
Subfractionation of soluble macronuclear chromatin and enrichment of specific genes as chromatin from *Euplotes eurystomus*

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ABSTRACT

Euplotes eurystomus is a hypotrichous ciliated protozoan possessing within one cytoplasm a transcriptionally-inactive micronucleus with chromosomal-size DNA and a transcriptionally active macronucleus with "gene-size" DNA fragments. The chromatin in the macronucleus can be isolated in a soluble form without prior treatment by nucleases. In this study, macronuclear, soluble chromatin was subfractionated using isokinetic sucrose density gradient ultracentrifugation in a buffer consisting of 50 mM NaCl, 1 mM Na₂ EDTA, 1 mM TEA HCl, pH 7.0, 0.1 mM TLCK and 0.1 mM PMSF. Fractions were collected and analyzed by DNA and protein gel electrophoresis, dot blot hybridization with specific gene probes, and modified Miller chromatin spreads. Analysis of the chromatin spreads showed that the sizes of the chromatin fragments in the various fractions correlate with the DNA size of the fragments. When dot blots of the fractions were hybridized with 5S rRNA, tubulin and rRNA gene probes we obtained about a 6 to 14-fold enrichment of hybridizable sequences in individual fractions. There appear to be differences in the non-histones present on each fraction as well as some overall similarities in histone and non-histone proteins.

INTRODUCTION

Ciliated protozoa possess two different kinds of nuclei within one cytoplasm: micronuclei, the germ line nuclei of the cells, which have important functions during sexual reproduction; and macronuclei (MAC), the somatic nucleus of cells which synthesize most, if not all, of the RNA. Studies of hypotrichous ciliated protozoa have shown that macronuclear DNA occurs in small linear molecules, while micronuclear DNA is of high molecular weight (1,2). The DNA molecules of the hypotrich macronucleus are large enough to contain only single or very few coding functions. For example, in *Oxytricha nova* a DNA molecule of 7400 base pairs (bp) codes for a single precursor RNA molecule that contains the 26S, 17S and 5.8S ribosomal RNAs (3). In *Oxytricha fallax* molecules of 1511 and ~1400 bp contain single actin genes (4,5); molecules of 2030 and 1880 bp carry single genes for β -tubulins; and a molecule of 2050 bp codes for a single α -tubulin gene (6). A molecule of 690 bp has been reported to code for 5S rRNA, but whether more than one

coding sequence is present is not known (7). In Stylonychia lemnae, two size classes of DNA molecules (1850 and 1730 bp) code for α -tubulin (8).

These "gene-sized" DNA fragments are small yet functional genetic units that must contain the sequence information required for regulation of their own expression and replication. Macronuclear DNA can be separated on agarose gels yielding a reproducible banding pattern with specific genes present in only one or a few bands. The macronuclear chromatin fragments are composed of nucleosomes (2,9-13), are soluble in low ionic strength buffers, and in both of the hypotrichs Oxytricha nova and Euplotes eurystomus migrate during gel electrophoresis in strict proportion to their DNA lengths (11,14). In the case of Euplotes eurystomus, a full complement of inner histones and a presumptive H1 are present in MAC soluble chromatin (11). The hypotrichs offer an excellent system to study the structure and composition of an eukaryotic gene as an intact soluble chromatin fragment.

Since macronuclear chromatin can be solubilized by merely lysing the nuclei in a low ionic strength buffer without the use of nucleases, procedures can be employed to fractionate this soluble native chromatin on the basis of size. Specific genes can be fractionated and enriched in their soluble chromatin form, and then assayed for composition and structural properties. MAC chromatin of two hypotrichs (Oxytricha nova and Stylonychia mytilus) has been fractionated using linear sucrose density gradient ultracentrifugation (9,14), and in the case of Stylonychia, a specific radiolabeled gene probe (rRNA) hybridized to the DNA of only a few of the fractions.

In this report, macronuclear soluble chromatin from Euplotes eurystomus was fractionated using isokinetic sucrose density gradient ultracentrifugation and the fractions were analyzed by DNA and protein gel electrophoresis, Southern and dot blot hybridization with three specific heterologous gene probes, and modified Miller chromatin spreads. The hybridization experiments show that specific genes are present in some of the fractions and that the fractionation resulted in differential enrichment for these specific sequences.

