

AN ABSTRACT OF THE THESIS OF

Christopher S. Szent-Györgyi for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on October 15, 1982.

Title: AN ELECTROPHORETIC INVESTIGATION OF NUCLEOPROTEIN ORGANIZATION IN YEAST CHROMATIN

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I have investigated nucleoprotein organization in chromatin of the yeast, Saccharomyces cerevisiae. My approach has been to prepare insoluble, minimally perturbed chromatin from a mitochondrial DNA-free strain of yeast, using a number of new techniques, and then digest this substrate with micrococcal nuclease. The soluble products are electrophoresed on a newly devised high resolution native nucleoprotein gel; DNA and protein constituents of the resolved complexes are analyzed by means of second dimension electrophoresis. I find that yeast nucleoproteins resolve into two bands for each DNA size class, lack a chromatosome, display a distinctive non-random pattern of nuclease excision for dinucleosomes and larger species, and have a markedly reduced complement of mononucleosomes when prepared in this manner. A single non-core protein of about 32,000 daltons appears to be bound to oligonucleosomes, but in modest amount, and

primarily on the trinucleosome. A very prominent 40 kilodalton protein is selectively released by RNase, but is not associated with oligonucleosomes during native electrophoresis. These findings are discussed, with emphasis on nucleosome-nucleosome interactions and mononucleosome stability.

An Electrophoretic Investigation of Nucleoprotein
Organization in Yeast Chromatin
by
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AN ELECTROPHORETIC INVESTIGATION OF NUCLEOPROTEIN
ORGANIZATION IN YEAST CHROMATIN

PREFACE

Studies of yeast chromatin have demonstrated a nucleosomal DNA repeat (1,2), and the existence of four inner histones (2,3), but little information exists as yet on the in vivo arrangement and composition of yeast deoxyribonucleoprotein complexes (4,5).

It is known that mono- and dinucleosomes, excised from mammalian or avian chromatin, have various non-core proteins associated with them. H1 and its subtypes, H5, and the high mobility group proteins (HMGs) 1,2, 14 and 17, are complexed with subsets of short oligonucleosomes released by micrococcal nuclease (6,7). The protein ubiquitin is covalently bound through an isopeptide linkage to a small percentage of the H2A and H2B molecules to form the conjugate proteins uH2A and uH2B (also termed A-24 proteins) in certain of these nucleosomes (8,9,10). In contrast, knowledge of the proteins associated with yeast oligonucleosomes is still lacking.

The presence of H1 in yeast has not yet been demonstrated (2,3,4,11). Although four polypeptides with typical HMG amino acid compositions have been isolated, no homology with mammalian HMGs has as yet been demonstrated, nor has it been shown that any of these polypeptides are specifically bound to mono- or oligonucleo-

somes (12). An isopeptidase activity that specifically cleaves the ubiquitin adduct from either free or chromatin-complexed mammalian uH2A is found in yeast as well as in other eukaryotes, but so far there is no report of these conjugates in yeast (13,14).

The principal method of investigating chromatin nucleoprotein heterogeneity is to resolve micrococcal nuclease digestion products on a low ionic strength non-denaturing gel, and subsequently to analyze either the protein or DNA constituents on various second dimension gel systems (6,15). Electrophoretic mobility in the first dimension native gel is dependent primarily on the nature and stoichiometry of the non-core proteins bound to the mono- or oligonucleosomes. A second order dependency of mobility on DNA length does not seriously interfere with nucleoprotein resolution (6).

Recently a subset of chicken erythrocyte nucleosomes enriched in transcriptionally competent DNA sequences has been isolated by HMG-affinity chromatography (16,17). These nucleosomes, which are specifically rendered DNase I sensitive by binding HMGs 14 or 17, have when stripped of non-core proteins, a different mobility in moderate ionic strength gels than do stripped bulk chromatin nucleosomes (17,18).

Yeast chromatin is reported to be uniformly DNase I sensitive (19), although chromatin from actively transcribing cells is digested more rapidly than the chromatin of

transcriptionally quiescent cells (20).

This thesis reports the analysis of micrococcal nuclease solubilized chromatin from growing yeast, using a new high resolution native nucleoprotein gel. I describe a method of preparing stable yeast chromatin that is designed to minimize structural perturbations. Chicken erythrocyte chromatin is analyzed in the same way in order to compare the compositional and structural differences between the two eukaryotes.

PART I: DISCONTINUOUS GEL ELECTROPHORESIS OF NATIVE
CHROMATIN NUCLEOPROTEINS

INTRODUCTION

Electrophoresis of native protein-nucleic acid complexes is a common method in the study of chromatin (21,22), where after a first dimension separation of complexes, a number of high resolution gel systems can be applied in a second dimension for the analysis of protein or DNA constituents (22,23,24). However, current native chromatin gels do not employ stackers. I show here that the use of a stacker greatly improves the resolution of nucleoproteins. A stacking gel is desirable not simply to improve resolution, but also to locally concentrate individual complexes, and thereby make easier the detection of minor species by techniques such as DNA hybridization (24,25), immunautoradiography (25), or in gel enzyme assays (26).

I have modified a discontinuous buffer system originally designed for the high ionic strength electrophoresis of proteins (27) to the low ionic strength electrophoresis required for chromatin nucleoproteins. Glycerol has been added to stabilize native nucleoprotein structure (28), and to prevent dissociation of the complexes.

The large size of chromatin nucleoproteins requires the use of a large pore stacker to avoid molecular sieving effects. I have found that low percentage agarose will

make an excellent stacker, provided one uses a number of specific techniques for the pouring and stabilizing of the gel.

The high capacity discontinuous gel system I describe here is capable of sharply resolving large anionic species that are released from chromatin by micrococcal nuclease digestion.

MATERIALS AND METHODS

Materials

Acrylamide, N,N'-methylene-bis-acrylamide, and xylene cyanole FF were purchased from Bio-Rad; 5,5'-diethyl barbituric acid, EDTA (free acid), and agarose (type VI, high gelling temperature) were obtained from Sigma Chemical Company. All other chemicals were reagent grade.

Gels were run on a slab apparatus, using 13 x 25 cm gel plates separated by 0.8 mm thick Teflon spacers. The Teflon well-former had teeth of dimensions 25 x 6 x 0.8 mm; adjacent teeth were separated by 6 mm.

The separating gel was 8.0 cm long, and the stacking gel was 3.3 cm long as measured from the bottom of the sample wells. A stacking gel of this length is necessary to fully stack sample volumes of 50 μ l in this apparatus; this corresponds to a stacker length of about 2.5 times the height of the sample load.

Clean gel plates were silanized in a fume hood by immersion overnight in 5% dimethyldichlorosilane in toluene, followed by a thorough rinsing, first with toluene, and then with water (29). Silanized plates were cleaned prior to each use with a mild detergent. Prolonged soaking in detergent was avoided because it led to loss of silanization.

Table I shows the composition of the separating and

stacking gels, and also the electrode and sample buffers.

Pouring of the Gel

The major separating gel components were equilibrated on ice prior to the addition of the polymerization catalysts. The mixture was stirred, poured between the plates at room temperature, overlaid with a few drops of 1 X separating buffer saturated with n-butanol, and polymerized for at least 30 minutes.

After removal of the overlay, the plates and separating gel were warmed to about 40° C in an oven. The agarose stacking mixture was melted by slowly bringing it to a boil in a foil-covered beaker on a stirring hotplate (heating in a microwave oven often led to volume changes). The hot stacking mixture was promptly poured between the plates, and the well-former quickly inserted so that the comb slots extended about 5 mm above the top of the gel. The gel was then cooled in a refrigerator or coldroom for at least one half hour.

The gel comb was removed while the plates were still cold and tightly clamped, and before the addition of electrode buffer. Excess agarose was trimmed off the gel top with a razor blade.

I found it necessary to avoid having the comb fully inserted; otherwise it is difficult to remove. Attempts to facilitate comb removal by wedging the plates slightly apart at one end destroyed the stacking capability of the

system.

Running of the Gel

The electrode buffer was poured into the apparatus reservoirs, and air bubbles and excess glycerol removed from the sample wells with a syringe having a fine needle. Equal volumes of experimental sample were loaded into each well; dummy samples (1 X sample buffer) of the same volume were loaded into any extra wells.

Electrode buffer was recirculated with a pump. Gels were run at 4° C towards the anode at a constant 75 volts, until the xylene cyanole marker had travelled about 7/8 of the length of the separating gel (about 40 hours). If the marker dye is run further than this, it suddenly diffuses abnormally. Increasing the voltage in an attempt to shorten the running time led to deformation of the stacking gel.

TABLE I

COMPOSITION OF GELS AND BUFFERS^{a,b}

	<u>Stock Solution</u>	<u>Volume % Used</u> ^c
SEPARATING GEL ^h	10 X separating buffer ^d	10.
	50% anhydrous glycerol (v/v)	60.
	acrylamide (T=30%, C=5%) ^e	20.
	distilled H ₂ O	9.10
	10% ammonium persulfate (w/v) ^f	0.75
	TEMED ^g	0.15
STACKING GEL ^j	10 X stacking buffer ⁱ	10.
	50% anhydrous glycerol (v/v)	60.
	distilled H ₂ O	30.
	agarose	0.70

Electrode buffer is 15 mM 5,5'-diethyl barbituric acid, 0.5 mM EDTA, adjusted to pH 7.0 with Tris base. This is always freshly prepared.

