

AN ABSTRACT OF THE THESIS OF

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Title: The 2- $\mu$ m Plasmid of *Saccharomyces cerevisiae*;

Partial Characterization of a Eukaryotic

Minichromosome

Abstract approved: Redacted for privacy

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I have shown that commonly used procedures for isolating 2- $\mu$ m minichromosomes from *S. cerevisiae* such as chromatography and sucrose gradient centrifugation do not eliminate significant genomic chromatin fragments. This contamination is not detectable under common electrophoresis conditions and could effect protein analysis and sedimentation properties. Due to the high copy number of rDNA in the genome, this contamination can be easily identified by hybridization to cloned rDNA.

When the minichromosome is electrophoresed in composite agarose-acrylamide particle gels, random genomic fragments are no longer detectable. The purified 2- $\mu$ m minichromosome contains the four core histones as well as HMG proteins S<sub>3</sub>, S<sub>4</sub>, and HMG a. Mobility in particle gels is sensitive to a cell division cycle mutation present in cdc 28. When the mutation is expressed, the minichromosome has a lower mobility in particle gels.

The 2- $\mu$ m Plasmid of Saccharomyces cerevisiae;  
Partial Characterization of a Eukaryotic  
Minichromosome

by

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## ABBREVIATIONS

- rDNA - ribosomal DNA
- Tris - tris(hydroxymethyl)amino methane
- EDTA - (ethylenedinitrilo)-tetraacetic acid
- NP-40 - nonidet P40
- PMSF - phenylmethyl sulfonyl fluoride
- SDS - sodium lauryl sulphate
- HMG - high mobility group protein
- kb - kilo base pair of DNA

THE 2- $\mu$ m PLASMID OF SACCHAROMYCES CEREVISIAE;  
PARTIAL CHARACTERISTICS OF A EUKARYOTIC  
MINICHROMOSOME

INTRODUCTION

Most strains of Saccharomyces cerevisiae contain 50-100 copies per cell of a circular plasmid which comprises about 4% of the total yeast DNA. It has been shown that this DNA has a monomeric circumference of two micrometers and has the same density as nuclear DNA (1, 2, 3). Strains lacking the plasmid are referred to as  $\text{cir}^0$ , the presence of the plasmid is indicated as  $\text{cir}^+$ .

Hybridization of labelled cloned 2- $\mu$ m plasmid to restriction fragments of linear yeast DNA (4) and total DNA from  $\text{cir}^0$  strains (5, 6) indicate that the plasmid is not integrated into the yeast genome.

2- $\mu$ m DNA exists as a heterogeneous population which consists of at least two types of molecules as shown in Figure 1. The molecules differ by an inversion of about  $1.6 \times 10^6$  daltons. Both types of molecules contain two nontandem repeated sequences,  $.45 \times 10^6$  daltons each, which are inverted with respect to each other and flank the  $1.6 \times 10^6$  dalton inversion (7, 8, 9). If 2- $\mu$ m DNA is denatured and self annealed, a dumb-bell structure is formed which consists of a double strand stem, arising from the  $.45 \times 10^6$  dalton inverted repeats, and two single stranded loops designated S(.67  $\mu$ ) and L(.86  $\mu$ ), resulting from the



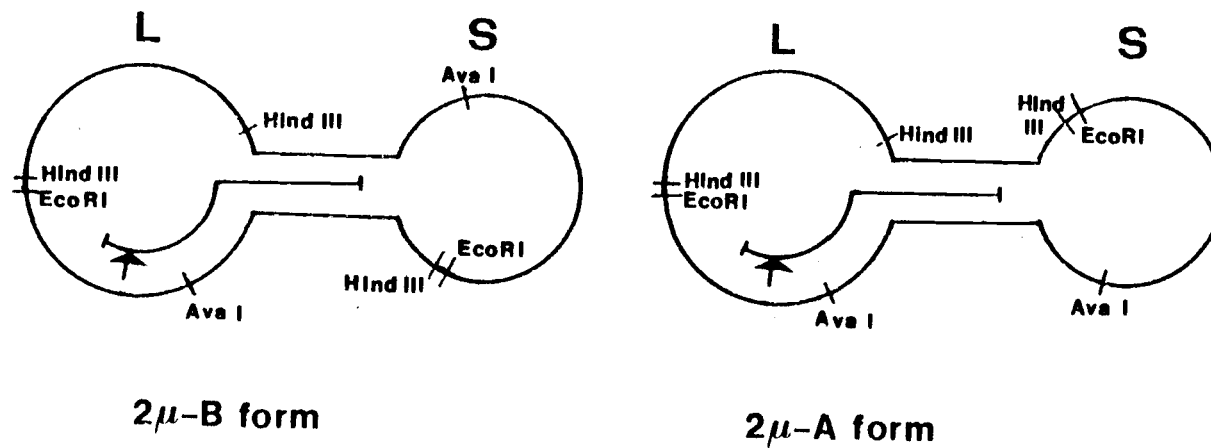


Figure 1. Representations of the two forms of 2-μm DNA after denaturing and self-annealing. Shown are the Eco RI, Hind III, and AVA I restriction sites. S and L refer to the small loop and the large loop respectively. The arrow indicates the region believed to be non-transcribed due to a lack of hybridization to poly(A)-RNA.

non-repeated sequences. A small percentage of molecules exist with two S loops or two L loops (9). In addition, a few species of dimers and trimers of the basic 2- $\mu$ m monomer exist.

It has been suggested that the two forms of 2- $\mu$ m DNA arise as a result of intramolecular reciprocal recombination between the inverted repeat regions. Bell and Byers (10) detected a cross strand exchange form (Holliday structure, half chiasma) of 2- $\mu$ m circle in cells undergoing meiosis. These cross strand exchange forms have been predicted as intermediates in the genetic recombination of eukaryotes.

Replication of 2- $\mu$ m DNA occurs by a semiconservative mechanism where each of the plasmid molecules replicates once each cell cycle. Replication is bidirectional from a single origin. Recent studies using density transfer techniques indicate that replication of the plasmid is in phase with chromosomal replication and occurs during the first third of S phase (11). It appears that the mechanism which controls the replication of each 2- $\mu$ m circle is identical to that which controls the replication of chromosomal DNA. This hypothesis is supported by another set of experiments with a series of cell division cycle mutants which arrest at different states of the yeast cell cycle. Incubation at the non-permissive temperature of *cdc 8* and *cdc 21*, both DNA chain propagation mutants (12), increases the

proportion of 2- $\mu$ m double branched replicative structures by a factor of ten. It has been concluded that at least five nuclear gene products (deficient in *cdc 8*, *cdc 7*, *cdc 28*, *cdc 4* and *cdc 21*) which are required for chromosomal DNA synthesis are also required for the synthesis of the plasmid (13, 14).

