces superhelical turns in relaxed circular DNA in the presence of a nicking-closing enzyme like histones, HMG, and HU.

MATERIAL AND METHODS

Isolation of Yeast Mitochondria. Different strains of *Saccharomyces cerevisiae*, grown at 28°C in 6 liters of a medium containing 2% galactose, 1% yeast extract, and 1% bactopeptone, were harvested in late exponential phase, yielding about 70 g of wet cells. The method used to purify mitochondria was adapted from Petzuch (12) as modified by Faye *et al.* (13) and can be summarized as follows: protoplasts were obtained from cells by incubation with Helicase (Industrie Biologique Française, Gennevilliers, France). Once the complete digestion of cell walls was achieved (1 hr at 30°C), protoplasts were thoroughly washed with sorbitol medium (1.08 M sorbitol/0.05 M citric acid/0.15 M K_2HPO_4, pH 5.4) and then resuspended in 0.7 M sorbitol solution containing 1 mM EDTA, 60 mM Tris·HCl (pH 7.4), and 0.4 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was homogenized in a Kenwood blender for 25 sec at full speed. Unbroken cells, cellular debris, and nuclei were discarded by two successive low-speed centrifugations (2000 rpm in a Sorvall GSA rotor for 15 min). The crude mitochondrial extract was then pelleted at 15,000 $\times$ g for 15 min and resuspended in 0.5 M sorbitol/50 mM Tris·HCl, pH 7.4/0.1 M EDTA/0.5 mM PMSF. Further purifications were performed in this buffer by differential centrifugation. For DNase I treatment, the mitochondrial pellet was suspended in 50 ml of 20 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$ and was incubated for 1 hr at 10°C with 50 μg of DNase I per ml (Sigma). Mitochondria were then thoroughly washed with the 0.5 M sorbitol solution.

Purification of HM Protein. The method used to isolate HM protein follows the purification procedure for the HU protein of *E. coli* (9), which was inspired by the protocol of Alberts and Frey (14). The purified mitochondrial pellet was resuspended into 10 ml of 20 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/2 mM $CaCl_2$/1 mM EDTA/0.5 mM dithiothreitol/0.4 mM PMSF, disrupted by sonication, and treated for 90 min at 10°C with 20 μg of DNase I per ml. After dialysis against 20 mM Tris·HCl, pH 8.0/5 mM EDTA/0.5 mM dithiothreitol/1 mM PMSF, the extract was made 10% glycerol and was chromatographed on

Abbreviations: bp, base pairs; PMSF, phenylmethylsulfonyl fluoride; NaDodSO₄, sodium dodecyl sulfate; SV40, simian virus 40.
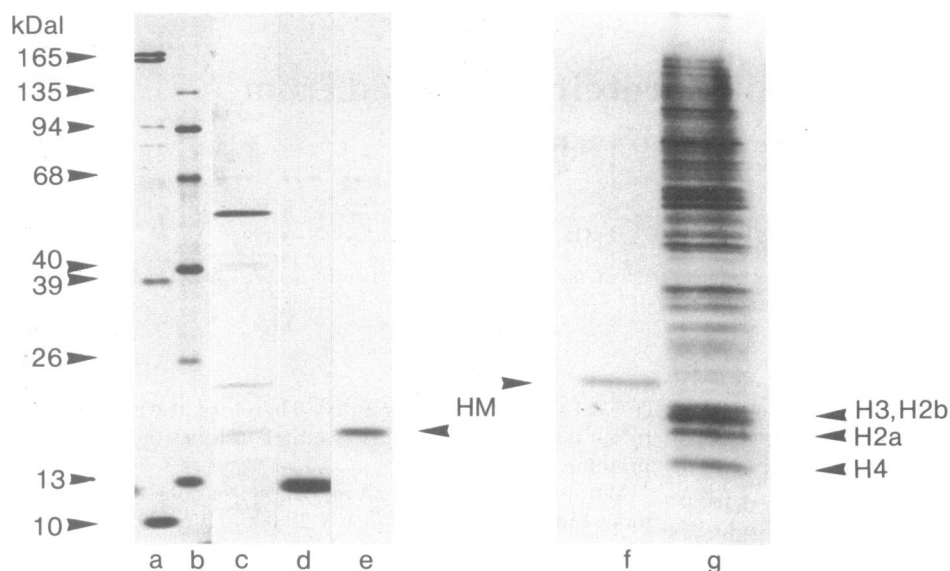
FIG. 1. Characterization of HM on NaDodSO₄/polyacrylamide gels. Lane a, *E. coli* RNA polymerase and *E. coli* HU; lane b, molecular weight markers; lanes c–e, fractions eluted from the DNA-cellulose column at 0.15 M (c), 0.4 M (d), and 2.0 M (e) NaCl; lanes f and g, separate gel: HM protein (f) and proteins extracted at 2.0 M NaCl from yeast nuclei (g). kDal, kilodaltons.

