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# Yeast may not contain histone H1: the only known 'histone H1-like' protein in *Saccharomyces cerevisiae* is a mitochondrial protein

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ABSTRACT

It is likely that histone H1 is involved in the condensation of chromatin in eukaryotes. However, both the presence of histone H1 in yeast and the extent of yeast chromatin condensation are controversial. A 20 kD protein copurifies with yeast chromatin and was shown by other investigators to have characteristics of histone H1 protein. In an attempt to obtain a positive identification of the 20 kD protein, we purified the protein to homogeneity and raised antibodies against it. We show here by immunofluorescence that the 20 kD protein does not localize to the nucleus but to cytoplasmic particles resembling mitochondria. Furthermore, we show by Western-blot analysis that anti-20 kD protein antibodies react to protein isolated from purified mitochondria. Finally, we present evidence based on size, charge, amino acid composition and immunological cross reactivity to suggest that the yeast 20 kD protein is likely to be the mitochondrial DNA-binding HM protein. This leaves no candidate for histone H1 in yeast.

## INTRODUCTION

In addition to the four core histones, most eukaryotic cells examined to date contain the linker histone H1 (1). This protein is associated with DNA between nucleosomes and is likely to be involved in chromosomal condensation (2). There is serious question whether the yeast, Saccharomyces cerevisiae, contains histone H1. Since this protein is often the most labile histone and certainly the most evolutionarily divergent, it is the most difficult histone to identify. The extent of chromosomal condensation in yeast is also unknown. However, there appear to be some differences in chromatin structure between yeast and other eukaryotes. Yeast chromatin is more sensitive to DNAase 1 than chromatin of other eukaryotes suggesting that it may exist in a less condensed conformation (3). Since H1 has been implicated in chromosomal condensation it is necessary to establish whether yeast contains histone H1.

As shown by Sommer (4) a 20 kD protein found in a partially purified yeast chromatin fraction has properties characteristic of histone H1. The protein copurifies with the core histones, and has the stoichiometry, size and

charge expected for H1 when compared to H1 of other eukaryotes. Sommer has also presented evidence that the protein can be released from the chromatin fraction with micrococcal nuclease suggesting that the 20 kD protein, like H1, binds to linker DNA between nucleosomes (5).

Other investigators have also analyzed the 20 kD, H1-like protein and suggested that the protein has characteristics of an HMG (high mobility group) protein (6,7). Weber and Isenberg found that conditions used in other organisms to purify HMG proteins resulted in the isolation of 4 HMG-like proteins from yeast. The predominant one was named HMG-a (7). The amino acid composition of this protein is similar to that of the 20 kD protein and it appears that HMG-a and H1-like 20 kD protein are the same protein (7).

In this paper we present evidence that the histone H1-like, HMG-like 20 kD protein is a mitochondrial protein.

## MATERIALS AND METHODS

### Isolation of Yeast Nuclei and Chromatin Fractionation

Baker's yeast nuclei were isolated using an unpublished procedure from P. Baum and J. Thorner (UC Berkeley, California), washed 3 times with buffer containing 0.5% NP-40 (21) and twice with RSB (.01 M Tris, pH 7.5, .01 M NaCl, 0.1 mM PMSF). Chromatin was resuspended at 2 mg/ml DNA and digested with micrococcal nuclease (Worthington) at 400 units/ml in RSB + 0.1 mM $CaCl_2$ for 10 minutes. The reaction was stopped by the addition of 0.02 M EDTA and the mixture was spun at 700 x g for 5 minutes. The supernatant was collected and the pellet was washed twice with 0.01 M Tris, 0.1 mM EDTA, pH 7.4. Pooled supernatants were loaded on a 5-20% linear sucrose gradient (0.01 M Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM PMSF) and the gradient was spun in a Beckman SW 40 rotor at 33,000 rpm for 15 hours. Fractions were collected from the bottom and the distribution of DNA was determined by measuring absorbance at 260 nm. Sizes of DNA in each fraction were determined by comparison to size standards upon electrophoresis on a 2% agarose gel. Fractions containing specific chromatin particles were pooled and aliquots containing 40 µg of DNA were loaded directly on 15% polyacrylamide-SDS gels (12).