3 X sample buffer is 90% anhydrous glycerol (v/v), 0.01% xylene cyanole FF (w/v), 1.5 mM EDTA, 45 mM Tris base, adjusted to pH 5.5 with H₃PO₄.

^a Modified from the discontinuous buffer of Williams and Reisfeld (27).

^b Glycerol use follows Todd and Garrard (28).

^c All percentages are v/v, except agarose (w/v).

^d 10 X separating buffer is 150 mM Tris base, 5.0 mM EDTA, adjusted to pH 7.5 with HCl.

^e Definition of T and C (30).

^f This is always freshly prepared.

^g N,N,N',N'-tetramethylethylenediamine

^h Final separating gel composition is 6% acrylamide (19:1 acrylamide:bis acrylamide), 30% glycerol (v/v), 0.5 mM EDTA, 15 mM Tris-HCl, pH 7.5.

ⁱ 10 X stacking buffer is 150 mM Tris base, 5.0 mM EDTA, adjusted to pH 5.5 with H_3PO_4 .

^j Final stacking gel composition is 0.70% agarose (w/v), 30% glycerol (v/v), 0.5 mM EDTA, 15 mM Tris- H_3PO_4 , pH 5.5.

RESULTS AND DISCUSSION

Figure 1 shows a gel of chicken erythrocyte chromatin partially digested with micrococcal nuclease. It can be seen that the gel is capable of high resolution over a wide range of sample loadings. The two chromatosomes and the core nucleosome have relative mobilities similar to the same nucleoproteins run on a low ionic strength gel system that does not contain glycerol (7). It is evident that a significant stacking capacity is retained even at a drastically reduced ionic strength of gel buffer (15 mM instead of 410 and 565 mM in the original stacking and separating gels), although the additional components (EDTA and glycerol) may also contribute in this regard.

I observe that the electrophoretic banding pattern is sometimes dependent on the amount of sample loaded; in particular, a heavily loaded band (about 2-3 μ g DNA as chromatin, on this apparatus) can distort the mobility of nearby minor bands to a point where resolution is largely eliminated.

Thirty percent glycerol has previously been used in a low ionic strength chromatin gel (28). The glycerol is reported to prevent the loss of non-inner histone proteins from nucleosomes (28), and also to mediate an apparent compaction of oligonucleosomes, when reconstituted with about 2 moles H1 per mole nucleosomes (31).

Glycerol has long been used to stabilize protein

preparations. It has been demonstrated that glycerol at this concentration range stabilizes the native tertiary structures of a wide variety of proteins (32), and that the formation of certain protein complexes is favored by its presence (33). Although the mechanisms by which glycerol exerts its effects on proteins are still being explored (32-34), its properties may make it especially applicable to the electrophoresis of chromatin.

Glycerol also affects the mechanical properties of this gel system. Both glycerol and silanized plates are essential to the function of the stacking gel; in the absence of either, sample streaking and sometimes stacker deformation are observed. The adhesion of low percentage agarose gels to glass plates is also improved by these steps.

Although the gel system I report here has been used exclusively for the study of chromatin, I see no reason why it may not also be useful in the study of other nucleoprotein complexes.

Figure 1. Nucleoprotein gel resolution as a function of sample load.

A) Discontinuous nucleoprotein gel of a serially diluted sample of chicken erythrocyte chromatin. Each well was loaded with 50 μ l of sample; DNA loadings were determined by the A_{260} of the initial digestion supernate. Soluble chromatin was prepared by digestion of nuclei for 60 minutes with micrococcal nuclease as described in Part II of this thesis. The digestion supernate, in a buffer of 5 mM Na-PIPES (piperazine-N,N'-bis[2-ethane sulfonic acid]), 0.5 mM $MgCl_2$, 0.05 mM $CaCl_2$, 1.0 mM EDTA, pH 6.5, was added directly to 0.5 volumes of 3 X sample buffer prior to storage at -20° . Stored samples were used directly for electrophoresis. Band 1 is the core nucleosome; bands 2 and 3 are chromatosomes (7,35) containing either H5 or two H1 subtypes, respectively, in addition to the inner histones (see Figure 11). The dinucleosome and trinucleosome regions are designated by 4 and 5.

B) An early redigestion of soluble chicken erythrocyte chromatin prepared by another method (36). Bands x and y are unidentified minor species.

C) The same erythrocyte samples seen in A), but now run on a non-stacking 6% acrylamide gel, incorporating 30% glycerol and the buffer of Todd and Garrard (28).

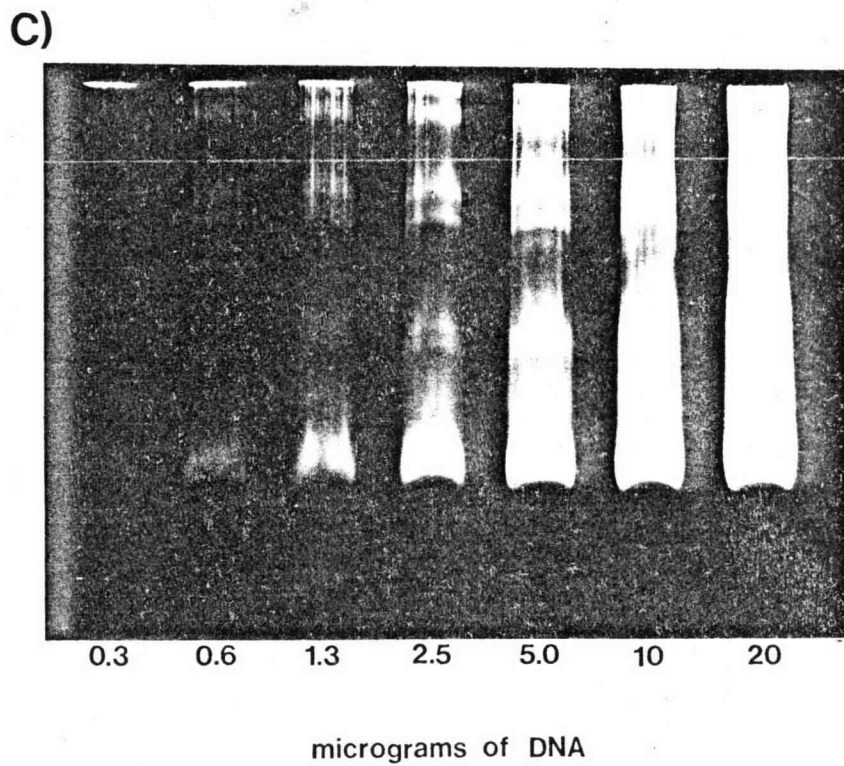
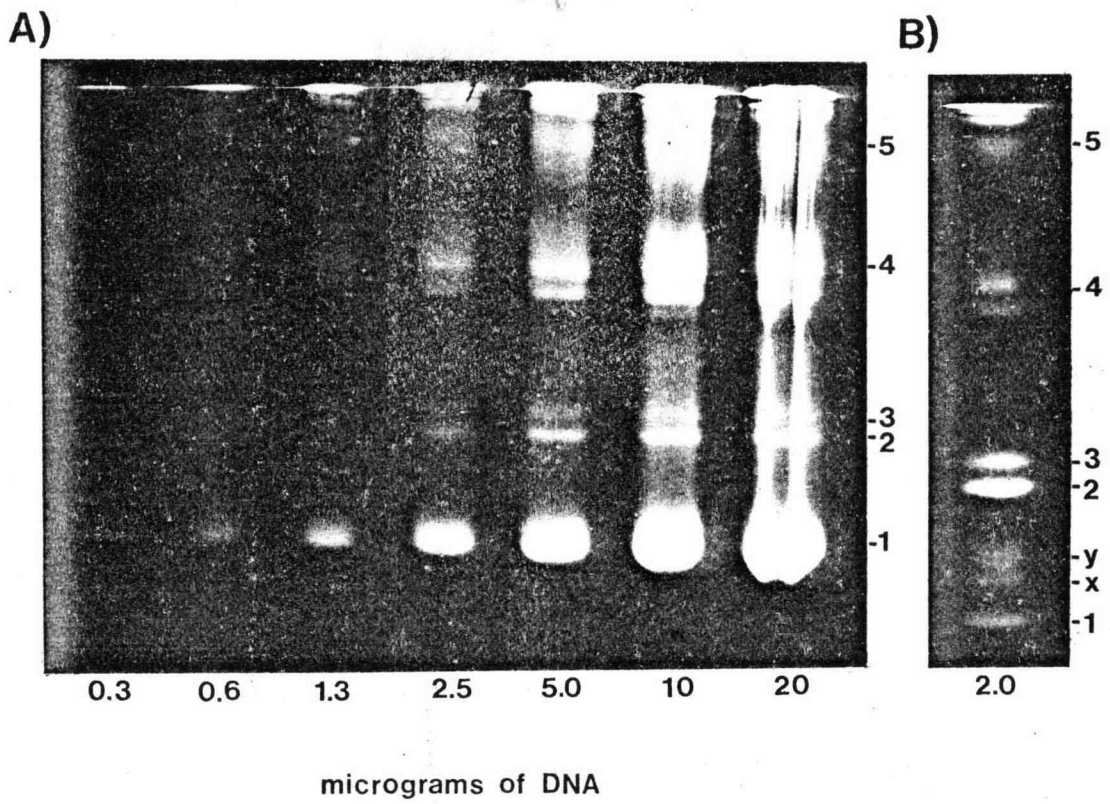


Figure 1.