MATERIALS AND METHODS

Cell Culture and Chromatin Isolation

The Euplotes eurystomus cell line was obtained from Carolina Biological, N.C. Cell stocks were maintained in 100 mm dishes in Carolina spring water containing 6-7 previously boiled wheat seeds. Large-scale cultures of

Euplotes were grown in large photographic trays containing 5 L of Pringsheim salt solution and were fed live algae, Chlorogonium elongatum (University of Texas, Austin, Culture College of Algae).

Macronuclear soluble chromatin was prepared as described previously (11). Prior to loading on a sucrose density gradient, the chromatin (A_{260} of about 10) was concentrated using a Centricon microconcentrator (Amicon Corporation) with a molecular weight cut-off of 10,000 daltons in a SS-34 rotor at 6,000 rpm for 1 hr. The final DNA absorbance at 260 nm of the chromatin was ~150. No obvious aggregation was observed in the concentrated chromatin.

Sucrose Gradients of Macronuclear Chromatin

Fractionation of macronuclear soluble chromatin was performed on a 5-27% isokinetic sucrose density gradient in 50 mM NaCl, 1 mM Na_2EDTA , 1 mM TEA HCl, pH 7.0 containing 0.1 mM PMSF, and 0.1 mM TLCK, with Ct=5%, Cr=27%, $V_m=9.4$ mL and a particle density of 1.51 g/mL at 28,000 rpm for 8 hours in a Beckman SW41 rotor at 4°C (15,16). Between 5-10 A_{260} in a volume of 55-100 μ L were layered on one gradient. Fractions of 0.4 mL were collected using an ISCO gradient fractionator and the absorbance at 254 was monitored. The A_{260} of each fraction was also measured in a Zeiss spectrophotometer to determine the DNA content of each fraction.

DNA and Protein Gel Electrophoresis

For DNA gels, aliquots of fractions from the sucrose gradients were removed and 1/4 volume of 5X DNA sample buffer (12.5% Ficoll 400 DL (Sigma), 2.5X TBE (0.22 M Tris-borate, 0.22 M boric acid and 5 mM Na_2 EDTA) buffer, 0.2% xylene cyanol, 0.16% v/v saturated bromophenol blue) was added. The agarose DNA gel consisted of 1.25 % agarose (Biorad-Ultrapur DNA grade) containing 40 mM Tris-HCl, 5 mM Na acetate, 1 mM Na_2 EDTA (pH 7.9) and 1.0% sarkosyl. Gels were run for 4 hours at 100 volts using water cooling and buffer exchange. Alternatively, samples were treated with Proteinase K (200 μ g/mL) at 37°C for 30 min, then phenol extracted, precipitated with EtOH and dissolved in 1X DNA sample buffer. When purified DNA was electrophoresed, the agarose gel did not contain sarkosyl.

Electrophoresis of proteins was performed according to Laemmli (17) in 15% SDS-polyacrylamide gels (SDS-PAGE) using 14.0 x 17.8 x 0.1 cm slab gels, with water cooling (at room temperature) and the modified electrode buffer of Thomas and Kornberg (18). Protein samples were prepared by precipitating chromatin from aliquots of the sucrose gradient fractions in microfuge tubes by the addition of 0.2M sodium phosphate buffer (pH 7.6) to a final concen-

tration of 5 mM, followed by $MgCl_2$ to 6 mM, and 0.7 volumes of 96% ethanol (19). The sample was left for a minimum of 30 min at $-20^\circ C$, and then centrifuged in a microfuge for 10 min at $4^\circ C$ to precipitate the nucleoprotein particles. The supernatant was discarded and samples dried in a vacuum dessicator. Then 1X SDS sample buffer (17) was added to the precipitate to dissolve each sample. The samples were boiled for 1 minute prior to SDS-PAGE.

