Proc. Natl. Acad. Sci. USA
Vol. 76, No. 8, pp. 3727–3731, August 1979
Biochemistry

Isolation of a condensed, intracellular form of the 2-μm DNA plasmid of *Saccharomyces cerevisiae*

(yeast plasmid chromosome/histone proteins)

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Communicated by Jerard Hurwitz, May 14, 1979

ABSTRACT **We have initiated an investigation of the proteins bound *in vivo* to the 2-μm DNA plasmid found in the yeast *Saccharomyces cerevisiae* by examining its intracellular form. To isolate 2-μm DNA without disturbing proteins bound to the plasmid, an extract was prepared from a strain lacking mitochondrial DNA and the nuclear chromatin was removed from the extract by centrifugation. When the DNA in this extract was sedimented through a sucrose gradient containing 0.15 M NaCl, plasmid DNA had a sedimentation coefficient of approximately 70. This S value is greater than the S value of 25 for naked, superhelical 2-μm DNA. Cosedimenting with the DNA were proteins of the same size as the histone proteins associated with yeast nuclear chromatin. Digestion of the plasmid DNA with micrococcal nuclease and electrophoresis of the resulting DNA fragments revealed that segments of discrete sizes are protected from degradation. Examination of the plasmid DNA protein complex by electron microscopy showed nucleosome structures. We conclude that 2-μm DNA exists as a condensed chromosome body within the cell.**

Small, circular DNA molecules, such as coliphage φx174 and simian virus 40 DNAs, provide excellent models for studying the mechanism of DNA replication. One model system that has not been exploited in this regard is the 2-μm DNA plasmid of the yeast *Saccharomyces cerevisiae*. It is a double-stranded, circular DNA molecule of about the same size as simian virus 40 DNA and exists in approximately 100 copies per cell (1). In addition to its size, circular contour, and copy number, all of which facilitate laboratory manipulation, 2-μm DNA is a particularly significant model because its replication is similar to the replication of yeast nuclear DNA. Although both biochemical (1, 2) and genetic (3) studies suggest that 2-μm DNA may not reside in the nucleus, genes that control the initiation and completion of S phase within the yeast nucleus are also needed for 2-μm DNA replication (4, 5). Therefore, both nuclear and 2-μm plasmid DNA must share enzymes or binding proteins involved in DNA replication. As a first step in defining the proteins needed for 2-μm DNA replication, we have determined that 2-μm DNA exists as a histone-bound chromosome as does yeast nuclear chromatin (6–9).

MATERIALS AND METHODS

Strain Selection and Cell Growth. Strain A364a and media for cell growth have been described by Hartwell (10). To produce a petite strain (one lacking mitochondrial DNA), strain A364a (*a* $ade_{1,2}$ ura_1 his_7 lys_2 tyr_1) was grown in YM-1 media with 100 μg of ethidium bromide per ml for 10 generations, and then individual cells were permitted to form colonies on yeast extract/peptone/dextrose plates. Colonies were initially tested for their inability to grow on glycerol as a carbon source, and the DNA from individual clones was fractionated by isopycnic centrifugation to ensure the absence of mitochondrial DNA. One such petite strain, designated A364a (rho°), was selected for additional studies.

Strain A364a (rho°) was grown at 28°C in rich medium (YM-1) or in minimal medium (5) supplemented with glucose and the necessary nutrients. To radioactively label the DNA, the concentration of uracil in the minimal medium was decreased to 2 μg/ml and either [6-^{3}H]uracil was added to 1 μCi/ml (1 Ci = 3.7×10^{10} becquerels) or [2-^{14}C]uracil was added to 0.5 μCi/ml. Cells were harvested in late logarithmic phase, 5–7 × 10^7 cells per ml for rich medium and 1–2 × 10^7 cells per ml for minimal medium.