PART II: NUCLEOPROTEIN ORGANIZATION IN YEAST CHROMATIN

METHODS

Conversion of Yeast to Mitochondrial DNA-free Strain

Saccharomyces cerevisiae strain Y-55 was made petite phenotype rho^o by growing the yeast for 24 hours in YEPD medium (1% yeast extract, 2% bacto-peptone, 2% dextrose) containing 10 µg/ml ethidium bromide (37), and using this culture as an inoculum for a further 24 hours growth in fresh medium under identical conditions.

Growth and Spheroplasting of Yeast

The Y-55 rho^o strain was grown on YEPD medium at 30° to a logarithmic growth phase density of about 5×10^7 cells/ml, and immediately made 0.5 mM in PMSF (phenylmethylsulfonylfluoride) by the addition of a stock solution of 100 mM PMSF in isopropanol. The cells were then harvested by centrifugation for 5 minutes at 5,000 g, and subsequently washed twice in cold distilled H₂O containing 0.5 mM PMSF. They were then washed for a third time in S₁ buffer (1.4 M sorbitol, 40 mM Na-HEPES, 0.5 mM MgCl₂, 1.0 mM PMSF, pH 7.5. HEPES is N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid.). Five liters of logarithmic culture gave a cell pellet of about 12 grams wet weight.

The spheroplasting enzyme, Lyticase (fraction II), was prepared according to Scott and Schekman (38), using

a glucan substrate derived from three alkaline extractions of yeast cells. Lyticase stored at 4° remained active for two years. Washed cells were suspended in S₁ buffer (4 ml/gram cells) containing 0.5% β-mercaptoethanol. An empirically determined volume of Lyticase, sufficient to completely spheroplast cells in about 90 minutes, was diluted with an equal volume of 2 X S₁ buffer plus β-mercaptoethanol, readjusted to pH 7.5, and then added to the cell suspension. Spheroplasting was at 30° with gentle agitation. Completion was assayed by subjecting a small drop of the suspension to gentle manual shearing between a microscope slide and coverslip, and visually determining that little or no cell wall remained in the lysate.

Isolation of Nuclei
and Preparation of Insoluble Chromatin

The isolation protocol incorporated elements of the methods of May, and of Bhargava and Halvorson as modified by Sajdel-Sulkowska et al. (39-41). All steps were on ice or at 4°; centrifugation steps were in a Sorvall HB-4 swinging bucket rotor. Upon completion of spheroplasting, the cell suspension was diluted by the addition of 2-3 volumes of S₂ buffer (1.4 M sorbitol, 20 mM Na-PIPES, 0.5 mM MgCl₂, 0.5 mM PMSF, pH 6.5) to a total volume of 180 ml, and centrifuged in 50 ml tubes for 10 minutes at 2,000 g. The pelleted spheroplasts were resuspended in about 10 volumes (105 ml per 10-12 g initial cell wt) 18%

Ficoll buffer (18% Ficoll 400 w/v, 20 mM Na-PIPES, 0.5 mM $MgCl_2$, 1.0 mM PMSF, pH 6.5) by adding 20-25 ml to each tube, covering it with parafilm, and mixing at high speed on a vortex mixer for 1 minute. The tube contents were pooled, and the lysate homogenized with one stroke of a motorized Teflon pestle homogenizer (Vitro 30 ml rotated at 1200 rpm). Twenty-five ml of the homogenate was then layered over 20 ml of Glycerol-Ficoll buffer (20% glycerol v/v, 7% Ficoll w/v, 20 mM Na-PIPES, 0.5 mM $MgCl_2$, 1.0 mM PMSF, pH 6.5) in each of four 50 ml tubes, and centrifuged 40 minutes at 25,000 g. The supernate was carefully removed by aspiration. The pellet at this stage consisted primarily of nuclei entrapped by cell membrane. The nuclei were released by suspending each pellet in 20 ml of 18% Ficoll buffer and mixing as before for 3 minutes on the vortex mixer. This was followed by centrifugation for 15 minutes at 3,000 g. The supernate, which contained the nuclei, was decanted into another tube, and spun for 15 minutes at 25,000 g. The pelleted nuclei were washed once by adding 20 ml of 18% Ficoll buffer to each tube, mixing for 2 minutes, and repelleting as above.

Nuclei were converted to insoluble chromatin by suspending the pellets with a spatula into a combined volume of 20 ml of digestion buffer (5.0 mM Na-PIPES, 0.5 mM $MgCl_2$, 0.05 mM $CaCl_2$ or 0.05 mM $SrCl_2$, 1.0 mM PMSF, pH 6.5), mixing until homogenous, and incubating with agitation for 10 minutes at 37°. After a further mixing

for 1 minute, the suspension was centrifuged for 15 minutes at 12,000 g, and the supernate removed. This cycle was repeated once again to produce the washed, insoluble chromatin pellet. The final yield was about 2.4-2.8 mg DNA as chromatin.

Yeast Nuclease Digestion

Insoluble chromatin was mixed on the vortex mixer into about 9 ml of digestion buffer until homogenous. Digestions were at 37° with about 400 units/ml micrococcal nuclease (Worthington Biochemical Corp.), and 300 µg/ml DNA as chromatin. Samples were withdrawn at designated times, and the reaction terminated by the addition, on ice, of 1/10 volume of 10 mM EDTA, 5mM Na-PIPES, pH 6.5. After at least 15 minutes on ice, the samples were centrifuged for 10 minutes at 4° in a Beckman microfuge. The supernate was removed, and divided into two aliquots, one of which was added to 0.5 volume of 3 X native nucleoprotein sample buffer (see Table I). All samples were stored at -20°.

The measurement of the DNA in the supernate to monitor the extent of digestion was essentially as in Lohr et al. (1).

In some experiments, a small portion of the insoluble chromatin was digested with a mixture of RNase T1 and bovine pancreatic RNase (0.04 Sankyo units/ml and 0.15 Kunitz units/ml; both Calbiochem-Behring Corp.). Diges-

tion and handling were as above.

Isolation and Digestion of Erythrocyte Nuclei

Chicken erythrocyte nuclei were isolated essentially as in Libertini and Small (36), except that five washes incorporating 1.0 mM PMSF were used. Nuclei were washed once in digestion buffer, centrifuged 10 minutes at 2,000 g, and resuspended in digestion buffer at about 1.4 mg/ml DNA. Digestion and sample handling were as with yeast.

Gel Electrophoresis and Analysis

A high resolution native nucleoprotein gel that was devised is the subject of the first part of this thesis. Other electrophoretic protocols, and the subsequent staining and quantitation of gels, are detailed in the figure legends. An outline of the analytic strategy is presented as a flowchart (Figure 2).

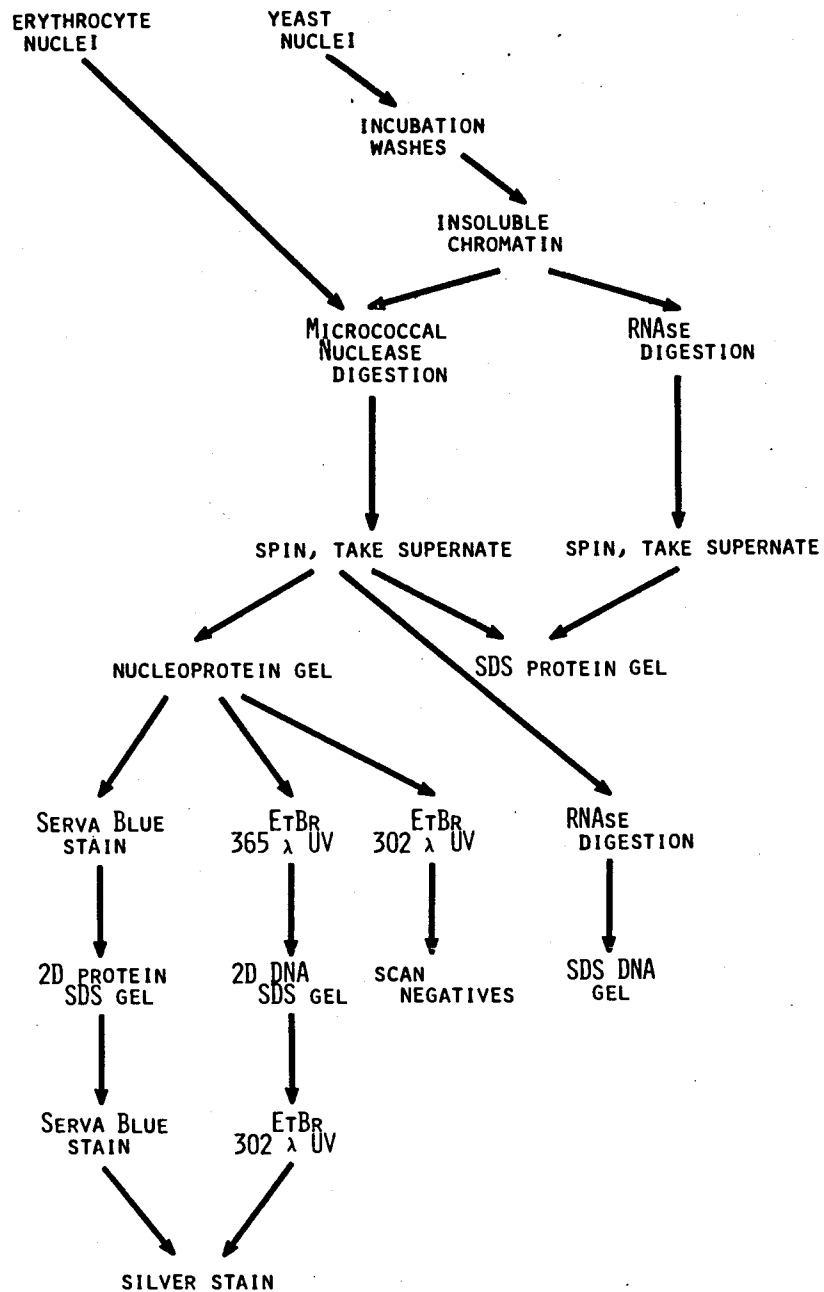


Figure 2. Flowchart of experimental procedure.