Dot and Southern Blot Procedures

DNA was transferred from agarose gels to nitrocellulose (BA-85 Schleicher and Schuell) using the procedure of Southern (20). Aliquots from sucrose density gradient fractions were examined for specific sequence contents also by direct dot-blotting of chromatin samples on nitrocellulose without prior DNA purification (21). First, Proteinase K was added to a sample to a final concentration of 100 $\mu g/mL$ and incubated at $42^\circ C$ for 30 minutes. NaOH was added to a final concentration of 500 mM and incubated 15 min at $25^\circ C$. Then HCl was added to neutralize the NaOH and 20X SSC added to a final concentration of 12.6X and samples were chilled at $4^\circ C$. Five hundred microliter aliquots (1 μg DNA) were spotted in each of three different nitrocellulose filters enclosed in a minifold apparatus (Schleicher and Schuell, Inc.). The filters were hybridized with three different gene probes: 1) pDP5 - this plasmid contains 30 base pairs of one 5S rRNA gene from MAC DNA of Tetrahymena thermophila and 90 bp of an adjacent 5S gene (22). The plasmid was a gift from David S. Pederson (NIH), 2) pmacC9 - this hybrid plasmid was constructed with pBR322 and full length macronuclear DNA from Stylonychia lemnae and contains the 1850 bp α -tubulin gene (8). This plasmid was kindly given to us by E. Helftenbein (University of Tübingen, Federal Republic of Germany), 3) pMS7.5 - a plasmid containing the ribosomal gene (~7.5 kb) from Euplotes aediculatus (3) which was generously provided by Marshall Swanton (University of Colorado, Boulder). The filters hybridized to the α -tubulin and ribosomal RNA gene probes were washed at $65^\circ C$ in 2X SSC, 0.1% SDS, while those that were probed with the 5S rRNA gene probe were washed at $42^\circ C$ in 6X SSC, 0.1% SDS. All filters were exposed to X-O-MAT film using two Cronex intensifying screens, and the spot densities were measured with an LKB 2202 Ultrascan Laser Densitometer interfaced to an Apple IIe computer.

Electron Microscopy

Soluble MAC chromatin fractions were diluted with 1 mM Na_2EDTA , 1 mM TEA HCl, 50 mM NaCl, pH 7.0 to an A_{260} of about 1.0, then were placed on a carbon

film which had been freshly glowd in the presence of amyl amine vapor for 30-60 seconds, rinsed in dilute photoflo, air-dried and stained with 0.1% uranyl acetate in glass distilled water (23). Chromatin was visualized in the darkfield mode on a Siemens Elmiskop 102 electron microscope operated at 80 kV (24). Nucleosomes were counted on 3 times magnified prints of negatives taken at an instrument magnification of 60,000 X.

RESULTS

Soluble macronuclear chromatin from Euplotes was fractionated on 5-27% isokinetic sucrose density gradients. Under the conditions employed, most of the chromatin sedimented in the bottom two thirds of the gradient. Figure 1(b) shows the DNA size distribution of the chromatin fragments of in each fraction after gel electrophoretic separation on a 1.25% agarose gel. One can observe a good correlation between the sedimentation position of the chromatin fragment and the size distribution of its DNA; since, for example, the most rapidly sedimenting chromatin fractions contain the highest molecular weight DNA. Figure 1(d) also shows darkfield electron micrographs of macronuclear chromatin from sucrose gradient fractions spread onto glowd carbon film. Micrographs were analysed to determine the range of the number of nucleosomes per chromatin fragment present in each fraction. Assuming a nucleosome repeat length of 186 bp for macronuclear chromatin (11), the distribution of DNA length was calculated for each chromatin fraction. The DNA size range data obtained from DNA gels and chromatin spreads was plotted against fraction number. The two sets of data (i.e. from chromatin spreads and DNA gel electrophoresis) correlate very well. There is a slight difference in the DNA sizes calculated for the fractions in the upper part of the gradient, which contain the slower sedimenting chromatin fragments (Fractions 9-12). This may be due to the fact that the chromatin spreads of those fractions were particularly dilute and we analyzed fewer molecules for Fractions 9-12 than for the rest of the fractions. Still, the DNA sizes of the chromatin fragments calculated using the DNA gel methods are very well within the range of those calculated by analysis of the spread chromatin molecules.