### Isolation of Mitochondria

Mitochondria were isolated as described (10). The postmitochondrial supernatant was called the cytoplasmic fraction. Protein concentrations were determined as described (22) using a commercial assay system (BioRad Laboratories). 5 µg protein from each fraction were boiled in gel-loading buffer (12) for 3 minutes and separated on a SDS-polyacrylamide gel (12). The

proteins were then transferred to nitrocellulose and incubated with anti-20 kD antibody as described below.

Western-blot Analysis of Chromatin Fractions

Separated proteins were transferred to nitrocellulose (Schleicher and Schuell) for 3 hours at 600 mA constant current using a Hoefer-Scientific transblot chamber (11). Under these conditions about 90% of the proteins transfer to the membrane as estimated by the transfer of dansylated bovine serum albumin visualized with long wave UV-light. All subsequent incubations were done at room temperature with shaking. The filter was incubated for 1 hour in 100 ml NET (0.15 M NaCl, 0.02 M EDTA, 0.05 M Tris, pH 7.4, 0.25% gelatin, 0.05% NP-40, 1% Normal Goat Serum) + 1% BSA. The filter was then incubated overnight with a mixture of anti-20 kD and anti-H2B serum diluted 1/1000 in NET with 0.01% sodium azide. Following a rinse in NET, the filter was incubated with goat anti-rabbit peroxidase at 1/1000 dilution in NET for 1 hour. The filter was washed with NET followed by TBS (0.05 M TRIS-HCl, pH 7.4, 0.15 M NaCl) and bound antibodies were visualized by the addition of 0.6 mg/ml 4-chloro-1-napthol and 0.025% $H_2O_2$ for 5 minutes. The filter was washed in TBS before photographing.

In some experiments, protein-antibody complexes were visualized with iodinated protein-A (Amersham). Proteins were transferred from SDS-gels and incubated with the first antibody as described above. The filters were then washed for 10 minutes each in 1 x TBS, 1xTBS, 0.1% NP-40 (Sigma) and 1x TBS. The filter was then reacted with 5 µCi iodinated protein A (30 µCi/µg) in 1x TBS containing 0.05% bovine serum albumin (BSA) for 1 hour at room temperature. The TBS-washes described above were repeated and the filter was then autoradiographed with an intensifying screen for 5 hours using Kodak XAR-5 film.

Purification of the 20 kD Protein

Chromatin was isolated from commercial bakers' yeast (Saccharomyces cerevisiae, "Redstar", Commercial Food Co., Milwaukee, USA) as described (4) with minor modifications. Histones were released from chromatin using micrococcal nuclease. 10 units enzyme were used per 80 O.D. (260 nm) of chromatin for 30 min at room temperature in 10 mM $CaCl_2$, 10 mM NaCl and 10 mM TRIS-HCl, pH 7.4. Undigested chromatin was pelleted at 12,000 x g for 30 min at 4°C in an Eppendorf microfuge. The supernatant was made 1 M NaCl and the 20 kD protein was further purified by reversed phase high-performance-liquid-chromatography as described (19) with minor modifications. A VYDAC C-18 column was used at room temperature for all purifications. The aqueous

elution buffer was 0.1% TFA (trifluoroacetic acid) in water and the organic phase was 90% acetonitrile and 0.1% TFA. The peak that contains the 20 kD protein was collected, the solvents were evaporated and the protein was analyzed on acid-urea gels (20). Amino acid analysis of the purified 20 kD was performed as described (23).

Immunofluorescence

Purified histone H2B and 20 kD protein were isolated as described (19) and used to raise antibodies in rabbits. Yeast cells (RH218: a, gal2, trp1-289, mal, suc2, cup1) were grown on agar plates containing YEPD for 2-3 days. The cells were spread onto a polylysine coated coverslip with the tip of a pasteur pipette. Before the polylysine dried the cells were fixed in 10% formalin in PBS. The coverslips were transferred to methanol at -20°C for 6 minutes and then placed in 0.5 mg/ml zymolyase, 10 mM Tris-HCl, 10 MM EDTA, pH 7.5 for 30 minutes at 37° to form spheroplasts. After the cells were washed in PBS for 30 minutes, they were incubated with the first antibody using 20 µl of a 1/20 dilution of the respective IgG (5 mg/ml) at 37°C. This was followed by three 20 minute washes in PBS. The coverslips were then incubated with fluorescein-labelled goat anti-rabbit IgG (1/40 dilution) (Cappel Laboratories) in a similar manner and washed with PBS as described above. Cells were viewed with a Nikon microscope equipped for epifluorescence with a 100X phase objective and a mercury illuminator.