Preparation of Extracts. Unless otherwise noted all steps were carried out at 0°C. Cells were harvested by centrifugation at 1500 × *g* for 5 min. The cell pellet was suspended in 1 M sorbitol/20 mM K PO_4 buffer, pH 7.5/20 mM EDTA and subjected to centrifugation at 12,000 × *g* for 5 min. The cells were suspended in buffer A [20 mM Na Hepes, pH 7.5/0.15 M NaCl/5 mM KCl/1 mM EDTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/0.2% (wt/vol) Triton X-100] containing 1 M sorbitol such that 1 ml of buffer was added for every 1 × 10^{10} cells. Zymolyase 60,000 (Kirin Breweries, Takasaki, Japan) was added to 2 mg/ml, and the mixture was incubated at 37°C for 1 hr at which time microscopic examination revealed that most cells had lysed. The lysate was subjected to centrifugation at 27,000 × *g* for 30 min. The supernatant solution (fraction I) containing 2-μm DNA was decanted, and up to 2 ml of this fraction was passed through a Sepharose 2B column (0.9 × 23 cm) equilibrated with buffer A. Fractions were collected, the 2-μm DNA was located by agarose gel electrophoresis in the turbid fractions appearing at the void volume, and the peak fractions were pooled. The pooled fractions (totaling 3 ml) were subjected to centrifugation at 27,000 × *g* for 30 min, the supernatant solution (fraction II) was saved, and the pellet was discarded. In some experiments fraction II was passed through the Sepharose 2B column a second time.

Nuclear Chromatin Preparation. To recover nuclear chromatin from the extracts, the pellet remaining after centrifugation of the lysate yielding fraction I was suspended in buffer A (1 ml for every 1 × 10^{10} cells in the initial lysate) and subjected to centrifugation at 3000 × *g* for 5 min. This washing was repeated once more. The pellet was suspended in 1 ml of buffer A, layered on 15 ml of buffer A containing 2.6 M sorbitol in a $\frac{5}{8} \times 3\frac{1}{2}$ inch centrifuge tube, and sedimented for 1 hr at 27,000 rpm in a Beckman SW27 rotor. The pellet containing nuclear chromatin was suspended in buffer A and used for either micrococcal nuclease digestion or histone extraction.

Sedimentation Analysis. Up to 2 ml of fraction II was layered on top of a 15-ml linear gradient of 5–30% (wt/vol) sucrose in buffer A, and centrifugation was carried out for 15 hr at 18,000 rpm in a Beckman SW27 rotor. The amount of radioactively labeled DNA was detected by acid precipitation after alkaline hydrolysis as described (10).

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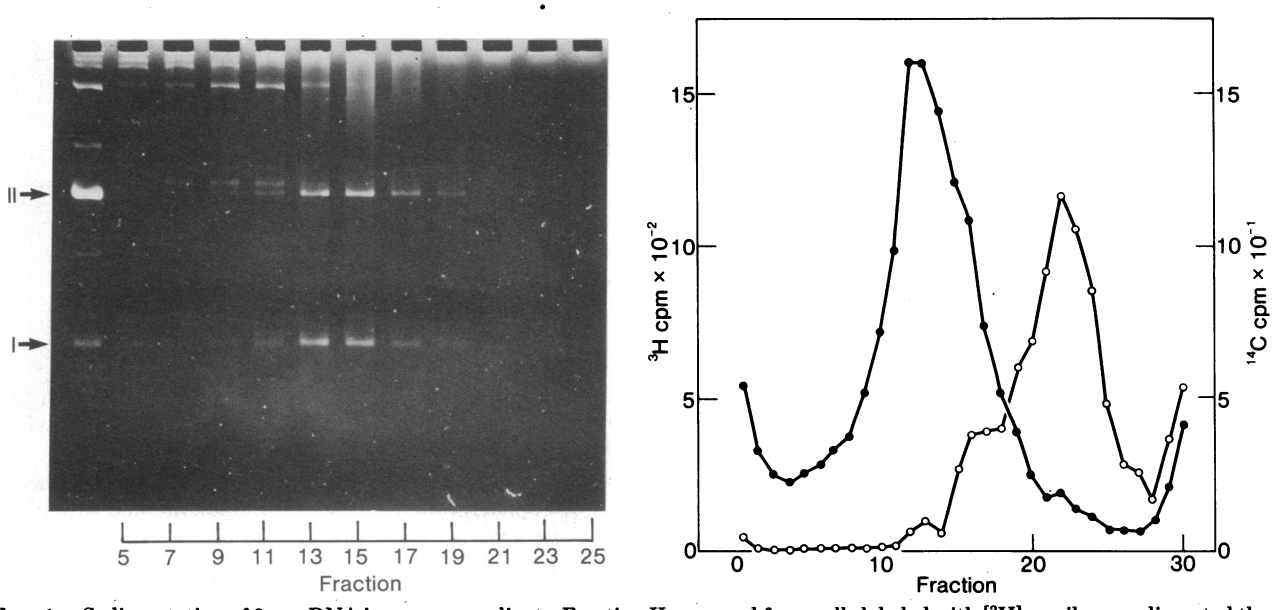