Figure 2(a) shows the results obtained when gel electrophoresis of Euplotes MAC DNA, separated on the basis of size, was transferred to a nitrocellulose filter by the Southern technique (20) to detect the DNA size of specific macronuclear genes using heterologous gene probes. On the right hand side of the figure is the ethidium bromide-stained DNA gel and the left

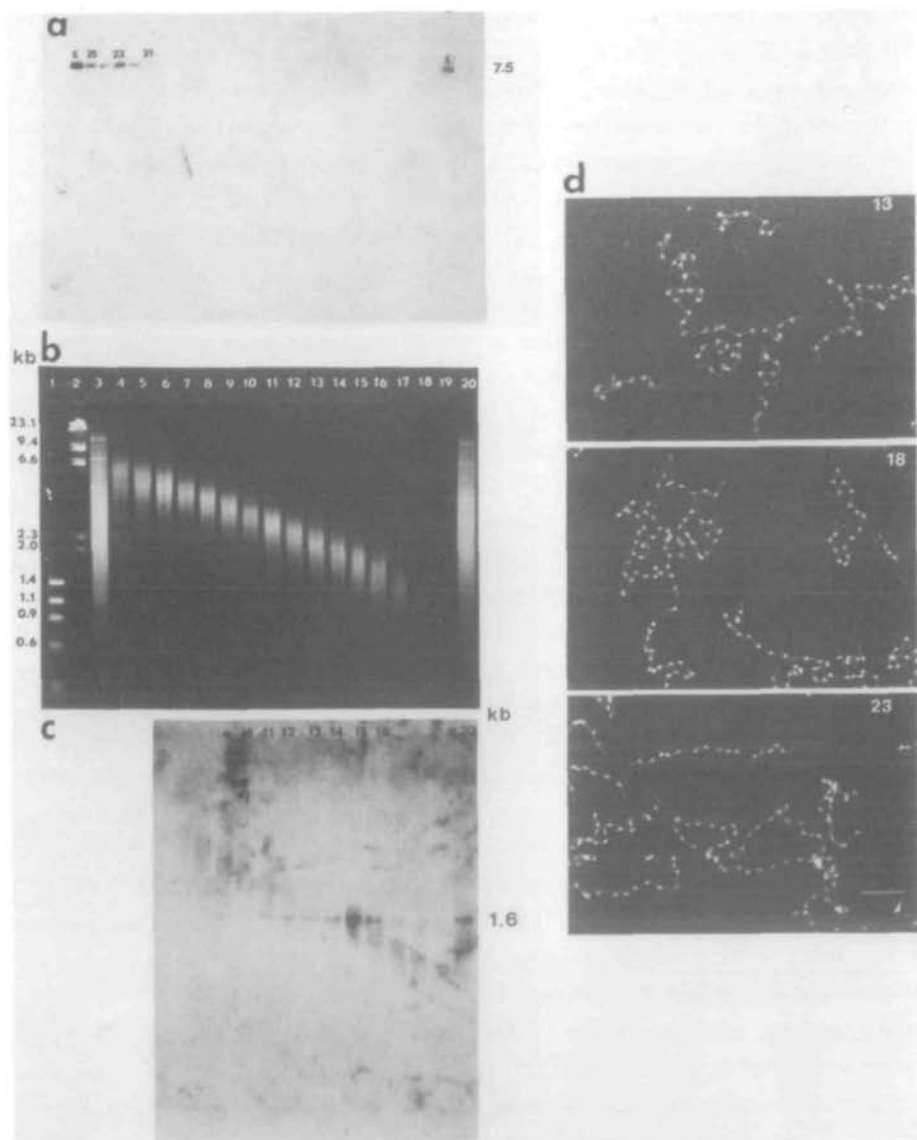


Fig. 1(a): Autoradiogram of Southern blot of a DNA gel hybridized with the rRNA gene probe from *Euplotes aediculatus*. Lanes labeled E, *Euplotes* macronuclei; lanes labeled 25-21, isokinetic sucrose gradient macronuclear chromatin fractions 25-21. (b): DNA gel electrophoresis of purified DNA from: lane 1, ϕ X 174 Hae III restriction fragments; lane 2, λ Hind III restriction fragments; lane 3 and 20, *Euplotes* macronuclear chromatin; lanes 4-19, macronuclear chromatin fractions 24-9. (c): Autoradiogram of