An in vitro replication system for 2- $\mu$ m DNA has been constructed (15). Cell free extracts from vegetatively growing yeast catalyze the synthesis of full length linear DNA strands, some of which become ligated and supercoiled by the extracts into supercoiled DNA.

Transcription of the 2- $\mu$ m plasmid occurs in vivo as evidenced by the presence in yeast cells of two major and approximately ten minor poly(A)-containing RNA species complementary to 2- $\mu$ m circle DNA (6, 16). Hybridization experiments with restriction fragments from 2- $\mu$ m DNA and total poly(A)-containing RNA indicate that transcription occurs from both strands of the entire 2- $\mu$ m circle, except from a small region around the *Ava I* restriction site in the L loop (6). On the basis of spatial relationships between the smaller transcripts and the larger ones, Broach *et al.*, postulate that the smaller RNA species arise from intramolecular splicing of the larger species.

The binding sites of Escherichia coli polymerase on 2- $\mu$ m DNA have recently been mapped (17). There are five binding sites on native 2- $\mu$ m DNA. There is only one

binding site which could function as a promoter site for transcription resulting in any of the poly(A)-containing RNA species isolated.

The poly(A)-containing RNA complementary to 2- $\mu$ m DNA (6) and the RNA produced from transcribing cloned 2- $\mu$ m DNA have been successfully translated in vitro and in E. coli minicells (18). The transcripts give rise to specific polypeptides of substantial size ranging from 33,000 to 48,000 daltons. There are approximately four polypeptides synthesized, the reported molecular weights of which are controversial (6, 18). To date, the in vivo presence of polypeptides resulting from 2- $\mu$ m circle transcription and translation has not been proven. There is no known function for 2- $\mu$ m DNA in yeast. However, early work of Guerineau et al. (19), indicated an association between oligomycin resistance and the presence of 2- $\mu$ m DNA. The nucleotide sequence of the 2- $\mu$ m plasmid has recently been determined (20).

Early attempts at determining the intracellular location of the plasmid indicated that it was of cytoplasmic origin (21, 22, 23). However, the procedures used for lysing of spheroplasts all are known to also lyse nuclei. Pilot experiments from our laboratory indicate a nuclear location.

2- $\mu$ m DNA has been shown to exist in a form condensed into nucleosomes as determined by micrococcal nuclease digestion patterns of native 2- $\mu$ m particles (24, 25). The

2- $\mu$ m and chromosomal digestion patterns are very similar, indicating that both are condensed into nucleosomes. The kinetics of digestion of 2- $\mu$ m chromatin and total chromatin are similar, and each has a repeat length of about 165 base pairs. The sedimentation constant of the minichromosome was reported to be 75S as compared to 25S for the naked DNA (13). However, as described in later sections, association with genomic chromatin may affect sedimentation properties of the minichromosome.

The 2- $\mu$ m minichromosome provides an excellent model system for investigations of the structure and function of eukaryotic chromatin, similar to the role played by the SV-40 minichromosome. In this thesis we examine previously-used purification procedures and present new techniques for the isolation of 2- $\mu$ m minichromosomes. 2- $\mu$ m DNA-associated proteins are identified, and the mobility of the minichromosome in particle gels after arrest in the cell cycle is investigated.

## MATERIALS AND METHODS

### Cells and Growth Conditions

For preparations of 2- $\mu$ m minichromosomes, S. cerevisiae strain a3-44, pep4-1 (26) was used. (Cir<sup>+</sup> indicates the presence of the 2- $\mu$ m plasmid, cir<sup>0</sup> the absence.) For control experiments, strain (cir<sup>0</sup>) NCYC74-CB11 (27) was used. For the cell division cycle experiments the following strains were used: parental strain A-364A with genotype a, ade 1, ade 2, ural, tyr 1, his 7, lys 2, gal 1, and three temperature-sensitive replication mutants, cdc 7, cdc 9, and cdc 28, isogenic with A364A except for the temperature-sensitive lesion (28).

Hybridization probes used were pYlrA12 containing 18S and 5.8S rRNA sequences (29) and p2 $\mu$ -141 containing the entire 2- $\mu$ m sequence inserted into pMB-9. For routine preparations of minichromosomes, cells were grown aerobically at 30°C in 1ℓ of YEPD medium containing 1% yeast extract, 2% bactopectone, and 2% dextrose. For the cdc experiments, cells were grown in YEPD plus 4 $\mu$ g/ml of both adenine and uracil. Cells were grown at the permissive temperature (25°C) until early log phase; for expression of the cdc mutations cells were shifted to the restrictive temperature (36°C).

Cells were harvested by centrifugation and suspended in a solution 4 ml/g cells of 1.1 M sorbitol pH 6.5, 0.4 mM  $\text{CaCl}_2$ , 0.5%  $\beta$ -mercaptoethanol, and 1.5 mg/ml Zymolyase 5000 (Kirin Brewery). Cells were incubated at 35°C for 20 to 40 minutes until spheroplasting was complete. Spheroplasts were harvested by centrifugation and washed once in a solution of 1.1 M sorbitol pH 6.5 and 0.4 mM  $\text{CaCl}_2$ .

#### Isolation of Nuclei

Spheroplasts were broken in 3 ml/g cells of 18% Ficoll, 1 mM PMSF. The suspension was gently homogenized with a loose fitting Teflon pestle in a 50-ml centrifuge tube. Nuclei were isolated by layering the 18% Ficoll lysate on an isopycnic density gradient of 1 M sorbitol, 0.5 mM  $\text{CaCl}_2$  dissolved in a solvent of 35% Percoll (Pharmacia) 65%  $\text{H}_2\text{O}$ , pH 6.5. The gradient was pre-formed before loading by spinning 34 ml of the gradient solution (in a 50 ml tube) in an SS-34 angle rotor at 27,000 g for 50 minutes. Six ml of the 18% Ficoll lysate was diluted with 6 ml 1 M sorbitol, 0.5 mM  $\text{CaCl}_2$  and then layered on this gradient. Nuclei were banded free from cell debris by a 7,500-RPM spin in a HB4 swinging bucket rotor for 15 minutes. The resulting band of nuclei was washed by dilution with 2 volumes 1 M sorbitol, 0.5 mM  $\text{CaCl}_2$ , pH 6.5 and pelleted at 4300 x g for 5 minutes. This procedure allows for isolation of intact nuclei free of lysed nuclei and cytoplasmic nucleases.