FIG. 1. Sedimentation of 2-μm DNA in sucrose gradients. Fraction II prepared from cells labeled with [^{3}H]uracil was sedimented through a sucrose gradient (*Right*). The direction of sedimentation is from right to left. ●, Alkaline stable, acid precipitable ^{3}H cpm from a gradient to which 1.5 ml of fraction II had been applied; ○, ^{14}C cpm from a sample of fraction II that had been treated with sodium dodecyl sulfate, ethanol precipitated, and taken up in 0.1 ml of 10 mM Tris·HCl, pH 7.4/1 mM EDTA before combination with the ^{3}H-labeled fraction and application to the gradient. The agarose gel (*Left*) shows the DNA recovered from fractions from the gradient. The lane to the far left contains purified 2-μm DNA, and the arrows point to the superhelical (I) and relaxed circular (II) forms of 2-μm DNA. Concatenates of plasmid DNA are responsible for the bands above these two forms (16). The 2-μm [^{14}C]DNA cannot be seen because it is present in less than visible amounts.

Gel Electrophoresis. DNA was precipitated from solution with ethanol and electrophoresed in an agarose gel as described (5). In some experiments the concentrated samples were not treated with pancreatic RNase so that ribosomal RNAs could be detected. Micrococcal nuclease digests were concentrated by ethanol precipitation as described above and electrophoresed in a gel containing 2.5% acrylamide and 0.5% agarose (11). Proteins were precipitated from solution with deoxycholate and trichloroacetic acid (12) and subjected to electrophoresis in an 18% polyacrylamide gel (13).

Micrococcal Nuclease Digestion. Fraction II was made 2 mM in $CaCl_2$ before addition of 0.05 units of micrococcal nuclease per ml (Sigma grade VI). Digestions were carried out at 37°C for various times and reactions were terminated by addition of EDTA to 10 mM and sodium dodecyl sulfate to 1% (wt/vol).

Electron Microscopy. Fractions from sucrose gradients were prepared for microscopy by addition of sodium sarcosinate to 0.02% and additional amounts of NaCl. The material was fixed with gluteraldehyde, placed directly on glow-discharged, carbon-coated grids, washed, and stained with uranyl acetate as described by Müller *et al.* (14). The grids were rotory shadowed at an 8° angle with 2 cm of Pt/Pd (80%:20%) wire wound around a tungsten filament (15). The current passing through the tungsten was sufficient to vaporize some of the tungsten. The material was viewed by using a Hitachi HU-11C transmitting electron microscope.

RESULTS

Purification of 2-μm DNA Chromosomes. The extraction prodedure was designed to release 2-μm DNA from cells without disturbing proteins bound to it. Cells were lysed in the presence of glucanases (Zymolyase) without prior recovery of spheroplasts. In such lysates the nuclear chromatin remains as viscous, high molecular weight material which is easily removed by centrifugation. As judged by examination of extracts electrophoresed in agarose gels (e.g., Fig. 3 *upper*), approximately 95% of the DNA in the supernatant solution was 2-μm plasmid DNA and its concatenated forms (16) and the remainder was nuclear chromatin DNA. This was a 400-fold purification of 2-μm DNA, which comprises only 3–5% of the cellular DNA (1). Greater than 50% of the 2-μm DNA was released by this procedure. Passage of the extract through a Sepharose 2B gel removed many of the ribosomes that sediment in sucrose gradients near the 2-μm DNA.