the Southern blot of the DNA gel in (b), hybridized with the α -tubulin gene probe from Stylonychia lemnae. Lane numbers are the same as indicated for the DNA gel in (b). (d): A sample of darkfield electron micrographs of fractionated macronuclear chromatin from Euplotes. Chromatin was placed on carbon film freshly glowd in the presence of amyl amine vapor and rinsed, dried and stained as indicated in Materials and Methods. (Bar = 0.1 μ m). The fraction number, corresponding to Fig. 1(b), of the individual samples are indicated on each micrograph.

hand side of the figure displays the autoradiographic results. When a radiolabelled heat shock (hsp 70) gene probe from Drosophila (25) (obtained from Carl Sirotkin, University of Tennessee), lane H, is employed for hybridization to MAC DNA, a single DNA band of ~2300 bp can be identified. When the α -tubulin gene probe from Stylonychia (8), lane T, was employed, a single DNA fragment of 1600 bp hybridized to it whereas when the Tetrahymena 5S rRNA gene probe (22) was utilized, lane 5, one band of 1100 bp was detected. The rDNA gene, lane R, from Euplotes aediculatus (3) hybridized to a single DNA band of 7500 bp. The genes detected in this manner compare very well with the sizes of these genes in the organisms from which the gene probes were derived. For example, molecules of Oxytricha fallax MAC DNA that contain sequences homologous to Tetrahymena thermophila 5S rRNA are 690 bp in length, while those homologous to rRNA are 6670 bp in length (7).

The individual sucrose gradient chromatin fractions were analyzed for the presence of specific gene sequences. Three different gene probes, the rRNA gene, the α -tubulin gene and the 5S rRNA gene (Figure 2(a) and refs. 3,8,22) were utilized in dot blot and Southern blot hybridizations. Figure 2(b) summarizes the dot blot hybridization results in terms of the fold enrichment of specific genes as chromatin fragments. The rRNA gene hybridizable sequences are present in the bottom part of the gradient (Fractions 17-bottom), while the 5S rRNA sequences are present in the upper part of the gradient (Fractions 6-9). The sequences that hybridized to the tubulin gene probe were present in the middle part of the gradient (Fractions 9-12). These results were consistent with the expected distribution of discrete gene sized chromatin fragments that vary in their DNA length.

The dot blot autoradiograms were analyzed densitometrically to determine the enrichment of specific sequences in individual fractions. The fold enrichment equals the hybridization intensity of fractionated DNA divided by the average hybridization intensity of all fractions. For the 5S rRNA gene we obtained a 6.9-fold enrichment of hybridizable sequences in fraction 7. The rRNA hybridizable sequences were enriched 14.0 fold in fraction 21 and