## RESULTS

### Purification of the 20 kD Protein

In order to determine the identity of the 20 kD protein we first repeated the chromatin purification procedure described by Sommer (4), except that we added high performance liquid chromatography to purify the protein to homogeneity. Several levels of characterization were employed to determine if we had isolated the same protein described by Sommer. We analyzed the mobility of the protein on acid-urea and SDS-polyacrylamide gels in order to compare size and charge. We also determined the amino acid composition of the 20 kD protein. Lastly, we analyzed the release of the protein from chromatin with micrococcal nuclease.

We obtained results very similar to those described by Sommer (4). The protein copurifies with histones as shown in Figure 1A. It is evident that the 20 kD protein is the major protein isolated along with the core histones as visualized on acid-urea gels. When compared to molecular weight standards,

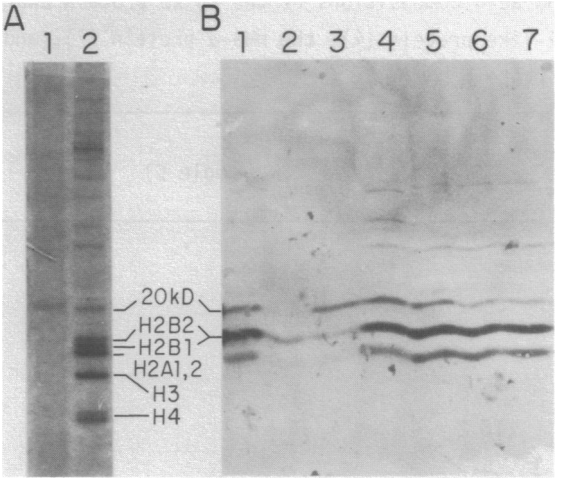


Figure 1. A) Separation of yeast chromosomal proteins on an acid-urea polyacrylamide gel.
Chromosomal proteins were released from chromatin as described in Materials and Methods and subsequently analyzed on an acid-urea gel (20). The resulting protein pattern, shown in lane 2, is similar to that observed by other authors (4,7,8). Lane 1 shows the HPLC purified 20 kD protein that was used to raise antibodies in rabbits.
B) Western-blot analysis of histone H2B and the 20 kD protein in sucrose gradient separated chromatin particles.
Proteins in fractions from the top to the bottom of a 5-20% sucrose gradient containing micrococcal nuclease-digested chromatin are shown. The Western blot of these proteins was incubated with a mixture of anti-20 kD and anti-H2B antisera.
Lane 1: Total micrococcal nuclease digested chromatin
Lane 2: Fraction A containing no detectable DNA
Lane 3: Fraction B containing DNA <100 base pairs
Lane 4: Fraction C containing mononucleosomes (about 165 base pairs DNA)
Lane 5: Fraction D containing dinucleosomes (about 330 base pairs DNA)
Lane 6: Fraction E containing trinucleosomes (about 495 base pairs DNA)
Lane 7: Fraction F containing tetranucleosomes and higher oligomers (>680 base pairs DNA)
The 20 kD protein is found predominantly in the mononucleosome and dinucleosome fractions and in the slowly sedimenting Fraction B. The latter does not contain intact nucleosomal particles and shows no evidence of histone H2B. The 20 kD protein is less abundant in the higher oligonucleosome fractions. Cross reaction to a low molecular weight protein and minor higher MW species is seen with the anti-H2B serum when large amounts of protein are loaded on the gels.

the protein has a molecular weight of 20,000 daltons (see Figure 3). The amino acid composition of the 20 kD protein is very similar to that described by Sommer (ref. 4, Table 1). We conclude from these data that the 20 kD protein we are examining is the H1-like protein studied by others (4,7,8).