a double-stranded calf thymus DNA-cellulose column. Step elutions were made in the same buffer at increasing ionic strengths. Fractions eluted at 150 mM, 400 mM, and 2 M NaCl were analyzed on sodium dodecyl sulfate (NaDoDSO₄)/polyacrylamide gels. All of the operations were performed at 4°C in the presence of PMSF.

Gel Electrophoresis. Proteins were analyzed by Na-DodSO₄/polyacrylamide gel electrophoresis. The gels contained a 10–25% linear gradient of acrylamide with a mono-to-bisacrylamide ratio of 30:0.17. Electrophoresis of proteins on acidic urea gels (15%) was performed according to Panyim and Chalkley (15).

DNA–Protein Association and Agarose Gel Electrophoresis. DNA and DNA-binding proteins were complexed by the procedure used to study the association process of HU to circular relaxed DNA (10). Each incubation mixture (40 μl), containing 0.3 μg of relaxed SV40 DNA (FIr), was incubated for 60 min (except when specified in the figure legend) with the appropriate DNA-binding protein (the protein:DNA ratio is given in each figure legend) in the presence of 2 μl of a chromatin extract from Krebs ascites cells containing a nicking-closing activity (extracted at 150 mM sodium phosphate) (16). The complexes were formed in 10 mM Tris·HCl, pH 8.0/1 mM EDTA and NaCl in the quantities indicated in each legend. The reaction was stopped by addition of 0.5% NaDodSO₄ (15 min at 55°C). The DNA was then analyzed by electrophoresis on 1% agarose gel as described (10).

RESULTS

Purification of HM Protein. The mitochondrial extract obtained from the wild-type yeast strain IL 8 8C (5) was chromatographed on a DNA-cellulose column. Fig. 1 shows the protein pattern of the different fractions eluted at 150 mM, 400 mM, and 2 M NaCl after NaDodSO₄/polyacrylamide gel electrophoresis. The protein that eluted at 400 mM NaCl (Fig. 1, lane d) was easily recognized as cytochrome *c* by its characteristic absorbance at 410 nm and by the fact that the analogous fraction obtained from a yeast mutant deficient in cytochrome *c* (J. Verdière and E. Pitrochilo, personal communication) did not contain this protein (data not shown). The protein that we call HM was the predominant protein eluted at 2.0 M NaCl (Fig. 1, lane e). In fact, this protein was eluted between 0.6 M and 0.8 M NaCl (data not shown). In the majority of the prep-

arations, HM was obtained practically pure after this step (Fig. 1, lanes e and f). When traces of low molecular weight contaminants were present, HM was further purified by phosphocellulose chromatography (10 mM Tris, pH 8.0/1 mM EDTA/NaCl gradient elution between 50 and 750 mM) (data not shown). Finally, we would like to underline the fact that PMSF is absolutely required all during the purification to prevent the degradation of HM (20,000 daltons) into a 16,000-dalton polypeptide.