Sedimentation Velocity Analysis. When the 2-μm DNA in fraction II was sedimented through sucrose containing 0.15 M NaCl, its velocity was greater than twice the velocity of naked 2-μm DNA prepared by a detergent wash and ethanol precipitation (Fig. 1). The velocity of 2-μm DNA in fraction II was also slightly greater than that of the 60S ribosomal subunit (data not shown). From these results the 2-μm DNA in fraction II is estimated to have an S value of 70 in contrast to an S value of 25 for naked superhelical 2-μm DNA.

As a control, we determined whether the addition of naked 2-μm DNA to an extract would alter its sedimentation velocity. The sedimentation rate of naked, ^{14}C-labeled 2-μm DNA added to fraction II (Fig. 1) did not differ from the velocity of the same DNA sedimented alone in a separate gradient (data not shown). Therefore, the 70S value for 2-μm DNA in fraction II does not derive from a change in the DNA brought about by combination with proteins in the extract. Furthermore, when the NaCl concentration was increased to 0.60 M in the gradients, the sedimentation velocity of 2-μm DNA in fraction II decreased to approximately 60S. This change in sedimentation velocity with salt has been observed also for simian virus 40 chromosomes (14, 17). The results of the sedimentation analysis suggest that 2-μm DNA may exist as a protein-condensed chromosome within the cell.

Proteins Cosedimenting with 2-μm DNA. To determine whether the core histones (H2a, H2b, H3, H4) are complexed with 2-μm DNA, fraction II was passed through the Sepharose 2B column for a second time and the resulting pool was sedimented through sucrose. Fractions from the sucrose gradient were analyzed for proteins by electrophoresis in an 18% polyacrylamide gel. The result, shown in Fig. 2, is that a group of

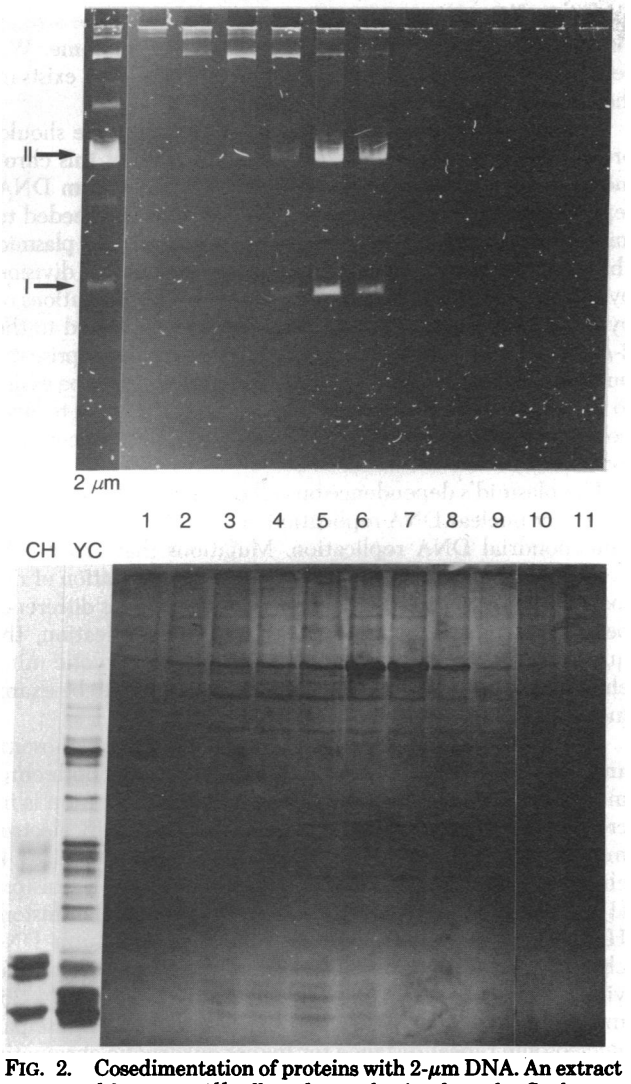

FIG. 2. Cosedimentation of proteins with 2-μm DNA. An extract was prepared from 1×10^{11} cells and passed twice through a Sepharose 2B column. The material was layered on four sucrose gradients and sedimented. Twelve equal fractions were collected from each gradient and the corresponding fractions from each gradient were combined. Two hundred microliters of fractions 1–11 were removed for analysis of DNA by agarose gel electrophoresis (*Upper*), and the remainder (4.0 ml) was precipitated and electrophoresed in a polyacrylamide gel (*Lower*). A standard of 2-μm DNA has been electrophoresed in the agarose gel in the lane labeled 2 μm (see Fig. 1 legend). Lane CH contains calf thymus histones (Sigma Type II-S); the core histones H2a, H2b, H3, and H4 are the dark bands near the bottom. Lane YC contains proteins removed from yeast nuclear chromatin. The numbers refer to the sucrose gradient fraction numbers. Sedimentation is from right to left.