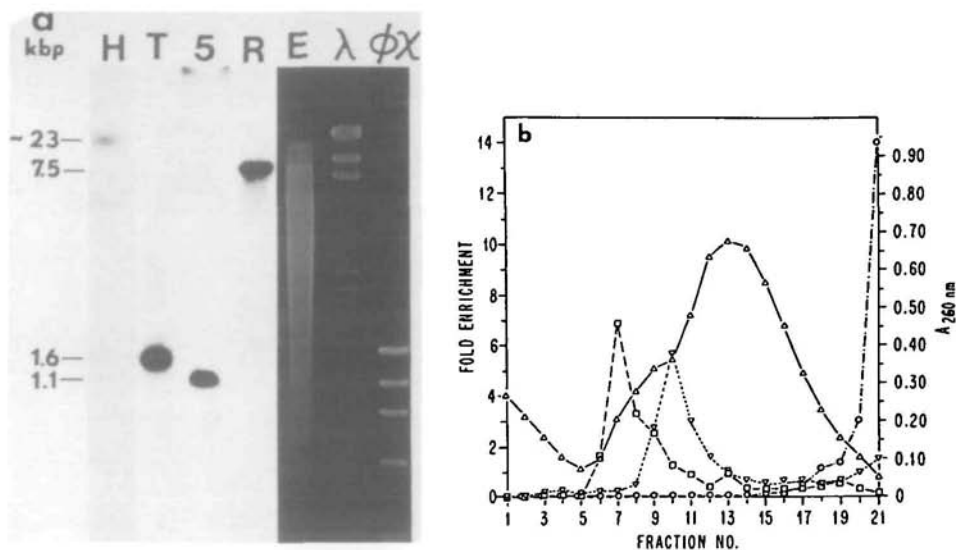


Fig. 2(a): Autoradiograms of Southern blots of macronuclear DNA hybridized with, from left to right: lane H, hsp 70 gene probe from *Drosophila*; lane T, α -tubulin gene probe from *Stylonychia*; lane 5, *Tetrahymena thermophila* 5S rRNA gene probe, lane R, Ribosomal RNA gene probe from *Euplotes aediculatus*. Right: DNA gel electrophoresis of *Euplotes* macronuclear DNA and molecular weight markers stained with ethidium bromide. From left to right: lane E, *Euplotes* macronuclear DNA; lane λ , λ Hind III restriction fragments; lane ϕ X, ϕ X 174 Hae III restriction fragments. (b): Fractionation of macronuclear soluble chromatin and enrichment of individual genes as chromatin molecules. Plot of: left ordinate, fold enrichment of specific sequences in individual fractions calculated as described in the Results section; right ordinate, absorbance at 260 nm of individual fractions; abscissa, fraction number. Triangles (Δ) represent the absorbance at 260 nm of individual fractions, squares (\square) represent fold enrichment of 5S rRNA hybridizable sequences, inverted triangles (∇) the fold enrichment of α -tubulin hybridizable sequences, and the hexagons (\hexagon) the fold enrichments of rRNA hybridizable sequences.

those sequences that hybridize to the α -tubulin gene probe were enriched 5.8 times in fraction 10.

To determine whether these specific DNA fragments remained intact during our fractionation procedure and to determine the sizes of the MAC DNA fragments that hybridized to the gene probes in the dot blots, DNA samples from individual fractions were run on agarose gels for native DNA separation and the DNA transferred to nitrocellulose. 32 P-labelled gene probes were utilized for hybridization. Autoradiograms showed (see Figure 1(a) for rRNA and Figure 1(c) for α -tubulin gene probe results) that the hybridizing sequences were present in a few of the fractions, and that the gene size of

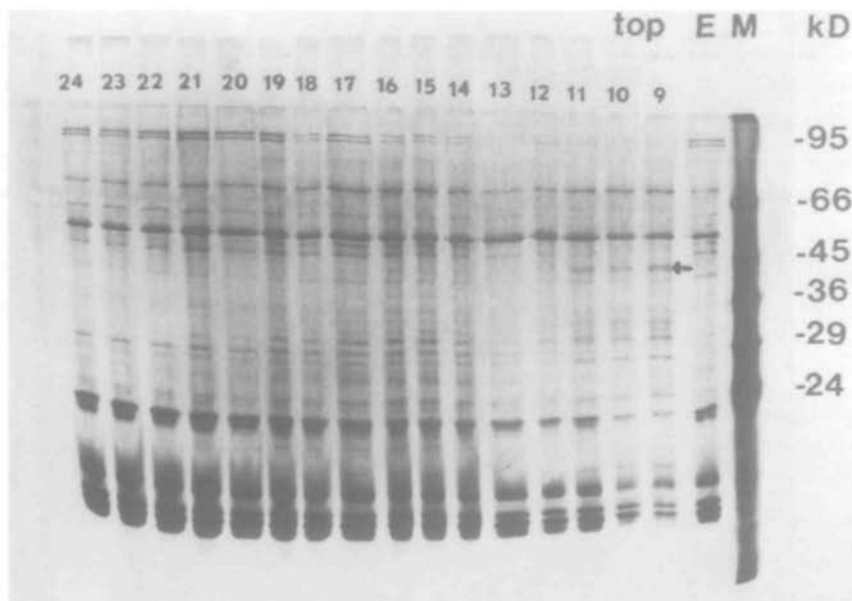


Fig. 3. SDS-polyacrylamide gel electrophoresis of macronuclear chromatin proteins. Chromatin from individual fractions was precipitated as described in Materials and Methods and precipitates dissolved in 1X SDS sample buffer. Samples were, from left to right: lanes labeled 24-9 correspond to chromatin proteins from fractions 24-9; lane E, *Euplotes* macronuclear chromatin proteins; lane M, protein molecular weight markers for SDS gel electrophoresis (Sigma).