Nuclei were lysed in 10 mM Tris-HCl pH 7.0, 2 mM EDTA, 0.2% Np-40, 2 mM PMSF. The nuclear lysate was centrifuged at 27,000 x g for 30 minutes. The supernatant, fraction I, was passed through an A-150 M agarose column equilibrated with 2 mM EDTA, 10 mM Tris-HCl pH 7 (buffer A). The fractions containing 2- $\mu$ m DNA were located by agarose gel electrophoresis and pooled (fraction II). Sucrose density sedimentation was performed on fraction II by layering 5 ml onto a 36 ml linear gradient of 10-40% (wt/vol) sucrose in buffer A, and centrifugation was carried out for 9.7 hr at 28,000 RPM, in a Beckman SW28 rotor. Two-ml fractions were taken and the 2- $\mu$ m chromatin was precipitated with 7 mM  $MgCl_2$ , then centrifuged at 15,000 x g for 5 min. The 2- $\mu$ m chromatin pellets under these conditions. For particle gel electrophoresis, fraction I was routinely used.

#### DNA Isolation and Gel Electrophoresis

DNA was isolated by redissolving the  $MgCl_2$  precipitate in up to 1 ml of 20 mM EDTA, 10 mM Tris-HCl pH 7.0, 100  $\mu$ g/ml pancreatic RNase (Schwartz/Mann), 100 U/ml  $T_1$  RNase (Sigma), and 0.1 U/ml  $T_2$  RNase (Sigma) and incubated at 37°C for 20 min. One-tenth volume of 22% Sarkosyl, 0.3 M sodium acetate, and 50  $\mu$ l of 10 mg/ml Pronase per ml of sample were added and the samples incubated for 3 hours at 37°C. DNA was extracted by a modified Marmur procedure (30) and precipitated with 3 volumes of 95% ethanol. After



centrifugation, the pellets were washed in 70% ethanol. The DNA was dissolved in one-tenth E buffer (E buffer - 40 mM Tris, 1 mM Na<sub>2</sub> EDTA, 20 mM sodium acetate pH 7.2).

DNA gels were 1% or 1.2% agarose in E buffer (40 mM Tris pH 7.2, 1 mM EDTA, 20 mM sodium acetate). In order to resolve genomic DNA from nicked circular DNA, 20 x 20 x 0.2 cm slab gels were electrophoresed until the supercoiled monomer of 2- $\mu$ m DNA was 2-3 cm from the bottom.

Particle gels were .5% agarose, 2.5% acrylamide, 30% glycerol, in 0.3E buffer. Electrophoresis was at 40 V for 10 hr in 90 x 70 x 1-mm slab gels. The lanes were cut out and ethidium bromide stained to visualize DNA.

For SDS acrylamide gel electrophoresis, a lane from the particle gel was soaked in SDS running buffer for 15 min. and layered onto the SDS slab gel.

For acetic acid-urea gels, a first dimension particle gel lane was soaked for 15 min in a solution containing 10% glycerol, 5%  $\beta$ -mercaptoethanol, 0.375 M potassium acetate pH 4.0, 2.5 M urea, and 1% protamine sulphate. SDS gels were run according to Laemmli (31) and acetic acid urea gels according to Panyim and Chalkey (32) with modifications (33). DNA gels were stained with 0.5  $\mu$ g/ml ethidium bromide and photographed under "blacklight" illumination on Polaroid Type 55 film. Protein gels were stained according to a modified silver stain technique (34) and photographed on a light box with Polaroid Type 55 film.

### Nick Translation

Two  $\mu\text{g}$  of DNA was reacted in 10  $\mu\text{l}$  containing .05 M Tris-HCl pH 7.5; 4  $\mu\text{g/ml}$  bovine serum albumin; 10 mM  $\text{MgCl}_2$ ; 10 mM  $\mu$ -mercaptoethanol; 20  $\mu\text{M}$  dATP, dGTP and dTTP; 200  $\mu\text{Ci}$  dCTP at 2000 Ci/mmol. DNase I (Worthington) was added to a final concentration of  $7.4 \times 10^{-4}$  units/100 ml, and the reaction mixture incubated at 14°C for 5 min. Six units DNA polymerase I (BRL, large fragment pol I) was added and incubated for 60 min at 14°C. The reaction was stopped with 0.1 vol 100 mM EDTA and 0.1 vol of 10X sulfopropyl-Sephadex buffer added [1X SP buffer - (sodium chloride 0.3 M, sodium acetate 0.1 M pH 5)]. The combined supernatant was then applied to a column of SP-25 Sephadex (Pharmacia) pre-equilibrated with 1X SP buffer. The column was eluted with three void volumes of 1X SP buffer. The nucleic acid elutes in the void volume free of protein and unreacted nucleoside triphosphates (35).

### Southern Transfers

The procedure used was a modification of the method of Southern (36). The ethidium bromide-stained gel was first irradiated on a UV transilluminator (UV Products, Inc., San Gabriel, CA). The gel was then processed for 30 minute washes in 1) 0.2 N NaOH, 0.6 M NaCl and 2) 1 M Tris-HCl pH 7.6 (Sigma 7-9), 0.6 M NaCl. The remainder of the procedure was

as described by Southern (36). The transfer was completed after 18 hr. The filter was air-dried and backed in vacuo at 70°C overnight or at 70°C for 2 hr.

### Hybridization

The filters were pre-hybridized for 30 min with a mixture ( $0.1 \text{ ml/cm}^2$ ) containing per ml 0.1 ml 10% Sarkosyl and .3 ml 20X SCP [20X SCP-(2 M NaCl, .6 M  $\text{Na}_2\text{HPO}_4 \cdot 7 \text{ H}_2\text{O}$ , 0.2 M  $\text{Na}_2\text{EDTA}$  pH 6.5)] and 0.6 ml carrier DNA in distilled  $\text{H}_2\text{O}$  ( $10 \text{ } \mu\text{g/cm}^2$  of salmon sperm or Agrobacterium DNA sonicated to 800-1000 bp average) which was thermally denatured for 10 min at 90°C. The hybridization mixture contained in addition the appropriate nick-translated DNA probes (specific activity  $2 \times 10^7 - 2 \times 10^8 \text{ cpm/g}$ )  $1 - 5 \times 10^5 \text{ cpm/ml}$  hybridization mix. Hybridization was carried out at 60°C overnight. The filters were washed twice for 10 min in each: 1) 2X SCP, 1% SDS at 50°C, 2) 2X SCP room temperature, and 3) 5 mM Tris-base pH 8.0 and air dried. Autoradiography was performed using Kodak NT-54 X-ray film with Kodak intensifying screens.

## RESULTS

### Initial Purification Schemes for 2- $\mu$ m Plasmid

Using the 2- $\mu$ m minichromosome as a system for probing eukaryotic chromatin structure requires a preparation free of contaminating genomic DNA and cellular proteins. In order to minimize the action of cytoplasmic proteases and nucleases, 2- $\mu$ m minichromosomes are routinely prepared from nuclei from a strain deficient in proteases. Nuclei isolated from pre-formed density gradients (Percoll) contain the majority of the 2- $\mu$ m DNA found in the cell. Figure 2 shows a typical nuclear preparation. It is not known whether the small amount of 2- $\mu$ m DNA found in the cytoplasmic fraction of the gradient results from lysis of the nuclei or whether a small fraction of 2- $\mu$ m DNA population has an extra-nuclear location. A more detailed study of 2- $\mu$ m DNA location is currently in progress.