Characterization of HM. The comparison of the band position of HM with a series of markers (Fig. 1, lanes a, b, and e) enabled us to assign to HM an apparent molecular weight of about 20,000. Fig. 1, lanes f and g, shows that histones are not present among the proteins eluted from the DNA-cellulose at 2 M NaCl and also that HM, which migrates on NaDodSO₄/polyacrylamide gels more slowly than H3, is not a histone. Fig. 2 shows that after electrophoresis on acidic urea gel, HM ran at a position slightly behind calf thymus H1, indicating that HM is probably a basic protein. Like the histones and HU, HM is thermostable but unlike these proteins, HM is not soluble in 0.1 M HCl (data not shown).
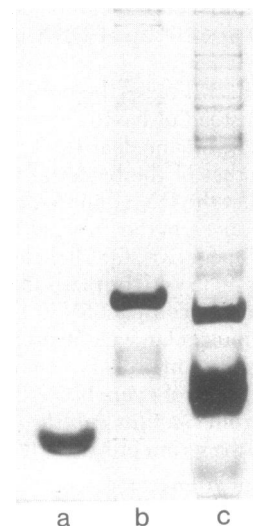

FIG. 2. Characterization of HM on acid-urea polyacrylamide gel. Lane a, *E. coli* HU; lane b, HM; lane c, calf thymus histones.

Table 1. Amino acid composition (mole %) of mitochondrial protein HM

	HM	HMG2	HU	H2B
Cys	1.7	Trace	0	0
Asx	9.4	9.3	8.9	4.8
Thr	3.8	2.7	6.8	6.4
Ser	8.3	7.4	4.5	11.2
Glx	12.4	17.5	9.5	8.0
Pro	4.0	8.9	2.5	4.8
Gly	13.3	6.5	7.2	5.6
Ala	7.5	8.1	18.5	10.4
Val	3.4	2.3	7.3	7.2
Met	0.8	0.4	1.2	1.6
Ile	4.6	1.3	6.6	4.8
Leu	7.4	2.0	6.9	4.8
Tyr	4.0	2.0	0	4
Phe	4.0	3.0	3.3	1.6
His	1.8	2.0	0.0	2.4
Lys	11.8	19.4	11.1	16.0
Arg	5.1	4.7	4.7	6.4

The amino acid composition was determined several times and was independent of the strains used. It is compared here to HU (17), to calf thymus HMG2 (18), and to H2B (19). No corrections were made for hydrolytic losses. Tryptophan and amides were not determined.

FIG. 3. Assembly of HM and DNA in the presence of a chromatin extract. Circular relaxed SV40 DNA (FIr) wàs incubated with different quantities of HM at 150 mM NaCl in the presence of a chromatin extract for 60 min at 37°C. Lanes b–f correspond to complexes with an HM-to-DNA ratio (wt/wt) of: b, 0.4; c, 0.6; d, 0.8; e, 1.0; and f, 1.2. The result of association of *E. coli* HU protein and FIr DNA at 50 mM NaCl and at a weight ratio of 1 is given in lane a. FI and FIr indicate the positions of supercoiled and relaxed SV40 DNA, respectively.

The amino acid composition given in Table 1 shows that HM is rich in Lys, Arg, Glx, and Asx. HM is thus similar in amino acid composition to several nonhistone proteins (compare to HMG2, also in Table 1) although its amount of hydrophobic residues is relatively high (25%) and closer to that of the histones and HU rather than to that of HMGs. In addition, HM (like HU) has a lower proline content than HMG2: 4% as compared to 8%. Both features suggest a more highly organized secondary structure in HM (and HU) than in HMG2.

Recently, different nonhistone proteins have been isolated from yeast (20, 21). One of them, isolated by Sommers (20), has an apparent molecular weight, as determined by NaDodSO₄/polyacrylamide gel electrophoresis, roughly identical to, and an amino acid composition that is similar to, HM. However, we do not think that we are dealing with the same protein mainly because the migration of these proteins on acid urea gel is very different (Fig. 2, lane b, and ref. 20).