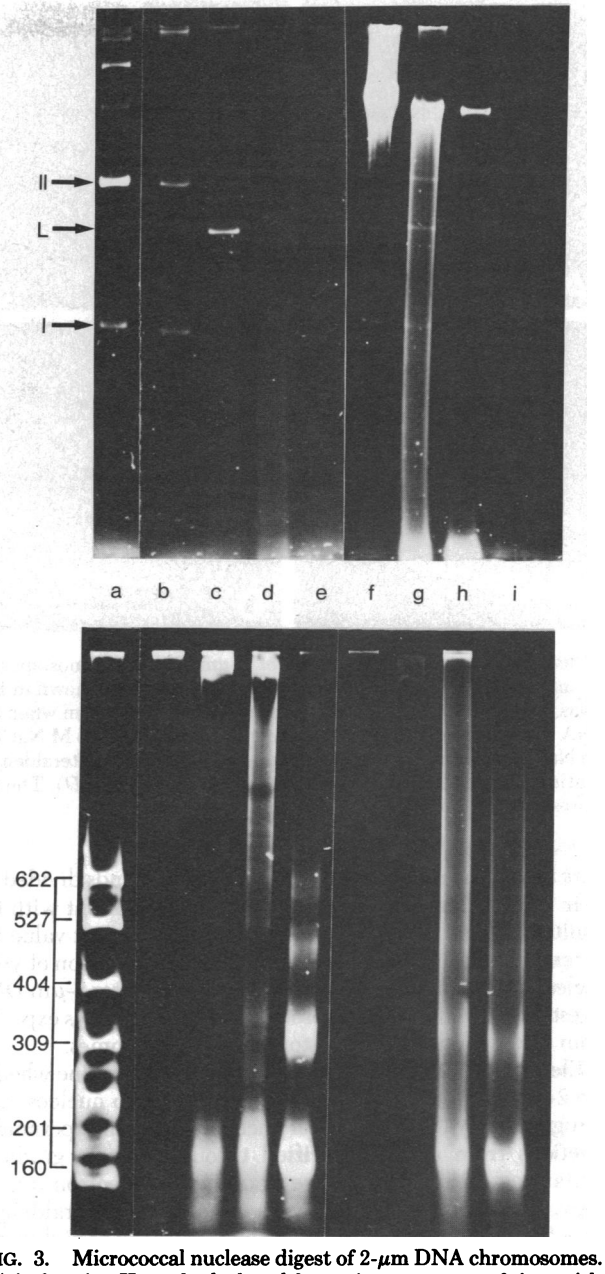

FIG. 3. Micrococcal nuclease digest of 2-μm DNA chromosomes. DNA in fraction II was hydrolyzed for various amounts of time with micrococcal nuclease and prepared for electrophoresis by ethanol precipitation and RNase digestion. Portions of each fraction were electrophoresed in a 0.7% agarose gel to display large DNA species (*Upper*) or in an acrylamide/agarose gel to reveal small species (*Lower*). Lane a contains a 2-μm DNA standard (*Upper*) or pBR322 DNA digested with the restriction endonuclease HpaII (*Lower*). Lanes b–e contain fraction II DNA digested for 0, 2, 10, and 30 min, respectively, with micrococcal nuclease, and lanes f–i contain nuclear chromatin digested for 0, 2, 10 and 30 min, respectively, with micrococcal nuclease. The arrows to the left of the agarose gel refer to the superhelical (I), relaxed circular (II), and linear (L) forms of 2-μm DNA. The numbers to the left of the acrylamide/agarose gel are the sizes in base pairs of some of the pBR322 restriction fragments.

small molecular weight proteins cosediment with 2-μm DNA. These proteins were similar to the proteins stripped from nuclear chromatin, which migrate at approximately the same rate as calf thymus core histones H2a, H2b, H3, and H4 (9, 18).