RESULTS

Characterization of Chromatin

Insoluble yeast chromatin appeared under the microscope as a fine granular material. The incubation washes removed a large number of proteins, including bands comigrating with a yeast 80 S ribosome preparation (Figure 3). No detectable inner histone or acid precipitable DNA was released by this procedure, although a small amount of acid soluble DNA, equivalent to 4-7% of the total DNA, was released in the first wash.

Chromatin, solubilized by micrococcal nuclease digestion, gave rise to a highly reproducible pattern of proteins on SDS gels (Figure 3). This pattern is similar to one from nuclei prepared by ultracentrifugation through a 30% Ficoll buffer (42). In contrast to previous reports, no obvious band is observed corresponding to a major defined non-histone protein, HMGa (11,12). The overall presence of the non-histone proteins closely correlates with the solubilization and subsequent precipitation of the inner histones as a function of nuclease digestion, with the single exception of a very prominent band of 40,000 daltons (SDS molecular weight). At low concentrations of released chromatin, Serva Blue R stains yeast H2A and H2B more intensely than H3 and H4, whereas erythrocyte histones stain almost equally (Figure 3). No evidence of proteolysis can be seen on SDS gels

Figure 3. SDS gel of nuclease released proteins. Equal volumes of supernate from each sampling point were loaded directly onto an 18% acrylamide Laemmli gel (49) (37.5:1 acrylamide:BIS). Gel was stained with 0.1% Serva Blue R (Serva Fine Biochemicals; a purified research grade of Coomassie Blue R-250) in 45% methanol, 9% acetic acid, and destained in 20% ethanol, 7% acetic acid. Most of the minor low molecular weight peptides released by micrococcal nuclease comigrate with bands from an 80 S ribosome preparation (obtained from J. Warner, see reference 50) (track 1). The washes remove almost all of the ribosomal proteins, as well as additional high molecular weight species (compare track 2 with tracks 5-8).

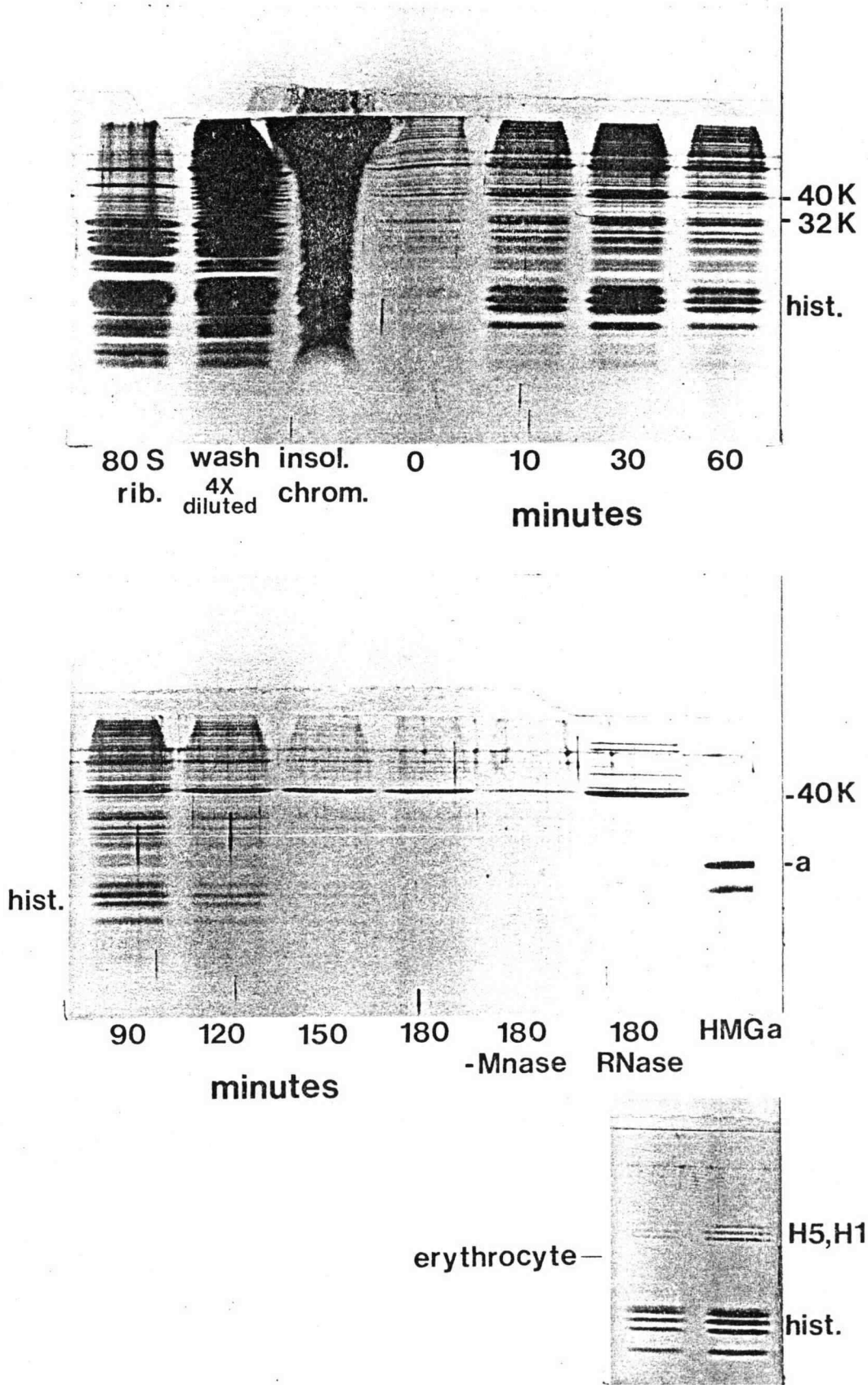


Figure 3.

after 2-3 hours of micrococcal nuclease digestion. A control incubation, for 3 hours without nuclease, releases neither protein nor DNA (Figure 3).

Sr^{++} , which has been reported to selectively induce DNase activity with micrococcal nuclease (43), was used rather than Ca^{++} in some digestions because yeast nuclei contain large amounts of RNA (44). Results with either nuclease activator were qualitatively and quantitatively similar.

RNase digestion (Figure 3) released only the 40,000 dalton protein and four minor higher molecular weight peptides. RNase, at low ionic strengths, can interact with core nucleosomes, and induce certain limited conformational changes (45). Digestions were therefore done at very low nuclease concentrations to avoid the possibility of displacement of non-histones by such non-catalytic binding.

Erythrocyte chromatin, solubilized by micrococcal nuclease (Figure 3), had a very simple composition in agreement with previous reports (46-48).

Kinetics of Yeast Chromatin Digestion

When the digestion of yeast chromatin is monitored by following the production of mononucleosomal and short oligonucleosomal DNA, two features stand out (Figure 4). (1) Mononucleosomal DNA is always present in much smaller amounts than any of the short oligonucleosomal DNA size

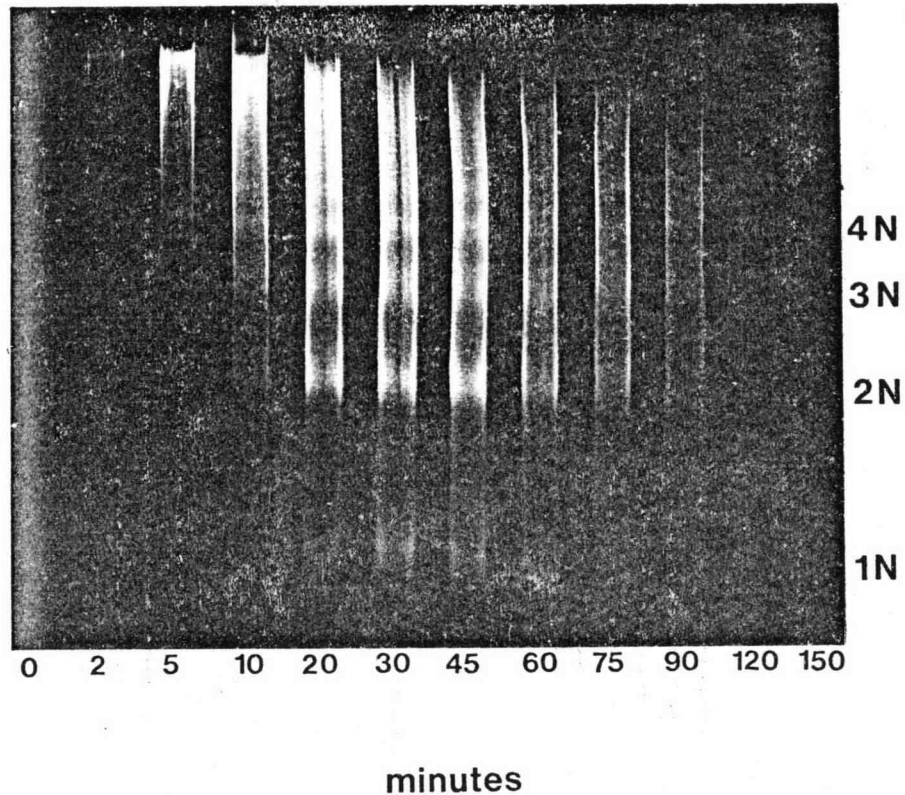


Figure 4. Time course of micrococcal nuclease solubilized yeast DNA. Equal volumes of RNased samples from a Ca^{++} activated digestion were resolved on a 6% acrylamide gel (20:1 acrylamide:BIS) using an SDS buffer system (15), and then stained with 1 $\mu\text{g}/\text{ml}$ ethidium bromide.

classes. (2) The DNA of the dinucleosome, trinucleosome, and tetranucleosome are present in a nearly constant ratio relative to one another throughout digestion.