Centrifugation and agarose column chromatography remove the majority of genomic chromatin and RNA species from the nuclear lysate. Further purification results from sucrose gradient centrifugation of the minichromosome preparation. The centrifugation and chromatographic steps are based on previously published procedures (24, 25).

The possibility of contamination from genomic chromatin in the final preparation was examined by subjecting a strain

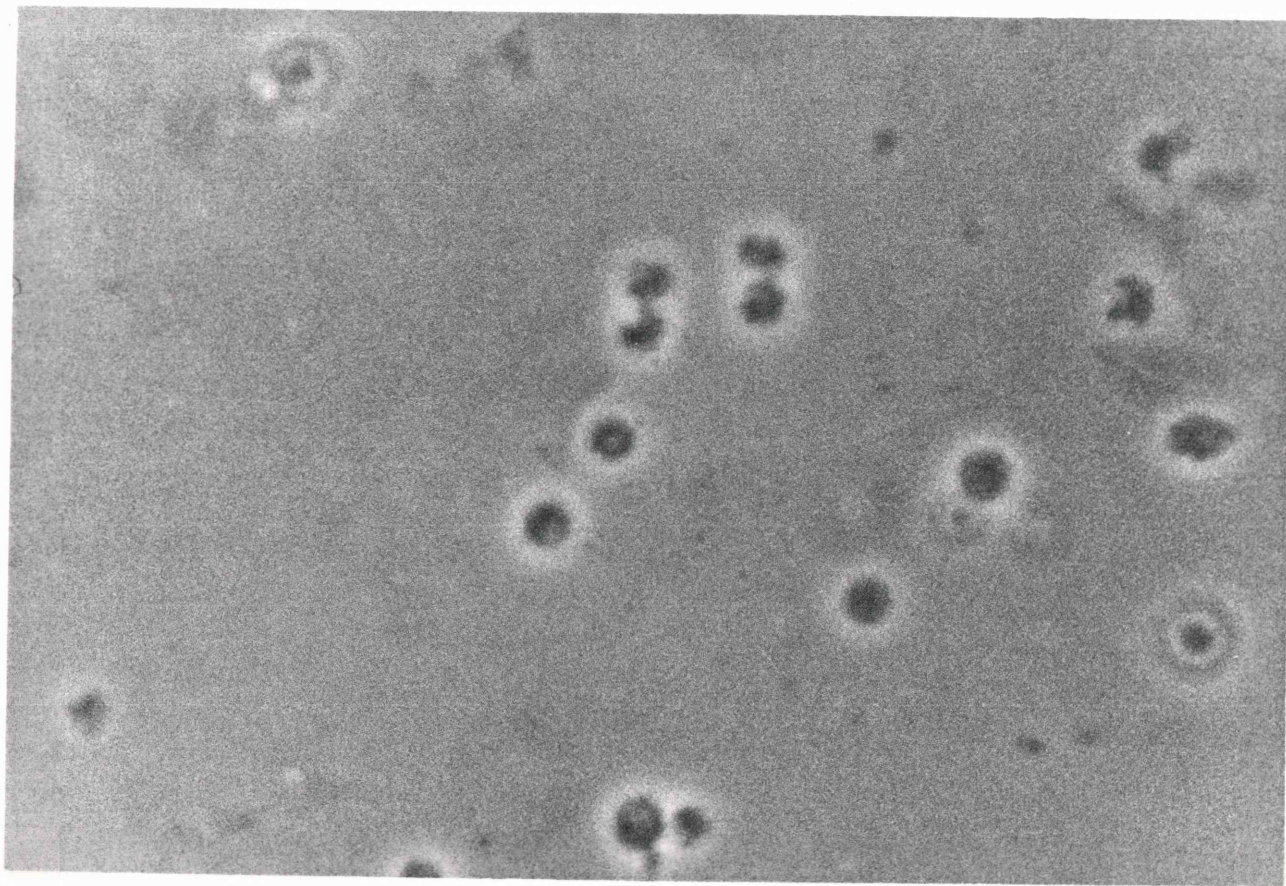


Figure 2. Nuclei prepared according to Materials and Methods. Phase-contrast microscopy, magnification 400X.

of yeast which lacks 2- $\mu$ m circular DNA, (cir<sup>0</sup>) CB-11, to the same purification procedure used to make 2- $\mu$ m minichromosomes. The cir<sup>0</sup> strain was found to contain a species of DNA which co-migrates during electrophoresis in agarose with the relaxed circular form of 2- $\mu$ m DNA. This DNA species does not hybridize to nick-translated cloned 2- $\mu$ m DNA and is present in the final 2- $\mu$ m minichromosome preparation. As shown in Figure 3, when DNA from the final fraction is analyzed by electrophoresis in 1 x 70 x 90-mm 1% agarose slab gels, a common migrating species is found in the cir<sup>0</sup> strain. The comigration of the DNA species with 2- $\mu$ m DNA demonstrates the need for caution in using electrophoretic migration in slab gels as a sole criterion for purity. In order to resolve the different DNA species, a 20 x 20 x 0.2-cm 1.2% agarose slab gel was electrophoresed until the supercoiled form of the 2- $\mu$ m DNA was a few centimeters from the bottom (at 80 V about 60 hr). Under these conditions, the various forms of the 2- $\mu$ m DNA remain as tight bands and the contaminating DNA migrates as a diffuse band, Figure 4a.

#### Identification of Contaminating DNA in 2- $\mu$ m Minichromosome Preparations

There are a number of size classes of circular DNA molecules in certain petite strains of S. cerevisiae which have been generated with ethidium bromide by the 'margin of

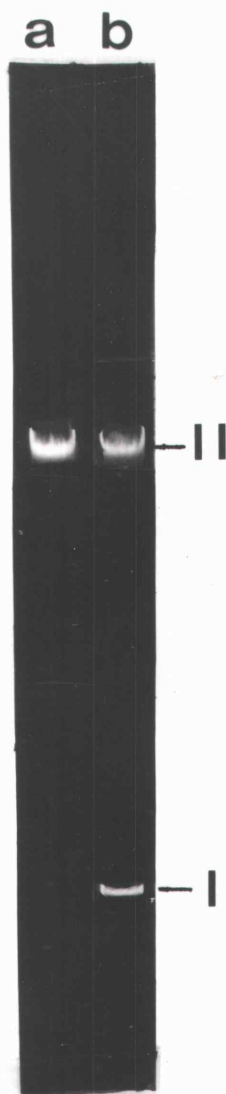


Figure 3. Gel electrophoresis in 9 x 7 x 0.1-cm 1% agarose. DNA from minichromosome preparations after A-150M agarose chromatography.