TABLE 1. Amino acid compositions of the 20 kD protein analyzed in this report, the H1-like protein (4), the HMG-a protein (7), and the yeast mitochondrial protein HM (10).

| | 20 kD | H1-like | HMGa | HM |
|---|---|---|---|---|
| | | (mole %) | | |
| CYS | - | trace | - | 1.7 |
| ASX | 10.8 | 10.0 | 8.5 | 9.4 |
| THR | 4.4 | 2.9 | 8.4 | 3.8 |
| SER | 9.6 | 7.1 | 7.5 | 8.3 |
| GLX | 16.2 | 15.5 | 15.6 | 12.4 |
| PRO | 5.3 | 5.9 | 5.9 | 4.0 |
| GLY | 7.7 | 7.7 | 3.6 | 13.3 |
| ALA | 6.8 | 7.4 | 8.8 | 7.5 |
| VAL | 3.2 | 3.2 | 2.3 | 3.4 |
| MET | trace | < 1 | - | 0.8 |
| ILE | 5.0 | 5.0 | 6.5 | 4.6 |
| LEU | 5.9 | 7.3 | 7.5 | 7.4 |
| TYR | 3.9 | 4.2 | 4.4 | 4.0 |
| PHE | 2.8 | 3.0 | 2.8 | 4.0 |
| HIS | 1.9 | 1.3 | 1.3 | 1.8 |
| LYS | 11.7 | 14.7 | 15.9 | 11.8 |
| ARG | 4.9 | 4.7 | 5.5 | 5.1 |

However, when chromatin was briefly treated with micrococcal nuclease and the products were examined with Western-blots using anti-20 kD antibody, we obtained results different from those previously reported (ref. 4, Figure 1B). We find the protein to be present in oligonucleosome fractions including dimers and trimers (lanes 5-7). We also find that the protein is present in even greater yield in the mononucleosome fraction (lane 4). Sommer has described an alternate distribution for the 20 kD protein that more closely resembles that expected for histone H1, with less of the 20 kD protein found with mononucleosomes than in oligonucleosome fractions (4). Our results suggest that the 20 kD protein is either bound to both oligonucleosomes and mononucleosomes or is a contaminant of the chromatin preparation.

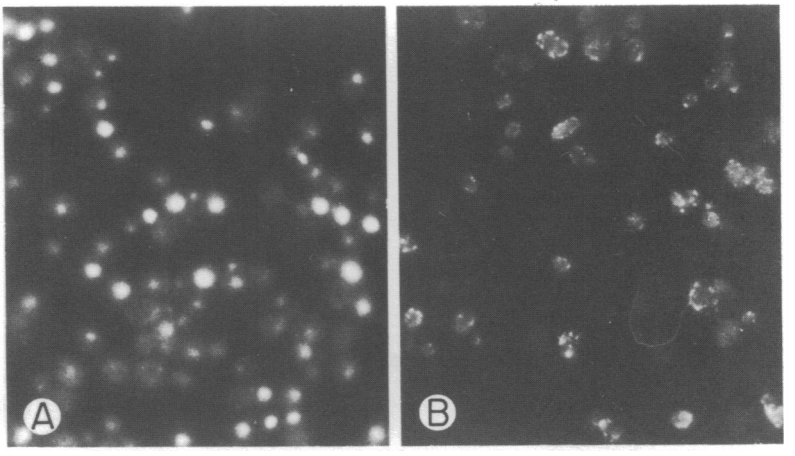


Figure 2. Indirect immunofluorescence microscopy of S. cerevisiae in order to determine the cellular localization of the 20 kD protein.
A. Cells were treated with anti-histone H2B antibody. B. Cells were treated with anti-20 kD protein antibody. The anti-20 kD antisera reacts only with proteins in the mitochondrial particles whereas the H2B antibodies react with protein in the nucleus.

## Cellular Localization of the 20 kD Protein

In order to further investigate the distribution of the 20 kD protein we raised antibodies to probe for the cellular localization of the protein by indirect immunofluorescence cytology. Antibodies against yeast histone H2B were used in parallel as a control. As shown in Figure 2, purified IgG to 20 kD protein localizes not to the nucleus but to discrete organelles in the cytoplasm. The distribution of these particles resembles the staining pattern of mitochondrial (9). Figure 2 also shows that IgG raised against histone H2B reacts specifically with protein in the yeast nucleus. These observations suggest that the majority of 20 kD protein molecules are located on or in the mitochondrial-like particles.