Electrophoretic Patterns of Nucleoproteins

A time course of released yeast nucleoprotein (Figure 5a) reveals a simple, persistent pattern of two closely resolved trinucleosomes, two well resolved dinucleosomes, and a mononucleosome.

No band that stains with ethidium bromide is seen in yeast in the chromatosome position; for erythrocytes, there is a prominent pair of such bands (7,35) (Figure 5a,b). However, as noted below, Coomassie Blue staining material is present in that position for yeast as well as erythrocytes.

The dinucleosomes, a and b, are continually present in a ratio of approximately 4:1 as assayed by scanning photographic negatives of nucleoprotein gels stained with ethidium bromide (Figure 6). The dinucleosomes together exceed the amount of mononucleosome throughout digestion (Figures 5a,6,7,8).

Bakayeva and Bakayev reported the electrophoretic resolution of two erythrocyte chromatosomes and the core particle (7). This is verified in the present study. The gels also resolve two dinucleosomes. Unlike yeast, the core size mononucleosome accumulates continuously relative to larger nucleoproteins during the course of digestion

Figure 5. Nucleoprotein gels stained with ethidium bromide. Gels were soaked for 30 minutes in 1% SDS, 1 X separating gel buffer, and then for 30 minutes in 1 μ g/ml ethidium bromide, 1 X separating buffer.

A) Tracks 1-7 are from a Ca^{++} activated yeast digestion, with sampling times as indicated. Track 8 is a 30 minute sample from a Sr^{++} activated digestion.

B) Tracks 1-6 show nucleoproteins released from erythrocyte nuclei by Ca^{++} activated digestion. Track 7 is a co-electrophoresed sample from the Ca^{++} activated digestion of yeast shown in A).

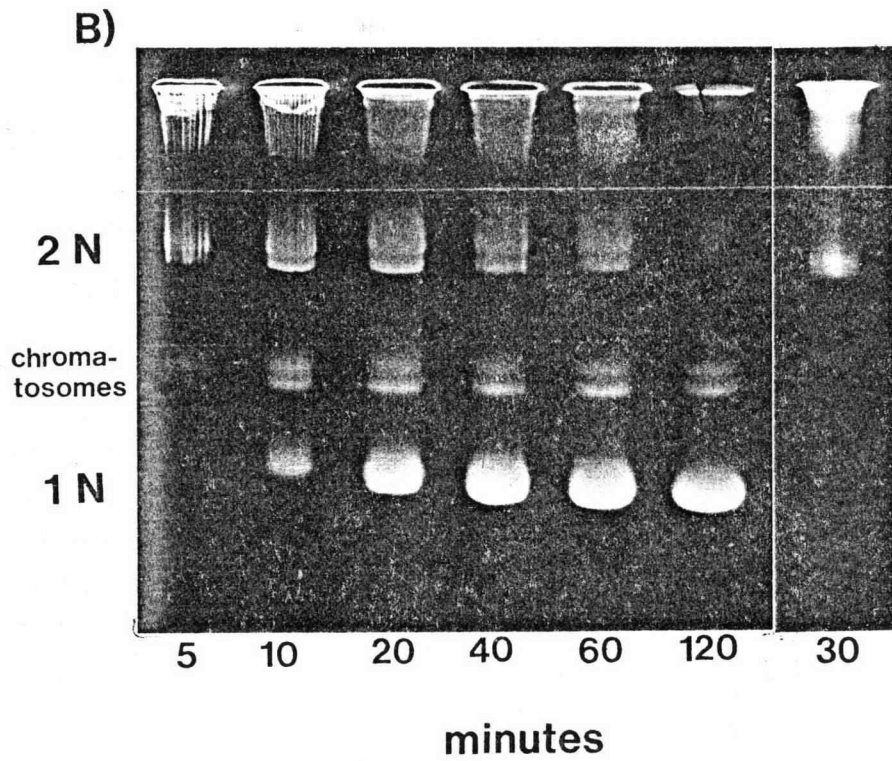
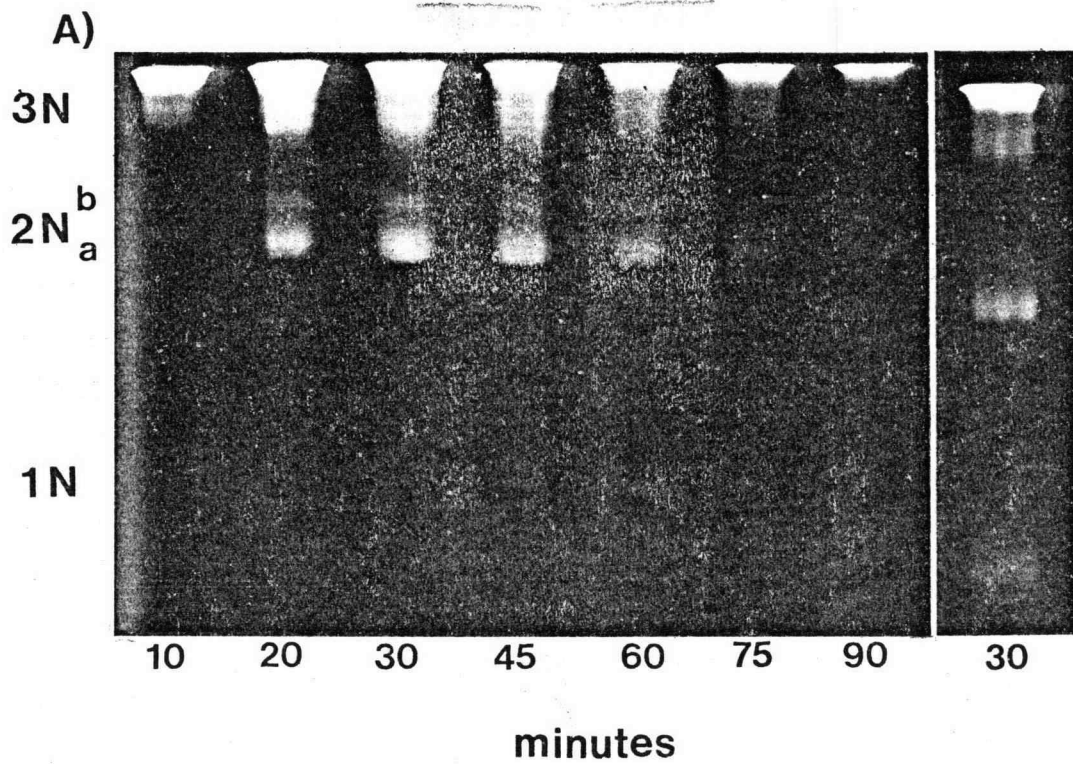


Figure 5.

Figure 6. Scan of yeast nucleoprotein gel stained with ethidium bromide. A Polaroid Type 55 negative was scanned on a Cary 219 spectrophotometer with scanning accessory. The sample is a 30 minute timepoint of a Sr^{++} activated digestion, but from a different experiment than that shown in Figure 5 (A).

The mononucleosome has a biphasic pattern; the shoulder at the leading edge becomes relatively more prominent late in digestion. This may represent a slight pause in the kinetics of digestion at the 146 bp DNA core particle (see Figure 9). There is a very weak and diffuse peak in the position corresponding to the minor mononucleosome; this feature is not always present. Note that this is also the position for a putative chromatosome. The two dinucleosomes give a very reproducible pattern.

This gel was not soaked in SDS prior to visualization with ethidium bromide. Ethidium bromide access to mononucleosomal DNA is about a third that of free DNA (5). A comparison of the peak areas of the mono- and dinucleosomes may therefore be inaccurate, if there are major structural differences. See figures 7 and 8 for the corresponding gel, but stained for protein.