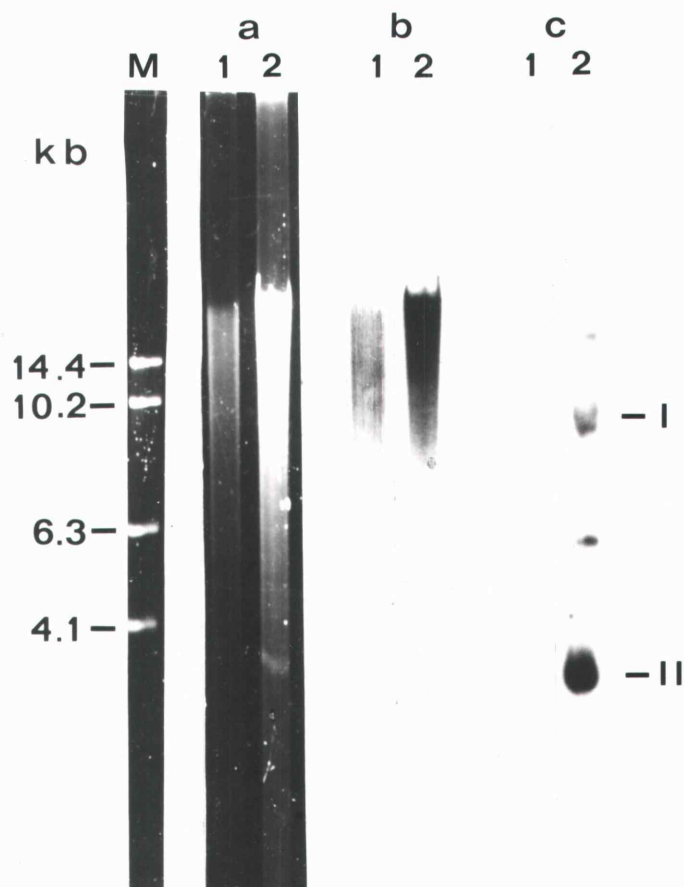


Figure 4. Gel electrophoresis in 20 x 20 x 0.2-cm 1.2% agarose. DNA from a minichromosome preparation after A-150M agarose chromatography. The three panels of the figure correspond to (a) ethidium bromide staining, and autoradiography of DNA bands transferred to a nitrocellulose filter and hybridized to (b)  $[^{32}\text{P}]$  rDNA and (c)  $[^{32}\text{P}]$  2- $\mu\text{m}$  DNA. Numbers represent (1) ( $\text{cir}^0$ ) CB-11 and (2) ( $\text{cir}^+$ ) pep-4. M is a marker track of adeno 2 DNA cut with Bam HI.



growth' technique (37) and lack detectable mitochondrial DNA. These contour lengths range from 1.9 to 5.8  $\mu\text{m}$  (38). Recently, it has been shown that some of these circular DNA's contain sequences hybridizing to 5S ribosomal RNA (39). In order to investigate the possibility that the contaminating DNA in our preparations co-migrating with nicked circular 2- $\mu\text{m}$  DNA contained ribosomal sequences, the total DNA from a 2- $\mu\text{m}$  plasmid fraction was electrophoresed in 1% agarose, Southern transferred, and probed with nick-translated DNA from a ribosomal clone. Figure 4b shows that the contaminating DNA visualized in 20 cm 1% agarose slab gels hybridizes to ribosomal sequences in both the  $\text{cir}^0$  strain as well as  $\text{cir}^+$  strains used to make 2- $\mu\text{m}$  minichromosomes. To determine whether the sequences hybridizing to rDNA were from isolated circular rDNA or from random genomic rDNA, the DNA from *pep-4* was cut with *Sma*I restriction endonuclease and probed with cloned rDNA. *Sma*I cuts the circular rDNA into a linear molecule of approximately 9000 bp. *Sma*I digestion of genomic rDNA also produces a 9000 bp fragment and a larger fragment of about 16,000 bp which disappears upon additional digestion (39). *Sma*I does not cut 2- $\mu\text{m}$  DNA. As can be seen in Figure 5b(2), digestion of DNA from our 2- $\mu\text{m}$  minichromosome preparation with *Sma*I results in 9000 bp and 16,000 bp fragments, but does not remove all of the contaminating DNA from the nicked circular 2- $\mu\text{m}$  region, Figure 5a(2). Therefore, we believe

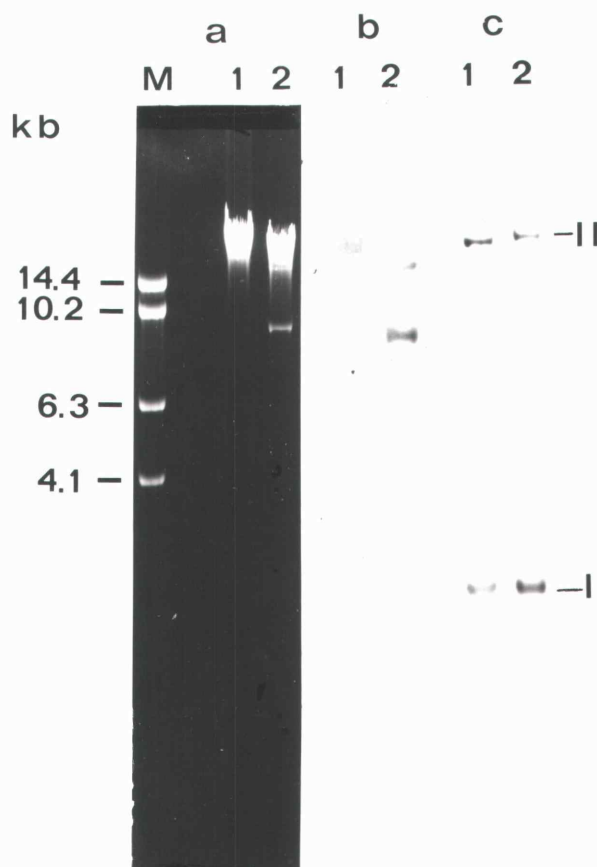


Figure 5. Gel electrophoresis in 20 x 20 x 0.2 cm 1.2% agarose. (1) DNA was prepared as in Figure 4, (2) the pep-4 DNA was cut with SmaI. Panel (a) shows the ethidium stained gel and (b) and (c) represent autoradiography after hybridization to  $[^{32}\text{P}]$  rDNA and  $[^{32}\text{P}]$  2- $\mu\text{m}$  DNA, respectively.

that the contaminant is random genomic DNA, some of which contains ribosomal sequences.