the DNA fragments that hybridized to the different gene probes was the same as those detected for purified macronuclear DNA [see Figure 2(a)]. No degradation of the DNA occurred during the fractionation.

The protein content of each fraction was analysed using SDS-PAGE. Figure 3 shows a typical gel: Lanes labeled 24 through 9 are proteins from fractions 24 to 9 (DNA size range of these same fractions shown in Figure 8). Lane E shows the proteins of unfractionated *Euplotes* macronuclear chromatin and Lane M shows molecular weight standards. There are many similarities and also some differences in the protein content of the different fractions. All fractions examined contain the core histone proteins, the 5% PCA extractable 17 kDa protein (11), the 18 kDa band, a prominent band at 53 kDa, and a 67.6 kDa band. Furthermore, two bands of about 100 kDa are present in all of the fractions in varying intensities. There are some differences in the nonhistone protein content of individual fractions. For example, fractions 9-11 have a 38 kDa protein that is absent in all the other fractions.

DISCUSSION

The analysis of the chromatin fractions obtained from isokinetic sucrose density gradient ultracentrifugation indicates that the sedimentation coefficient of the chromatin molecules correlates with their DNA size, as determined using DNA gels and electron microscopy measurements. The technique maintains protein integrity (since no degradation is observed in SDS-PAGE gels) and maintains nucleosomal chromatin structure (since the nucleosomal organization of these molecules can be visualized in the EM using modified Miller spreading techniques). This fractionation procedure can be used to enrich for specific genes as intact soluble chromatin molecules.

The data clearly illustrate the advantages that the chromatin of the hypotrich macronucleus offers for the study of individual genes in their soluble chromatin form. To obtain gene-size fragments from most other eukaryotic nuclei the chromatin must be digested with nucleases. This digestion may perturb the chromatin organization, with specific genes cleaved into a wide range of sizes and with little register, whereas the fragments containing each type of macronuclear gene are apparently homogeneous in length and sequence, and terminated with native telomeres (26).

In the present work we have utilized these advantages to achieve enrichment of specific gene sequences as chromatin molecules. The protein content of individual fractions indicate that several proteins are common to all gene-sized chromatin fragments. Core and putative linker histones and several nonhistone proteins are present in all of the fractions examined. The significance of the differential nonhistone protein content of individual fractions is not known at this time, and will be the subject of future studies in this lab. Antibody probes are being developed (27) to identify the many nonhistone proteins associated with macronuclear chromatin and in individual subfractions.

The data indicate that specific genes are enriched in a few of the fractions. These results make possible the study of individual genes in an intact manner, in their soluble chromatin form. Further purification of specific gene-containing chromatin molecules by recycling the fractions with repeated isokinetic density gradient ultracentrifugation could result in higher enrichment yields. It is also feasible that other means of chromatin fractionation, like ECTHAM-cellulose chromatography (28), can be used to enrich for active vs. inactive chromatin molecules of specific genes. The biophysical and biochemical properties of individual chromatin fractions could be studied also (11,29-31).

This subfractionation method has considerable potential for the study of chromatin organization in active vs. inactive chromatin. Possible applications of this method include assaying for structural changes of chromatin associated with gene expression and monitoring the addition or removal of nucleoproteins. For example, chromatin from heat-shocked and control cells could be fractionated and analyzed to determine whether heat shock induced genes are sedimenting at the same rate in treated vs. untreated cells. In addition, DNase I hypersensitivity assays could be performed to determine whether individual fractions are enriched in DNase I hypersensitive chromatin, which has been shown to be a characteristic of active chromatin (32).

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