The immunological affiliation of HM with HMG proteins, histones, and HU will be of interest. We know already that HM does not crossreact with a serum prepared against *E. coli* HU (data not shown).

Further Evidence for Localization of HM. Although the mitochondria used in our experiments have been extensively purified, it was necessary to eliminate the possibility that HM is present as part of a nuclear contaminant. Suspensions of mitochondria are, in fact, contaminated with variable amounts of nuclear DNA, which never exceeded 10% as judged by analysis on CsCl density gradients; contamination is usually of
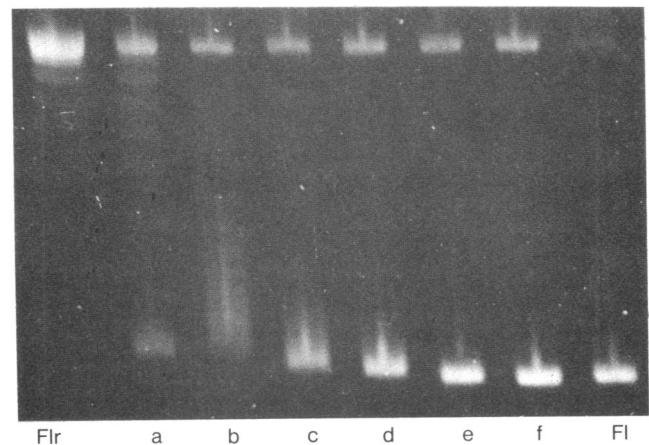
the order of 1% ʹdata not shown). To eliminate the possibility of nuclear contamination, we have treated the mitochondrial suspension with DNase I (see *Materials and Methods*) prior to any protein or DNA extraction. After treatment, the DNA analysis on CsCl gradients revealed the total absence of nuclear DNA and the persistent presence of mitochondrial DNA (data not shown). Moreover, the amounts of HM protein and mitochondrial DNA recovered do not drastically change (see Table 2). These results indicate, first, that HM is not bound to a nuclear DNA contaminant and, second, that mitochondria are intact up to the point that mitochondrial DNA is protected from the action of DNase I. We conclude that HM is localized inside mitochondria.

Mutant Analysis Shows that HM Is Coded by Nuclear DNA. The cytoplasmic "petite" mutation of *S. cerevisiae* is now well characterized at the molecular level. It consists of a deletion of any part of the mitochondrial genome followed by an amplification of the remaining DNA segment. Two such mutants retaining genetic markers have been well characterized. One, A72, is made of many tandem repeats of a 1000-bp DNA sequence that is a part of the 23S rRNA gene (22); the other, ja, is composed of 3300-bp inverted repeats that are located inside the mosaic organized cytochrome *b* gene (23). The common feature of these two mutant strains is the complete absence of mitochondrial ribosomal machinery. Moreover, if they could still code for one protein, it is very unlikely that both code for the same protein because the two fragments amplified originate from different regions of the genetic map (6). Table 2 shows that HM is present in these two mutants in amounts similar to

Table 2. Recovery of HM in different yeast strains from purified mitochondria

Strains	Purified HM, μg		Purified mitochondrial DNA, μg
	− DNase I	+ DNase I	
Wild-type IL8-8C	50–150	40–100	100–250
"Petite" mutants A72 and ja	50–150	ND	100–250