We did not quantitate the amounts of the presumed core histones bound to 2-μm DNA. The proteins of the 2-μm DNA chromosome must represent a very small fraction of the total cellular protein because attempts to radioactively label these proteins failed. Nevertheless, based on the assumption that chromatin contains nearly equal weights of DNA and histone proteins, the amount of presumed core histones observed in Fig. 2 is consistent with the amount of 2-μm DNA present and could not have resulted from contamination by nuclear chromatin.

Protection of DNA from Micrococcal Digestion. Indicative of histone interaction with DNA is the protection of the DNA from digestion with nucleases. When fraction II was digested with micrococcal nuclease and the DNA was displayed by electrophoresis in gels, a series of bands were seen (Fig. 3 *lower*). The bands were the same sizes as those obtained by digestion of yeast nuclear chromatin with micrococcal nuclease. The smallest fragment from the 2-μm DNA digest was 165 base

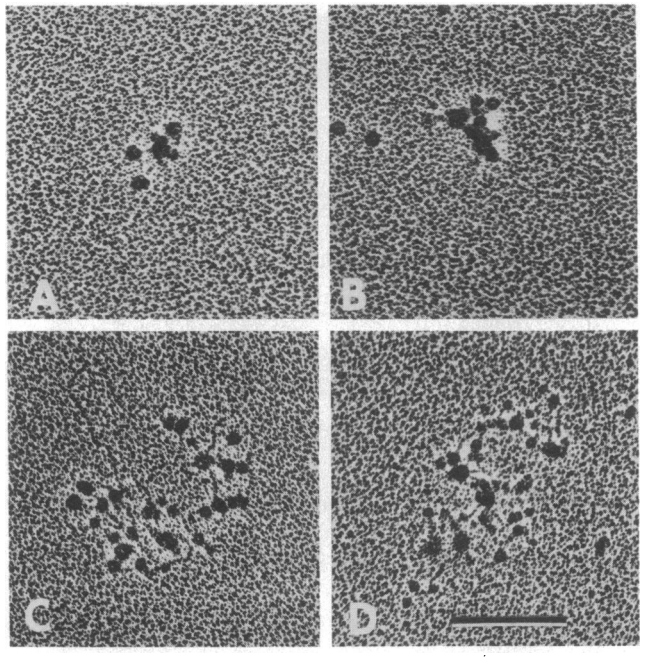

FIG. 4. Electron micrographs of 2-μm DNA chromosomes. A 0.1-ml sample of fraction 5 from the sucrose gradient shown in Fig. 2 was prepared for microscopy. (*A* and *B*) Molecules seen when the DNA was spread directly from gradients containing 0.15 M NaCl. If the NaCl concentration was increased to 0.50 M before gluteraldehyde fixation, the beaded nucleosomes could be seen (*C* and *D*). The bar represents 0.2 μm.

pairs in length and the average for all visible bands divided by their integral size was 162 base pairs, in agreement with the results of others (9, 19) who have obtained a repeat value between 160 and 165 base pairs after nuclease digestion of yeast nuclear chromatin. The pattern of bands seen after 2-μm DNA digestion showed a periodic protection of the DNA, as expected from DNA condensed by histones into nucleosomes.

Electron Microscopic Examinations. To determine whether the 2-μm DNA chromosome is condensed into nucleosomes, as suggested by the micrococcal nuclease digestion experiments, fractions from extracts purified through sucrose gradients containing 0.15 M NaCl were examined by electron microscopy. When the molecules were fixed with gluteraldehyde directly from gradient fractions, they appeared as highly condensed structures without a clear beaded definition as shown in Fig. 4 *A* and *B*. When additional NaCl was added before fixation, the molecules were more spread out and individual beads became visible. A concentration of 0.5 M NaCl was judged to be optimal, because at 0.4 M NaCl the molecules were only partially extended whereas at 0.6 M NaCl the molecules frequently contained stretches of filament lacking beads. At 0.5 M NaCl filaments containing a median value of 28 beads with a range between 21 and 34 beads were observed (Fig. 4 *C* and *D*). The diameter of these molecules is $\approx$0.2–0.3 μm, which is similar in size to the simian virus 40 chromosome under similar conditions (14, 17). Electron microscopy confirms that 2-μm DNA is condensed into nucleosomes.