This genomic DNA occurs in preparations of 2- $\mu$ m plasmid based on previously published procedures even if great care is taken not to shear DNA during isolation. Therefore, it is unclear whether prior analyses of 2- $\mu$ m associated proteins may be misleading due to the contamination with genomic chromatin. In addition, the genomic chromatin cosediments with 2- $\mu$ m plasmid in sucrose gradients and appears only in fractions which also contain 2- $\mu$ m DNA (Figure 6a, b). It is possible that this co-migration with genomic chromatin alters the sedimentation constant of the minichromosome.

#### Analysis of 2- $\mu$ m Minichromosome Proteins

Recently it has been reported that structures bearing a resemblance to nucleosomes can be assembled by incubating calf thymus high mobility group proteins (1 + 2) with closed circular DNA (40). These HMG proteins are capable of associating with closed circular DNA to form the characteristic 'beads on a string' appearance of nucleosomes as visualized by electron microscopy. Although these complexes were formed in vitro, the results indicate that in addition to other methods a careful analysis of proteins is necessary for the identification of particles as nucleosomes, especially when dealing with closed circular DNA's.

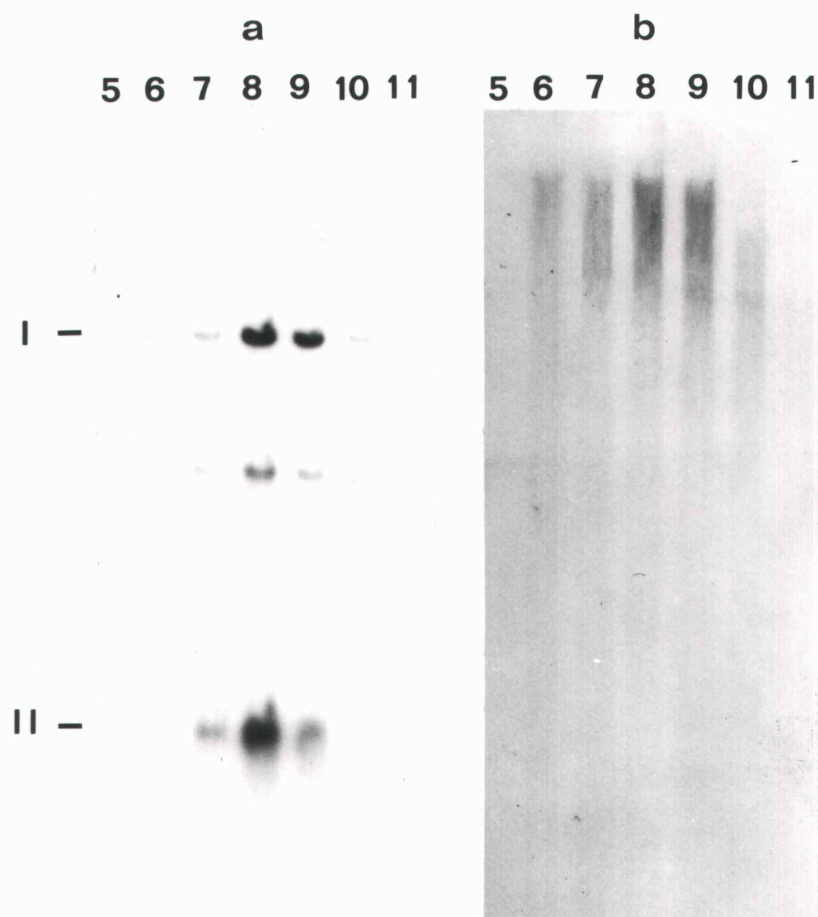


Figure 6. Gel electrophoresis of DNA from a ( $\text{cir}^+$ ) pep-4 minichromosome preparation after sedimentation in sucrose density gradients. Autoradiography of DNA after hybridization to (a) [ $^{32}\text{P}$ ] 2- $\mu\text{m}$  DNA and (b) [ $^{32}\text{P}$ ] rDNA. Numbers refer to 2 ml fractions from the gradient. There was not enough DNA present to visualize by ethidium bromide staining.

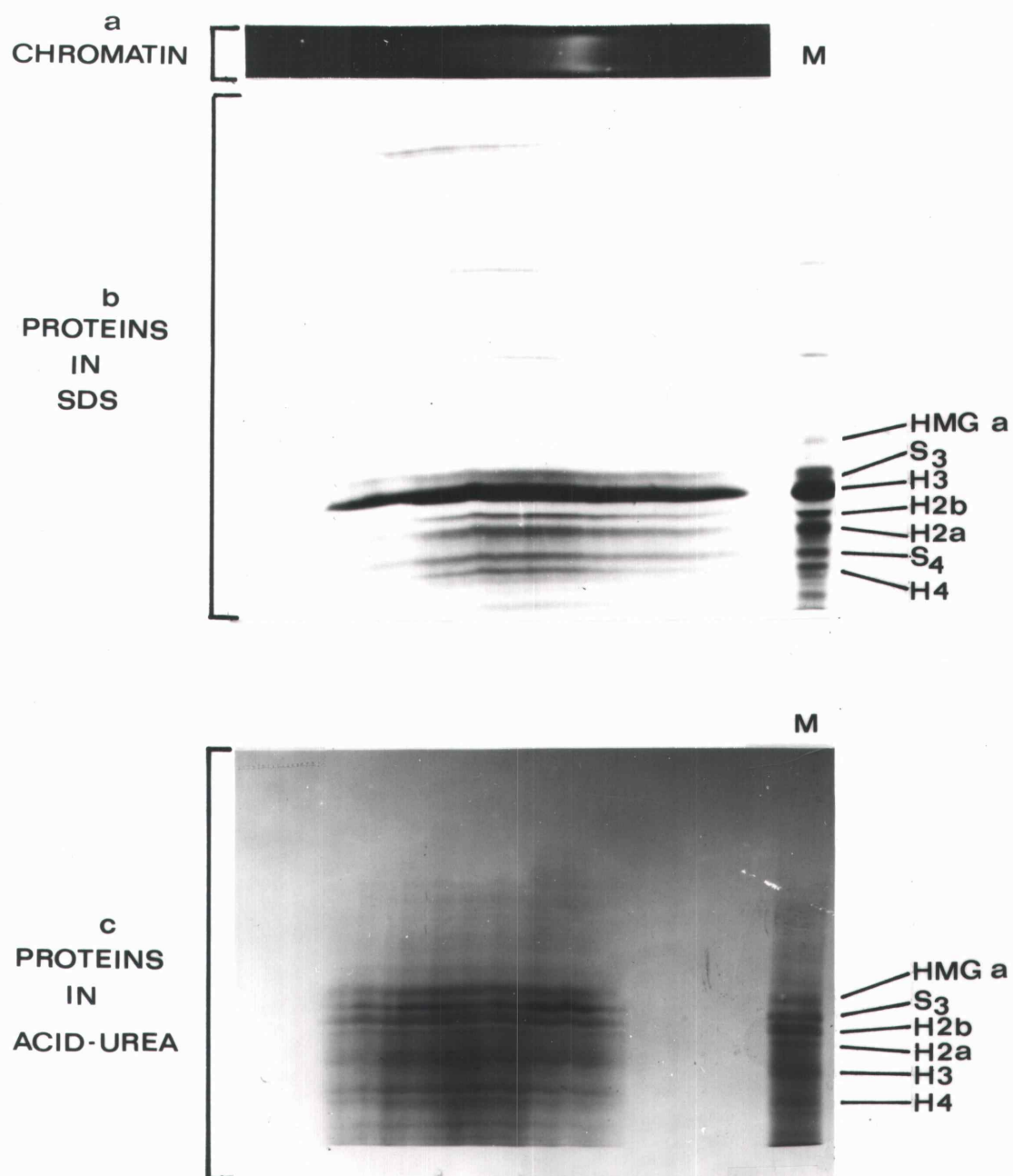
In light of the possible genomic chromatin contamination of previous 2- $\mu$ m minichromosome preparations and the possibility that association of 2- $\mu$ m DNA with other proteins besides histones could result in some of the characteristics of nucleosomes, new purification procedures were utilized to identify proteins associated with 2- $\mu$ m DNA. The most successful of the methods investigated is electrophoresis of 2- $\mu$ m chromatin in composite agarose-acrylamide particle gels as described in Materials and Methods. Figure 7a shows the electrophoretic pattern of 2- $\mu$ m chromatin visualized by ethidium bromide staining.