To obtain highly purified mitochondria we used the procedure of Caron et al. (10). Protoplasts were lysed and fractionated by differential centrifugation into a mitochondrial and a cytoplasmic fraction. To verify the mitochondrial fractionation we have also shown immunologically that the mitochondria-specific cytochrome oxidase subunit 6 localizes specifically to our mitochondrial fraction (data not shown). Nuclei were isolated using an unpublished procedure from P. Baum and J. Thorner (see Materials and Methods). Similar amounts of total protein from each of these fractions were

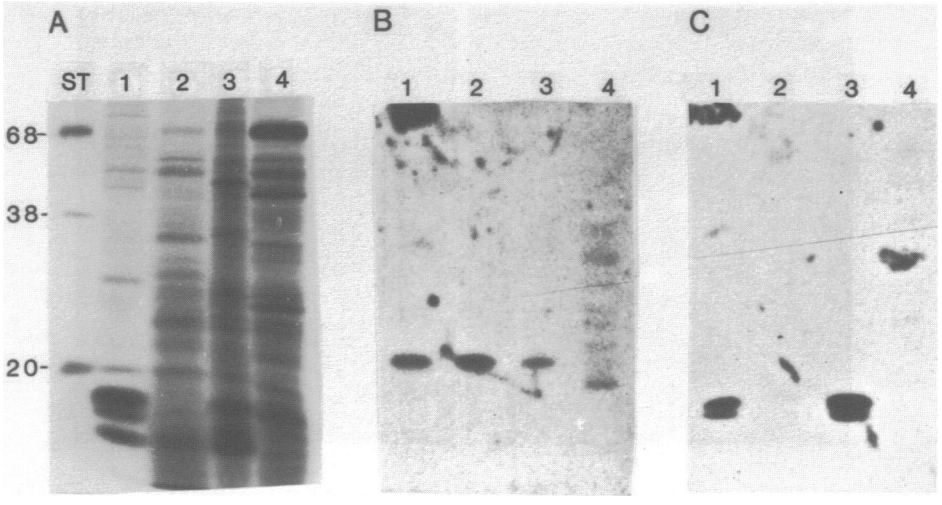


Figure 3. Localization of the 20 kD protein in different cellular fractions by SDS gel electrophoresis and Western blot analysis using anti-20 kD serum. Panel A: Coomassie blue staining. Lane ST, size standards (molecular weights are in kD); lane 1, chromatin isolated by the procedure of Sommer (4); lane 2, mitochondria isolated by the procedure of Caron et al. (10); lane 3, nuclei isolated by an unpublished procedure of Baum and Thorner; and lane 4, postribosomal supernatant of yeast lysates (cytoplasmic fraction). Panel B: Western blot. Lane 1, chromatin fraction; lane 2, mitochondrial fraction; lane 3, nuclear fraction; and lane 4, cytoplasmic fraction. Panel C: Western blot. Fractions as in panel B were probed with antibodies to yeast histones H4 and H2A in order to show that our mitochondria preparation is not contaminated with nuclear proteins. Protein antibody complexes in this experiment were visualized using iodinated protein A as described in Materials and Methods.

then boiled in SDS (sodium dodecyl sulfate) and separated on SDS-polyacrylamide gels. The proteins were then transferred to nitrocellulose filter paper for Western-blot analysis (11) using antibody to 20 kD protein and iodinated protein A to visualize antibody/protein complexes. The same samples and size standards were run in parallel and stained with Coomassie blue in order to visualize the proteins.

The results are shown in Figure 3. In Panel A, lane ST, we ran commercial size standards. In lane 1 we analyzed proteins that were released from chromatin with extensive digestion using micrococcal nuclease. The procedure followed was that described by Sommer (4). Lane 2 contains the mitochondrial, lane 3, the nuclear and lane 4 the cytoplasmic fraction. From the staining pattern it is obvious that a 20 kD protein is present in the chromatin, mitochondrial and nuclear fractions. It is evident in panel B that

the anti-20 kD antibody reacts to a greater extent with the mitochondrial (lane 2) than with the nuclear fraction (lane 3). These data strongly support the immunofluorescence cytology experiment (Figure 2) and make it likely that the 20 kD protein is mitochondrial. The presence of the protein in the nuclear fraction is most likely due to the difficulty in preparing pure yeast nuclei (13). Antibodies to yeast histones H4 and H2A reacted only with proteins in the chromatin and nuclear fraction suggesting, within the limits of detection that our mitochondrial preparation is not contaminated with nuclear proteins (Figure 3, panel C).