DISCUSSION

We have begun an investigation of the intracellular form of 2-μm DNA by partially purifying the DNA as a small chromosome. The 2-μm DNA chromosome has a sedimentation velocity expected for a condensed chromosome, yields a set of protected fragments characteristic of chromatin when digested with micrococcal nuclease, and reveals nucleosomes when spread for electron microscopy. Proteins of the same size as histones cosediment with the 2-μm DNA chromosome. We believe that these results demonstrate that 2-μm DNA exists in the cell as a histone-condensed chromosome.

The ability to purify the 2-μm DNA chromosome should prove beneficial to an examination of the state of this chromosome during the cell division cycle. Because 2-μm DNA replication requires the same set of genes that are needed to initiate and to complete nuclear DNA replication (4, 5), plasmid chromosomes may be examined in cells arrested in cell division by temperature-sensitive cell division cycle (*cdc*) mutations or by other means (20). Presumably, the proteins bound to the 2-μm DNA chromosome are a subset of those that comprise the nuclear chromatin. In fact, nonhistone proteins may be easier to detect than histone proteins because ^{35}S can be used to label nonhistone proteins but cannot be used to label yeast core histones (18).

The plasmid's dependence on cell division cycle genes necessary for nuclear DNA replication (4, 5) is in contrast to yeast mitochondrial DNA replication. Mutations that arrest cells before they initiate S phase do not inhibit the initiation of mitochondrial DNA replication (21–23). In light of this difference between nuclear and mitochondrial DNA replication, the question of whether or not yeast and other eukaryotic mitochondrial DNAs are condensed by histones needs to be examined.

The physical properties of the 2-μm plasmid chromosome and the simian virus 40 chromosome are similar. Both chromosomes appear to unfold when the salt concentration is increased, as observed by sedimentation analysis and by electron microscopic examination. In the case of the simian virus 40 chromosome, this is the direct result of loss of the H1 histone (17). Additional work is needed to determine whether histone H1 loss is responsible for the unfolding of the 2-μm DNA chromosome. The number of nucleosomes seen on the simian virus 40 chromosome averages between 20 and 24 (14, 24–26), in agreement with a 5200-base pair length of a 200-base pair nucleosome repeat distance for higher eukaryotic chromatin. For the 2-μm plasmid chromosome we observed a median average of 28 beads with a maximum of 34 beads, which is smaller than predicted for a 6200-base pair length and a nucleosome repeat distance in yeast of nearly 165 base pairs (9, 19). Possibly, in our preparations the chromosomes are not unfolded sufficiently to permit observation of every nucleosome or some nucleosomes have fallen off during preparation. A more precise value for the number of nucleosomes would be helpful in corroborating the evidence from nuclease digestions that the yeast nucleosome repeat distance is smaller than that in higher eukaryotic organisms.

Finally, we note that the 2-μm plasmid chromosome is a unique chromosome. The DNA of eukaryotic nuclear chromosomes as well as the mammalian virus DNAs that replicate in the nucleus are condensed by histones. The yeast 2-μm plasmid is different from these other DNAs in that it is retained during mitotic and meiotic cell division as an extrachromosomal element (3). In order to be maintained through both mitosis and meiosis, virus DNAs or nucleolar DNAs that are also condensed by histones are dependent on an integrated state within the nuclear DNA. Although attempts to find an integrated state of 2-μm DNA have been made, the attempts have proven futile (27, 28). Although the chromosomal nature of the plasmid once again focuses attention on its unknown cellular location and function, the existence of a yeast plasmid condensed as a chromosome demonstrates that small chromosomes, such as the simian virus 40 chromosome, are not limited to the cells of higher eukaryotic organisms.

We thank Sharon Robinson of the Electron Microscopy Laboratory, (St. Paul, MN) for her help and advice. We also thank James Bodley for critically reviewing the manuscript and Nancy Martin for her suggestions through the course of this work. This work was supported by National Science Foundation Grant PCM77-18705.

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