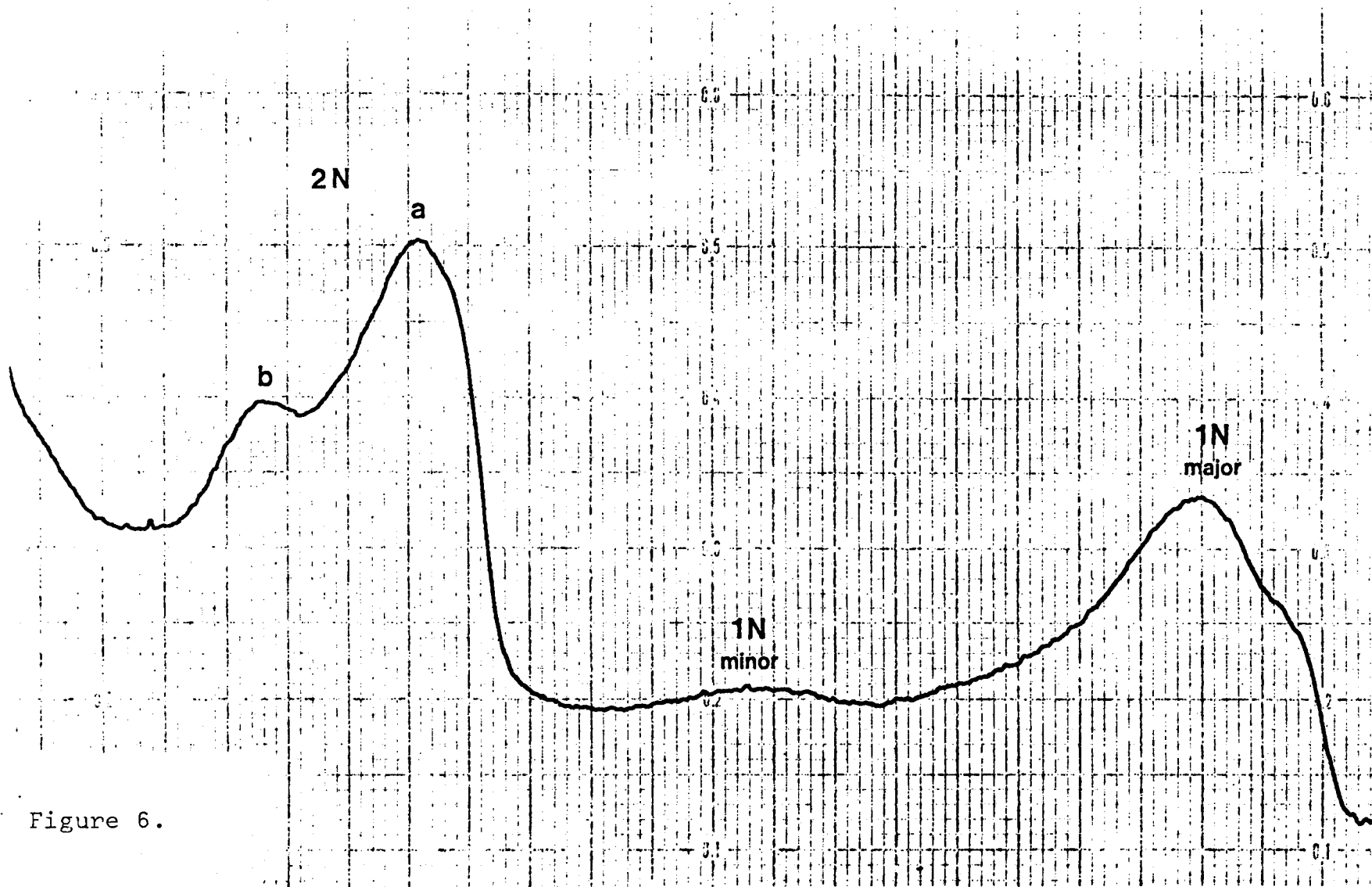


Figure 6.

Figure 7. Nucleoprotein gel stained with Serva Blue R. Tracks 1-10 follow a time course of a Sr^{++} activated yeast digestion. Tracks 11 and 12 are erythrocyte nucleoproteins prepared by redigesting chromatin already solubilized by EDTA treatment of a mild micrococcal nuclease digest. The erythrocyte buffer is 10 mM Tris-HCl, 0.2 mM EDTA, 1.0 mM CaCl_2 , pH 7.8 (36). Yeast bands X, Y, and Z do not stain with ethidium bromide. Band Y contains the prominent 40 kilodalton protein.

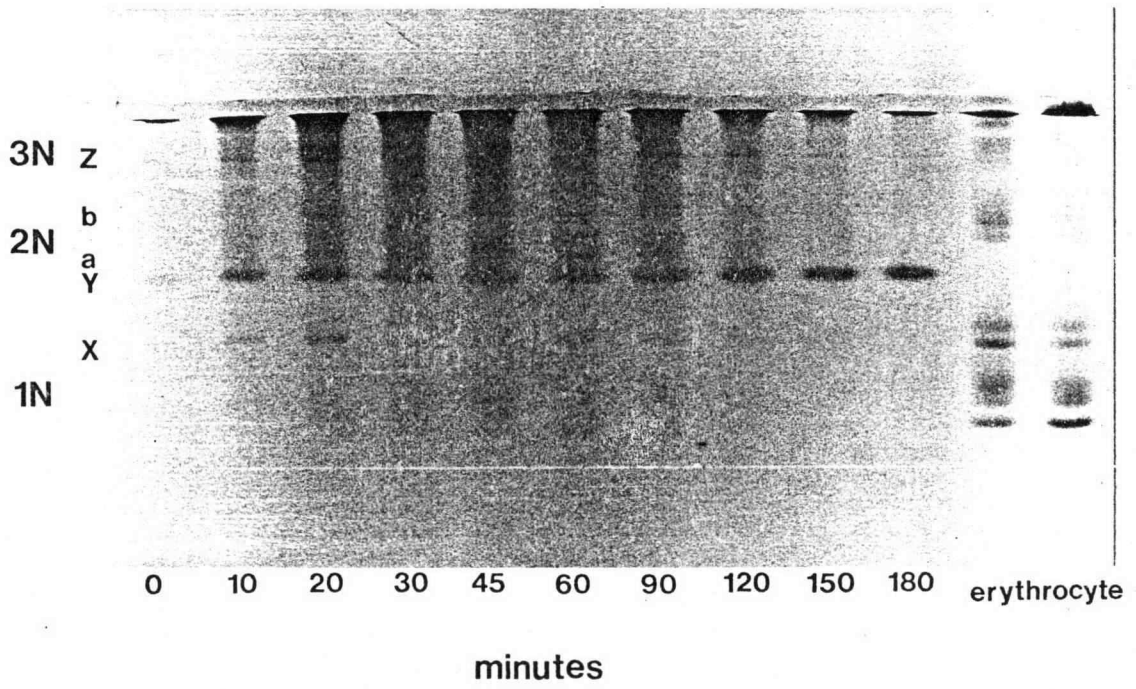


Figure 7.

Figure 8. Scan of yeast nucleoprotein gel stained with Serva Blue R. Destained gels were equilibrated with distilled H₂O, and excised gel tracks placed in a custom made 1.3 mm pathlength quartz gel boat. This was then filled with distilled H₂O, and scanned at 552 nm on the Cary 219 spectrophotometer. The sample is the 30 minute Sr⁺⁺ activated digestion shown in Figures 6 and 7. Sample loading is twice that of the ethidium bromide stained gel.

The major and minor dinucleosome peaks are labelled a and b. The minor features marked by the arrows are reproducible on Serva Blue stained gels. It is not known whether they are minor members or kinetic intermediates of a family of yeast oligonucleosomes, or whether they represent other co-electrophoresing species.

Band Y is the moiety that contains (or is) the 40 kilodalton polypeptide. Band X is still uncharacterized; considering the intensity of its staining, it is surprising that no non-histone proteins or node of inner histones can be seen in a corresponding position on a second dimension SDS protein gel (see Figure 10).