Evidence Showing that the 20 kD Protein is the Mitochondrial HM Protein

There is only one protein which has been shown to be an abundant DNA-binding protein in yeast mitochondria. This histone-like protein is called the HM protein. Caron et al. (10) have shown that it has a molecular weight of 20,000 daltons and runs in the position of the 20 kD protein on both SDS and acid-urea polyacrylamide gels. As described in Figure 3, we purified yeast mitochondria by the procedure described by Caron et al. (10). Proteins isolated from the mitochondrial fraction were electrophoresed on SDS-polyacrylamide gels and stained (12) to display the HM protein and analyzed by Western blot analysis (11). As shown in Figure 3B, lane 2, the anti-20 kD antibody reacts against the HM protein.

In Table 1 we compare the amino acid compositions of the H1-like protein described by Sommer (4), the HM protein (10) and the 20 kD protein. With few minor deviations the three proteins have similar amino acid compositions. The most extreme difference is in the glycine content. It is 13.3% in the HM protein (10) compared to 7.7% in the 20 kD protein. Since antibody to 20 kD protein clearly cross-reacts strongly with the HM protein we are led to believe that the minor differences in amino acid composition between HM and 20 kD protein are due to other factors. For example, elevated glycine content is a common artifact of amino acid composition analysis due to contamination with other proteins containing this abundant amino acid. We conclude therefore, that the H1-like (HMG-like) protein (4), the 20 kD protein (this paper) and the HM protein (10) are likely to be the same molecule.

## DISCUSSION

We have presented evidence that the yeast histone H1-like, HMG-like protein may in fact be the mitochondrial HM histone-like protein. Most isolation procedures for obtaining purified yeast chromatin involve washes of crude chromatin with the non-ionic detergent NP-40. Sommer (4) found that chromatin preparations where NP-40 was omitted contain much less of the

H1-like protein and he suggested that yeast chromosomes were presumably attached to the nuclear membrane which has to be solubilized in order to release the chromosomes. However, our data suggest that the mitochondrial DNA-HM protein complex co-sediments with the nuclear chromatin and that contaminating mitochondria are also lysed by the detergent.

These data have important implications in our understanding of yeast chromatin structure. The H1-like protein was the only known candidate for an H1 protein in yeast. Since it is mitochondrial, and unlikely to also be a nuclear protein (the immunofluorescence cytology shows no evidence of localization of 20 kD protein to the nucleus) this leaves no new candidate for H1 in yeast. How then does yeast condense its chromatin

Yeast chromosomes are very small, some not much larger than bacteriophage T4 (14). Therefore, their condensation at mitosis has not been seen cytologically. They have been counted cytologically only by counting synaptonemal complexes at meiosis as viewed under the electron microscope (15). While there appears to be an approximately 100 fold condensation at this stage (higher eukaryotes condense their chromosomes several thousand fold), it is not known to what extent the synaptonemal complex contributes to this condensation. Electron microscopy of yeast chromatin shows a tiny fraction of possibly solenoidal chromatin (16), however, this data is not conclusive. The presence of solenoidal chromatin was not shown by any other technique (e.g. sedimentation). Furthermore, no experiments were done to show the transition to solenoidal chromatin as a function of increasing salt concentration (16). Therefore, the extent of yeast chromosome condensation is still unknown.

Finally, yeast chromatin is much more sensitive to DNAase I than the chromatin of higher eukaryotes (3). This has been interpreted to mean that more of the yeast chromatin is in an open, less condensed conformation. This may be correlated with the higher percentage of the yeast genome which is transcribed (40% in yeast vs. 5% in higher eukaryotes) (17,18). Since the yeast chromosomes are likely to be less condensed than chromosomes of higher eukaryotes, it is difficult to escape the conclusion that yeast may have no need for histone H1.

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