Amounts of HM were estimated from the amino acid analysis of an aliquot assuming that the molecular weight of this protein is 20,000. The values obtained for mitochondrial DNA are the results of routine experiments on a large number of strains. The indicated ranges of amounts of purified HM and mitochondrial DNA reflect the variability of the yield of purified intact mitochondrial from a starting volume of late exponential phase culture that was always 6 liters. ND, not done.
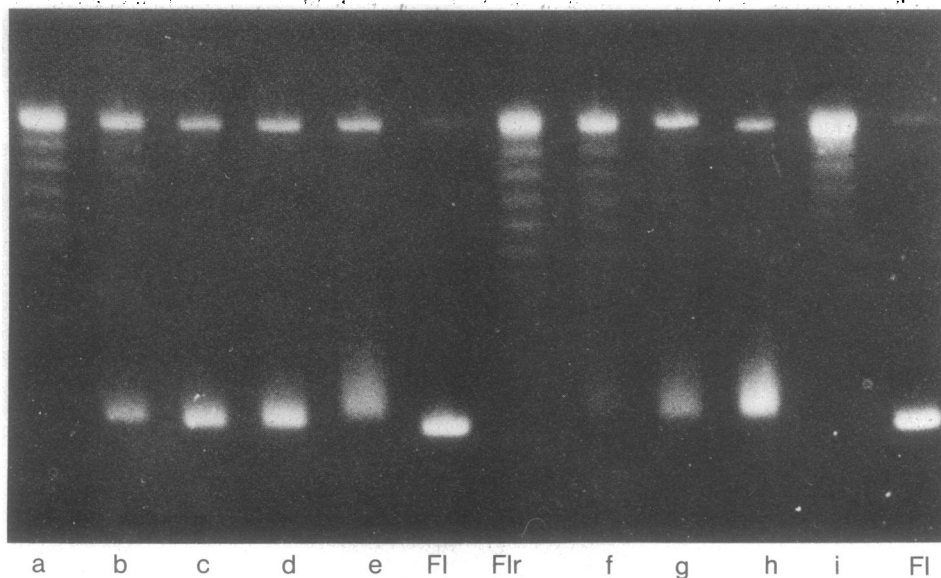
FIG. 4.   Effects of incubation time and of ionic strength on HM–DNA assembly. HM or HU protein was incubated with FIr DNA in the presence of a chromatin extract at various NaCl concentrations for different periods of time. HM–FIr complexes (ratio 1.5) were incubated in 150 mM NaCl for: lane a, 5 min; lane b, 15 min; lane c, 30 min; lane d, 60 min; and lane e, 90 min. HM–FIr complexes (ratio 1.5) were incubated for 60 min in (lane f) 50 mM NaCl or (lane g) 100 mM NaCl. HU–FIr complexes (ratio 1.0) were incubated for 60 min in (lane h) 50 mM NaCl or (lane i) 150 mM NaCl.

that of the wild type. Therefore, HM is coded by nuclear DNA and is translated in the cytoplasm.

Finally, the fact that the amount of HM recovered per mitochondrial preparation is roughly equal in weight to the DNA in the mitochondria (Table 2) suggests that HM may have a structural role in the organization of the mitochondrial genome.

DNA–HM Interactions. The *E. coli* HU protein, like the four core histones, forms beaded compact structures with DNA (10). This association is easily followed by measuring the introduction of negative super helical turns in relaxed DNA in the presence of a nicking-closing enzyme. It was of interest to see whether the HM protein belongs to this class of histone-like protein.

Relaxed SV40 DNA was incubated for 60 min with HM, at different ratios of proteins to DNA, in the presence of a chromatin extract that contained a nicking-closing activity (see *Material and Methods*). Fig. 3 shows that after deproteinization the number of superhelical turns introduced into the relaxed DNA molecules increases with the amount of HM, reaching a maximum at an approximate weight ratio of 1 (lanes e and f). In contrast to HU (lane a), which introduces at most 18 bands, HM (lane f) seems to be able to introduce a degree of supercoiling similar to that characteristic of the SV40 minichromosome *in vivo* or generated by histones *in vitro* (16).

The supercoiling efficiency under these conditions is also a function of ionic strength. Fig. 4 clearly illustrates that HM is more efficient in introducing superhelical turns at 150 mM NaCl than at 100 or 50 mM (compare lane d with lanes g and f), whereas the situation is reversed for HU (compare lane h, 50 mM NaCl, and lane i, 150 mM NaCl). In this aspect, HM association to DNA resembles the histone–DNA association (16). Fig. 4 also shows that the association between HM and SV40 DNA is not immediate. The reaction is complete (lanes c and d) only after 30–60 min of incubation at 37°C. Finally, HM protects supercoiled DNA (FI) against the action of the nicking-closing enzyme contained in the chromatin extract (data not shown). These last two properties have already been seen in both histones (16) and HU (10).