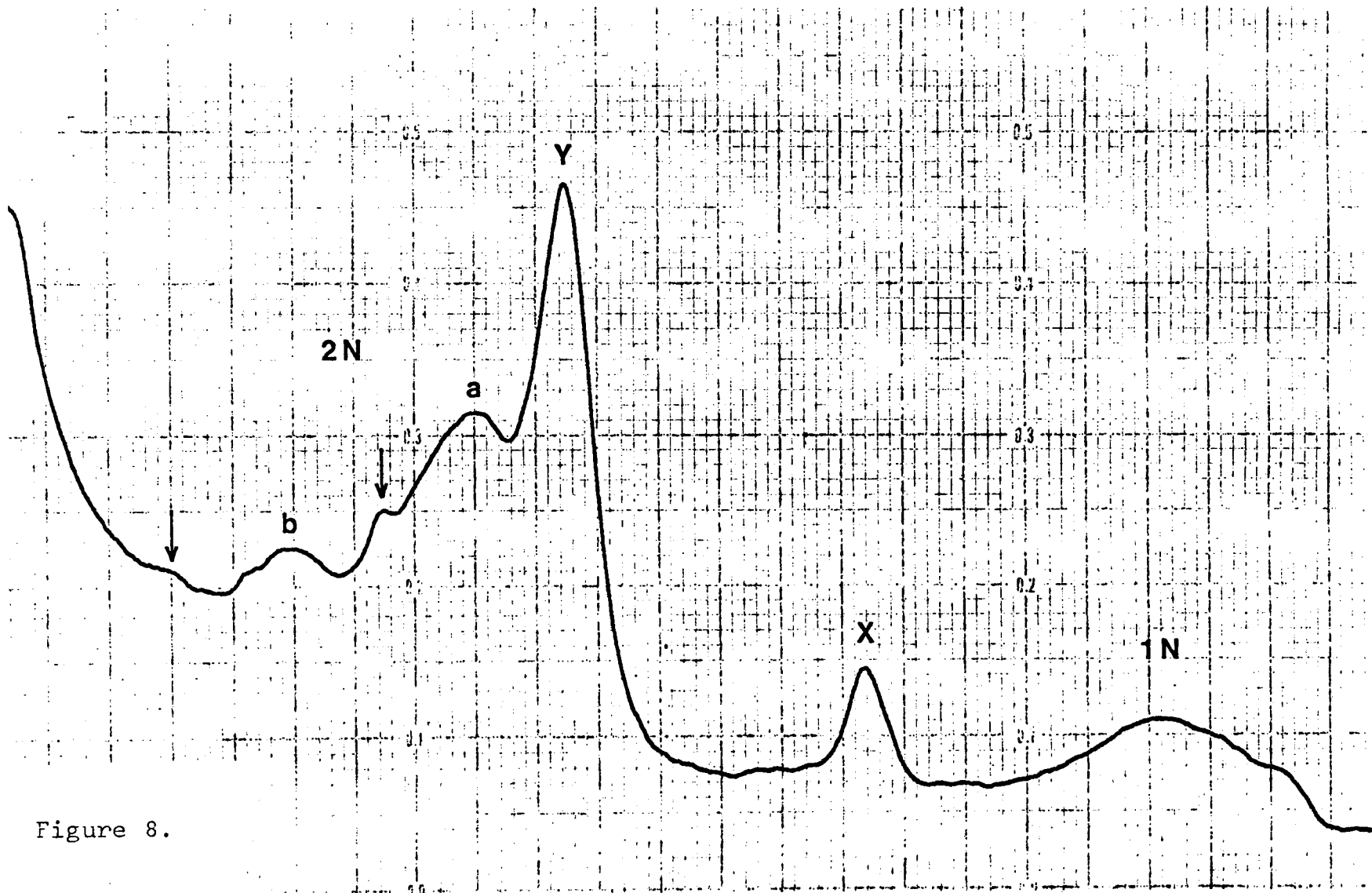


Figure 8.

(Figure 5b).

Analysis of Nucleoprotein DNA

A silver stained gel of yeast DNA, electrophoretically removed from nucleoproteins, reveals a minor DNA band corresponding to a second mononucleosome (Figure 9). The DNA length is very similar to that of the major mononucleosome. The lower size limit of mononucleosomal DNA is about 146 bp (basepairs), but core size DNA never appreciably accumulates. In both mononucleosomes the DNA runs on a diagonal; therefore, the precise mobility of each nucleoprotein complex in the gel directly correlates with the length of its component DNA. The DNA of the major species gradates at its higher molecular weight end into a roughly parallel DNA streak of diminishing intensity that is displaced toward lower nucleoprotein mobility, giving the region an overall zig-zag appearance. It is noteworthy that the DNA lengths of this mononucleosome tail (170-270 bp) greatly exceed the reported average yeast repeat length of 160-165 bp (1,2). The average DNA size of the two dinucleosomes is roughly the same, centering on about 330 bp, although the major species has a broader range. This data is consistent with previous work (1,2).

Analysis of Nucleoprotein Protein

A minor non-histone protein (SDS molecular weight of

Figure 9. Two dimension DNA gel from yeast. An ethidium bromide visualized nucleoprotein track from a Ca^{++} activated digestion (see 45 minute time-point in Figure 5) was used as the first dimension in a modified two dimension SDS gel system (15). The first dimension gel strip was soaked for 30 minutes at 50° in running buffer plus 1% SDS (15), and then equilibrated for 10 minutes at room temperature in running buffer plus bromophenol blue. The strip was polymerized onto a 6% acrylamide gel (20:1 acrylamide :BIS) with the same acrylamide mixture. After the second dimension was run, the gel was sequentially stained with ethidium bromide and silver (51).

pBR322 plasmid was digested with the restriction enzyme HpaII to provide DNA size markers.

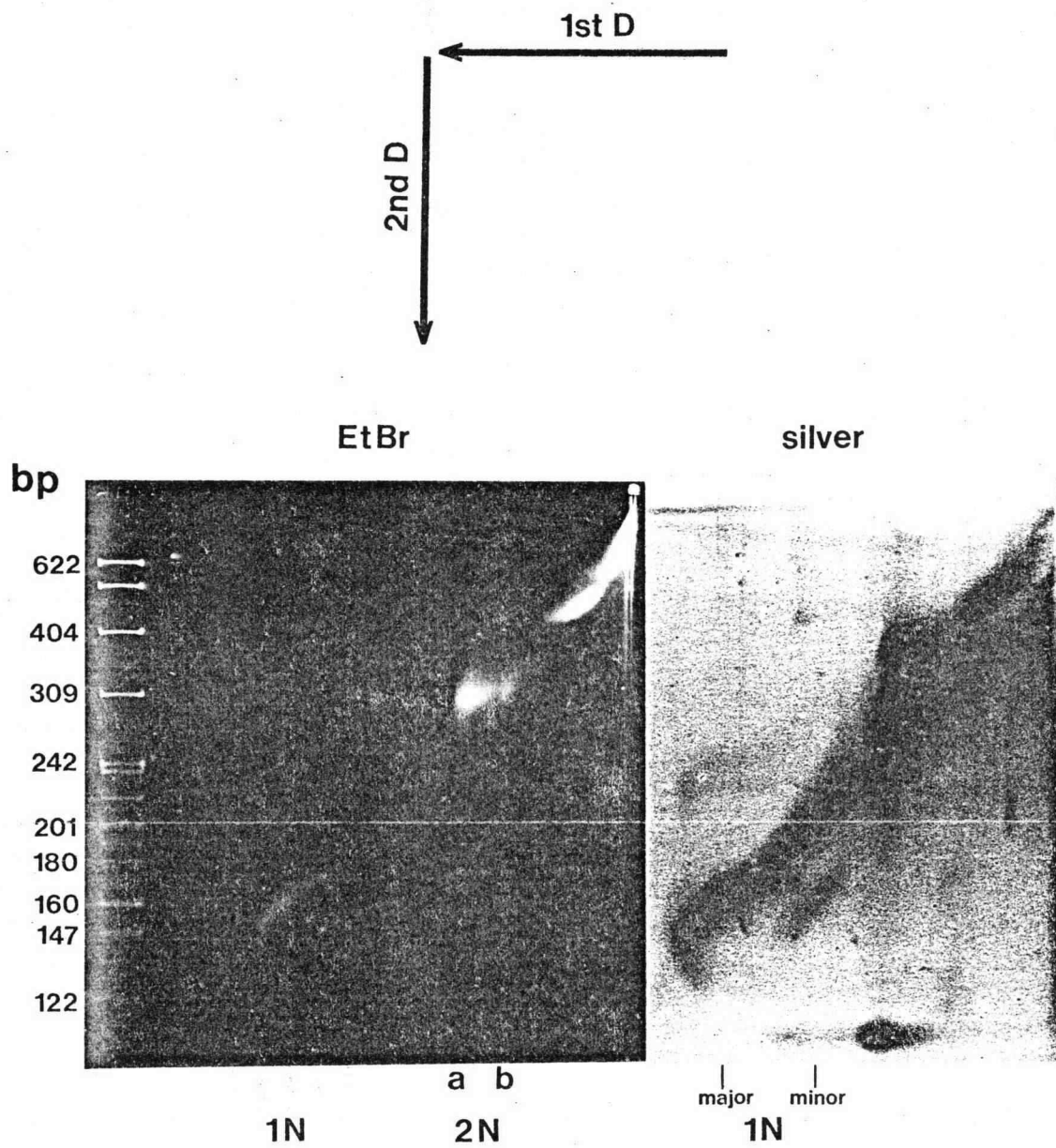


Figure 9.

about 32,000) appears to be associated with yeast nucleoproteins because it is correlated in two dimensional electrophoresis (Figure 10). The position of the peptide corresponds with the location of the trinucleosome, and in some experiments, also apparent subsets of the dinucleosomes. When chromatin is extensively digested so that the dinucleosome is depleted, then the correlated non-histone band is also depleted.

Erythrocyte chromatosomes contain H5 in the leading nucleoprotein band, and the two H1 subtypes in the trailing component as previously noted (7) (Figure 11).