The migration of 2- $\mu$ m chromatin in particle gels is not affected by a 3-fold change in ionic strength; however, electrophoresis in the absence of glycerol results in a smearing of the 2- $\mu$ m chromatin band. Todd et al. (41) observe a similar phenomenon in experiments with electrophoresis of mononucleosomes in particle gels. They postulate that certain proteins may be stripped off during electrophoresis without glycerol, resulting in a heterogeneous class of migrating particles. A concentration of 30% glycerol in the gel matrix seems to stabilize the protein DNA complex.

The bands shown in Figures 7a and 8a hybridize to cloned 2- $\mu$ m DNA, but not to cloned rDNA (Figure 8b, c). When the particle gels are run for a shorter period of time, a diffuse region of ethidium bromide staining material

Figure 7. Particle gel electrophoresis of a minichromosome preparation from (*cir*<sup>+</sup>) *pep-4* (a) stained with ethidium bromide and second dimension protein analysis in (b) SDS acrylamide and (c) acetic-acid urea. Proteins were visualized by a modified silver stain technique. The marker track (M) is a histone preparation from strain Y-55. Due to the high sensitivity of the silver stain procedure, HMGS and other nuclear proteins in the marker sample are also visualized.





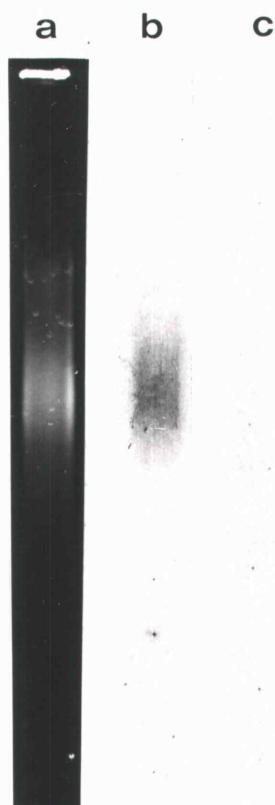


Figure 8. Particle gel electrophoresis of a minichromosome preparation from pep-4. (a) shows the ethidium bromide stained gel, (b) and (c) represent autoradiography of DNA after hybridization to [ $^{32}\text{P}$ ] 2- $\mu\text{m}$  DNA and [ $^{32}\text{P}$ ] rDNA, respectively. The rDNA did not hybridize to the minichromosome region.



migrates before the 2- $\mu$ m chromatin band and some material does not enter the gel matrix. A mock minichromosome preparation from strain (cir<sup>0</sup>) CB-11 subject to electrophoresis in particle gels gives rise to a rapidly migrating diffuse region in front of the 2- $\mu$ m band, but no ethidium bromide staining species co-migrates with the 2- $\mu$ m minichromosome (data not shown).

There is no difference in the mobility of the 2- $\mu$ m minichromosome before and after chromatography in A-150M agarose or sucrose gradient centrifugation. Therefore, in order to minimize preparation time, the 2- $\mu$ m minichromosome prepared from the supernatant fraction obtained after centrifugation of lysed nuclei is resolved directly on particle gels without prior purification.

2- $\mu$ m plasmid-associated proteins are analyzed by electrophoresing the particle gel into a second dimension SDS acrylamide or acetic acid-urea gel, Figure 7b, c.

The protein gels were stained with a modified silver staining procedure (34) based on the method developed by Switzer et al. (42). Conventional dyes such as Coomassie Brilliant Blue are not sensitive enough to detect the proteins in the second dimension. If the load on the first dimension particle gel is increased to allow detection of 2- $\mu$ m DNA proteins with Coomassie Blue, the migration pattern shown in Figure 7a is no longer maintained, but smearing and contamination result from overloading. The

drawback of the silver stain technique is that the intensity of staining is relatively nonlinear with respect to protein concentration. Oakley et al. (34) find that the minimum range of detectability for the silver stain procedure is from as low as  $0.06 \text{ ng/mm}^2$  to  $0.25 \text{ ng/mm}^2$ . In addition, experiments with serial dilutions of protein standards indicate a 50-fold difference in load concentration is difficult to detect. Therefore, it is impossible to analyze the stoichiometry of proteins bound to 2- $\mu\text{m}$  DNA by this technique.

As can be seen in Figure 7b, c, 2- $\mu\text{m}$  DNA is associated with both the four core histones and high mobility group proteins HMG a,  $S_3$ , and  $S_4$ . HMG a was identified by co-electrophoresis of purified standard. Proteins  $S_3$  and  $S_4$  were identified by comparison of mobilities to yeast histones as described by Weber and Isenberg (43).