Unlike bacteria, where HU is the predominant protein that

binds to double-stranded DNA-cellulose, we have found two major DNA-binding proteins in mitochondria: HM, which elutes at 2.0 M NaCl, and cytochrome *c*, which elutes at 0.4 M (Fig. 1). This result was unexpected since we had previously observed that horse cytochrome *c* is not retained by double-stranded DNA-cellulose when applied at an ionic strength of 50 mM NaCl (unpublished data). In contrast to HM, yeast cytochrome *c* does not introduce superhelical turns into relaxed DNA in the presence of the chromatin extract.

However, yeast cytochrome *c* seems to affect the migration of a fraction of the DNA slightly even in the absence of the nicking-closing enzyme.

DISCUSSION

Although mitochondrial and nuclear genomes both coexist in the same cell, the results presented here strongly support the idea that they have different structures. Indeed, nuclear DNA interacts with histones to give the nucleosomal subunit, whereas mitochondrial DNA interacts with at least one protein, HM, and not with nuclear histones. This follows from the fact that we did not isolate nuclear histones along with HM and that we found the amount of HM recovered per mitochondrial preparation to be roughly the same as the amount of mitochondrial DNA. Also consistent with this notion are digestions of mitochondria with micrococcal nuclease under conditions that have been used for nuclear chromatin (24): we did not find any indication of a protected DNA fragment of 140–165 bp (unpublished results). We verified that the level of nuclear contamination was very low (less than 10%) and found that, by DNase I treatment of mitochondria, nuclear contamination could be totally eliminated without affecting the results.

Biochemical characterization of HM has revealed the following features. HM amino acid composition is rich in Lys, Arg, Glx, and Asx and in this respect could suggest classification of HM along with nuclear nonhistone proteins. However, its content of hydrophobic residues, its high affinity for double-stranded DNA, and its ability to induce as many superhelical turns as histones at 150 mM NaCl lead us rather to consider HM as a histone-like protein. Indeed, eukaryotic nonhistone proteins are usually loosely bound to DNA (25), whereas HM requires

a NaCl concentration much higher than 0.4 M to dissociate it from DNA. Also, HMG1 and 2 seem to be, on a weight ratio basis, less efficient in introducing superhelical turns than HM (26). However, electron microscopic studies are necessary to prove that HM can form beaded compact structures as do the histones or HU.

The analysis of "petite" mutants has clearly shown that HM is coded by the nucleus and translated in the cytoplasm. In fact, HM may be an example of a protein carrying nuclear coded signals for the expression of the mitochondrial genome.

Recently, a model of organization for the mitochondrial genome has been proposed (27). This model presents the yeast mitochondrial genome as a system of interspersed genes transcribed monocistronicallly with repetitive sequences and, for this reason, it was argued that the mitochondrial genome has a eukaryotic type of organization rather than a prokaryotic one. Although this model has not been confirmed, it is supported by the recent observations (23, 28, 29) that at least two mitochondrial genes have a mosaic organization. Our results show that this eukaryotic-like organization of yeast mitochondrial DNA sequences does not appear to be associated with a nucleosomal structure. The presence of only one DNA structural protein, HM, suggests that the structural organization of the mitochondrial genome may more closely resemble that of the *E. coli* genome. In fact, HM has an affinity for DNA intermediate between that of histones and that of HU. Regarding the number of superhelical turns introduced into SV40 DNA and the effect of ionic strength upon the introduction of superhelical turns, HM more closely resembles the core histones than it does HU. Because the origin of mitochondria is still a subject of controversy the problem of DNA packaging in mitochondria may provide a clue, to its evolutionary history (30).

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