Mobility of 2- $\mu\text{m}$  DNA Plasmid in Particle Gels  
is Subject to Cell Cycle Control

Replication of the 2- $\mu\text{m}$  DNA plasmid occurs during the S phase (11) and is controlled by genes that control nuclear DNA replication (14). The 2- $\mu\text{m}$  DNA does not replicate at the restrictive temperature in cells bearing the cell division cycle mutations *cdc 4*, *cdc 7*, *cdc 8*, and *cdc 28* (13, 14). The mobility of 2- $\mu\text{m}$  minichromosomes purified from the cell division cycle mutants in particle gels and associated proteins was investigated. Minichromosome

preparations from cdc 7, cdc 9, and cdc 28 grown at the restrictive temperature were subject to particle gel electrophoresis and second dimension protein analysis. The only difference in mobility found occurs in cdc 28 grown at the restrictive temperature. As shown in Figure 9c, the mobility of 2- $\mu$ m chromatin in cdc 28 is different from both 2- $\mu$ m chromatin from pep-4 and cdc 28 grown at the permissive temperature. The other cdc mutations tested did not alter 2- $\mu$ m chromatin mobility. The second dimension SDS electrophoresis of cdc 28 2- $\mu$ m plasmid and subsequent silver staining did not reveal qualitative differences in protein patterns. However, due to the nonlinearity of the silver stain staining procedure, relatively large differences in stoichiometry would not be detected.

The product of gene cdc 28 is required for both bud emergence and initiation of DNA synthesis (44). To date, only one specific protein has been identified with the cdc mutations; cdc 9 is deficient in DNA ligase (45).

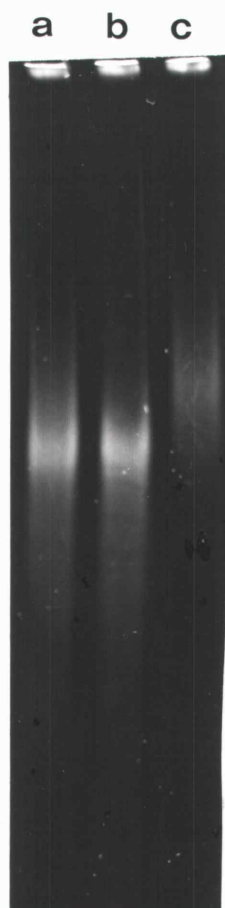


Figure 9. Particle gel electrophoresis and ethidium bromide staining of minichromosome preparations from (a) cdc 28 at the permissive temperature, (b) pep-4, and (c) cdc 28 at the restrictive temperature.

## DISCUSSION

The 2- $\mu$ m minichromosome serves as an excellent model system for studying eukaryotic recombination, gene control, chromosomal replication, and chromatin structure at a molecular level.

Isolation procedures used previously for the 2- $\mu$ m plasmid do not eliminate genomic chromatin contaminants. These fragments appear to be similar in length and, under certain conditions, co-migrate with the nicked circular form of 2- $\mu$ m DNA, making detection by ethidium bromide staining difficult. The presence of this contaminant requires a re-evaluation of 2- $\mu$ m chromatin proteins and also the development of new purification methods for the minichromosome. The majority of the contaminant is not circular rDNA as evidenced by hybridization to cloned rDNA after removal of ribosomal sequences from genomic DNA with SmaI. Because of the large copy number in the genome, 100-140 copies/haploid (46), rDNA serves as an excellent probe for contamination in 2- $\mu$ m minichromosome preparations.

Electrophoresis in particle gels separates the 2- $\mu$ m minichromosome from genomic chromatin.

When the minichromosome is electrophoresed into a second dimension protein gel, 2- $\mu$ m DNA appears to be associated with the four core histones, HMB proteins, and other proteins which are abundant in the yeast nucleus.

The possibility exists that certain proteins become associated with the plasmid during the isolation procedure which are not removed during electrophoresis. This possibility seems unlikely for two reasons. First, it has already been shown that free 2- $\mu$ m DNA does not associate with histones during typical isolation procedures such as chromatography and centrifugation (25, 47). Second, if analogies can be made between yeast chromatin and chicken erythrocyte chromatin, HMG 14 and 17 bind specifically to active nucleosomes in a 10-20 fold excess of inactive nucleosomes (48). Non-specific binding occurs only at a 2 fold weight excess of HMG's to nucleosomes. It is unlikely that a large excess of free HMG's or other nuclear proteins is available to non-specifically bind to 2- $\mu$ m minichromosomes during isolation.

The 2- $\mu$ m DNA plasmid is associated with folded chromosome isolated from logarithmically growing cells (47). When cells arrest at the cell cycle due to nitrogen starvation or the *cdc 28* gene product is inactivated at the non-permissive temperature, the plasmid is largely removed from the folded chromosome complex. It is interesting that, of the *cdc* mutations tested, only the *cdc 28* gene product affects both the mobility of 2- $\mu$ m DNA plasmid in particle gels and the association with the folded chromosome in sucrose gradients.

The reason for the change in electrophoretic mobility of 2- $\mu$ m chromatin when the cdc 28 mutation is expressed is not known. Such a significant change in mobility could only arise from either a major change in conformation or a significant change in composition. An association of a large fraction of the nucleosomes in the plasmid with HMG proteins could result in an unfolding of the minichromosome thus altering both sedimentation properties and electrophoretic mobility. HMG proteins or other chromatin modifications may be responsible for an overall conformational change in the minichromosome as well as an alteration of monomer nucleosome conformation.

Monomer nucleosomes appear to have subtle differences in conformation which allow them to be fractionated in particle gels (48). HMG 14 and 17 are associated with the slowest-migrating monomers. This association is not the sole source of heterogeneity since removal of HMG 14 and 17 does not affect nucleosome mobility. The differences in conformation appear to be induced by the  $\text{NH}_2$ -terminal highly basic segments of H3 and H4, since removal of about 30 residues (as well as HMG 14 and 17) results in a disappearance of mononucleosome heterogeneity.

Certain modifications of core histones such as acetylation could result in an altered conformation of chromatin, and it has been argued that histone acetylation is necessary for gene expression (50, 51). When chromatin is

fractionated into active and inactive regions following DNase II digestion, histone H4 appears to be hyperacetylated in the active fraction (52). Preliminary evidence from our laboratory indicates that sodium butyrate inhibits the deacetylase activity in yeast. In order to investigate the role of histone acetylation, a 2- $\mu$ m minichromosome preparation was isolated in the presence of sodium butyrate; however, no difference in 2- $\mu$ m chromatin mobility was observed (unpublished results).

The lack of change in mobility of 2- $\mu$ m minichromosomes isolated in the presence of sodium butyrate does not necessarily prove that histone acetylation is not a factor in 2- $\mu$ m chromatin conformation. Inhibition of deacetylase activity during isolation of 2- $\mu$ m chromatin from an asynchronous culture may have only a minor effect, if only a small percentage of the histones in minichromosomes are acetylated in the first place. Therefore, the possibility still exists that histone acetylation is a factor contributing to the change in electrophoretic mobility of 2- $\mu$ m chromatin when isolated from a synchronous culture arrested at the cdc 28 mutation.

Since it is 2- $\mu$ m chromatin that is electrophoresed in particle gels, the altered mobility due to the cdc 28 mutation could be a result of modifications to the proteins, modifications to the DNA, or an altered mode of interaction between the two.



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