Additional Electrophoretic Species

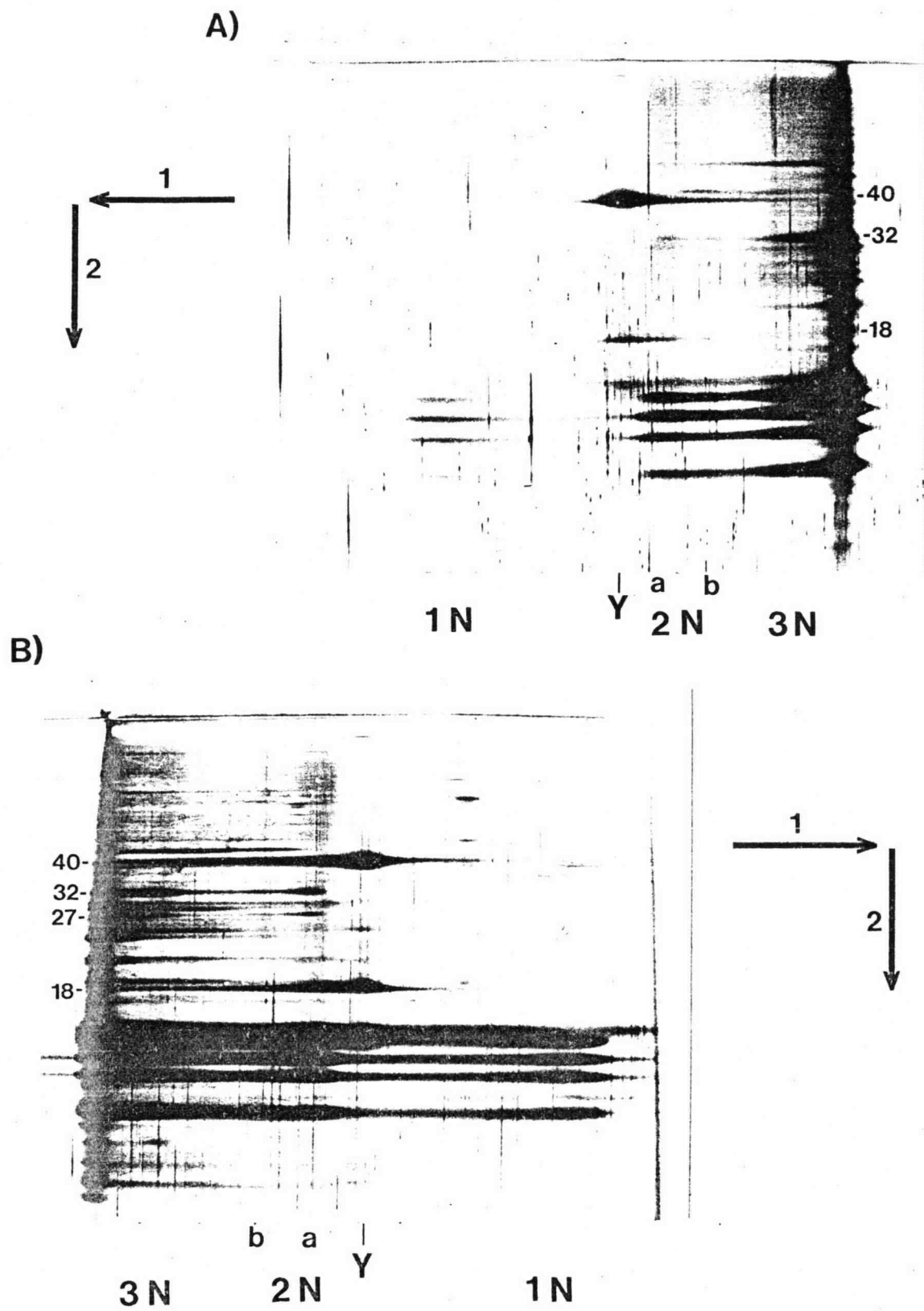
Micrococcal nuclease digestion in yeast also generates entities that migrate on a nucleoprotein gel, and perhaps do not contain DNA or RNA (Figures 7,8). Most prominent is a band that stains intensely for protein and migrates just ahead of the leading dinucleosome. Second dimension SDS protein gels reveal that it consists primarily of the 40,000 dalton peptide that is released, but not reprecipitated, during the course of both micrococcal nuclease and RNase digestions (Figure 10). A minor component is a peptide of about 20,000 daltons that apparently is not solubilized by RNase. Silver staining for DNA and RNA, which is 20-100 times more sensitive than ethidium bromide (51), detected no nucleic acid corresponding to this band (the lower size limit of DNA detectable on these

gels was about 70 bp). Fainter bands in the trinucleosome and chromosome regions have not been further characterized.

Figure 10. Two dimension SDS protein gels from yeast. Serva Blue stained nucleoprotein tracks were equilibrated for 30 minutes in 0.125 M Tris-HCl, pH 6.8, plus bromophenol blue, and polymerized onto 6% acrylamide stacking gels (37.5:1 acrylamide:BIS) with additional stacking mixture. The separating gels were 18% acrylamide (37.5:1). The buffer system was that of Laemmli (49), except that 0.01% mercaptoacetic acid was freshly added to the running buffer as an electrophoresable anionic reducing agent (52).

A) The 32,000 dalton protein is seen primarily on the trinucleosome of this silver stained (53) gel from a Sr^{++} activated digestion of 45 minutes (see Figure 7). The 40,000 dalton protein is always very prominent. It is not known whether the minor component of about 18 kilodaltons, which co-electrophoreses in the first dimension with band Y, is complexed with the 40 kilodalton protein in vivo, or is possibly the result of degradation in the first dimension gel.

B) A gel from an early timepoint of a Sr^{++} activated digestion of late growth phase yeast (1-2 cell divisions short of stationary density). The bimodal association of the 32 kilodalton protein with the two dinucleosomes can be clearly seen. The minor peptide (about 27 kilodaltons) that runs just beneath the 32 kilodalton protein has not been seen in other experiments, all on early to middle log phase yeast.



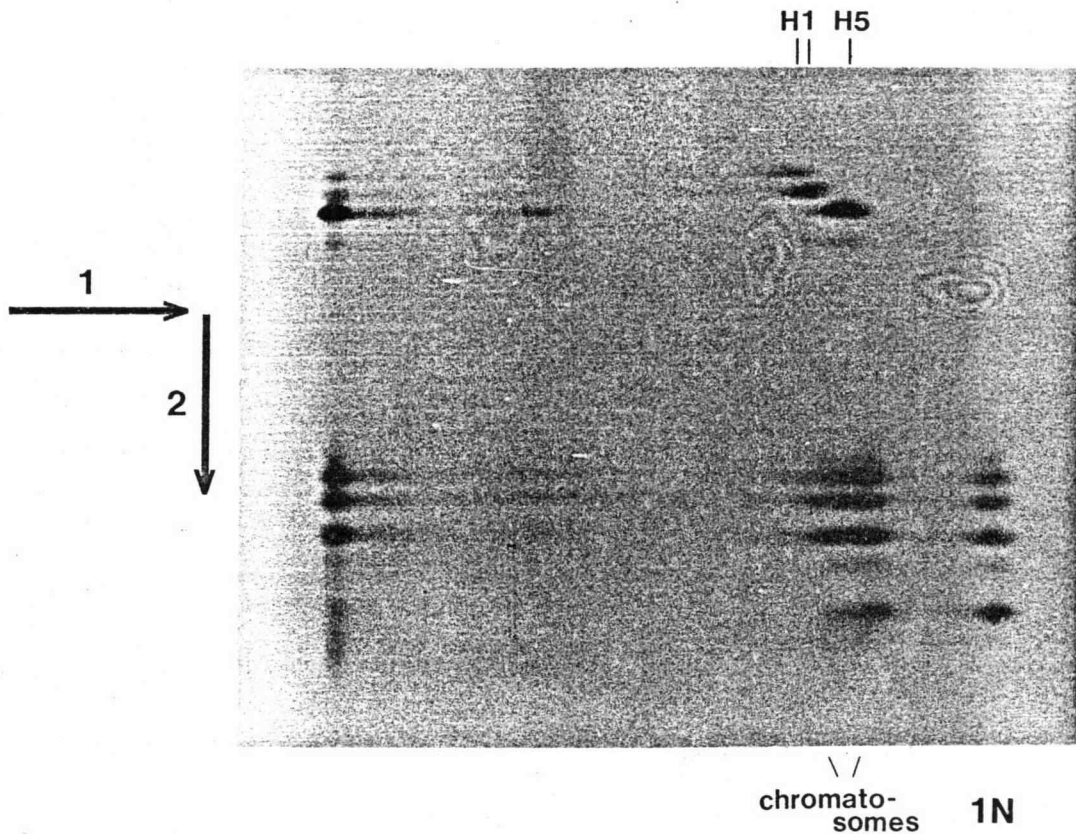


Figure 11. Two dimension SDS protein gel from erythrocyte. Gel is stained with Serva Blue R. The sample is from a redigestion of soluble chicken erythrocyte chromatin prepared by the method of Libertini and Small. The leading chromatosome contains H5; the trailing chromatosome band is actually a composite of two different complexes, each containing one of a pair of H1 subtypes.

DISCUSSION

A striking feature of the released nucleoproteins is that they are electrophoretically resolved into two bands for each DNA size class, mononucleosome to at least trinucleosome. This dualism is most easily examined experimentally for the case of the dinucleosome. Electrophoresis in a second dimension reveals no stoichiometric additional proteins that might readily account for mobility differences. The existence of nucleoprotein pairs in each size class that are resolvable on the basis of unknown features does not necessarily imply that each member of a given doublet falls into one of two functional domains. A proof of this would require either a direct correlation of nucleoprotein with function by various DNA hybridization experiments (15,25), or a demonstration of a precursor-product relationship between one member of a given doublet, and a corresponding member in a doublet of a smaller oligonucleosome (28).

The relative resistance of species as small as the dinucleosome to micrococcal nuclease degradation has interesting implications for nucleosome-nucleosome interactions. DNase studies have demonstrated that much, and possibly all, of yeast chromatin has a spacer length that is phased at 5,15,25... base pairs, a feature not found in erythrocyte or HeLa (54). From this information, it is hypothesized that yeast nucleosomes pack edge-to-edge in the chromatin fiber in a structure with a dinucleosomal

repeat (54,55). For the dinucleosome in these digestions to be so nuclease resistant, the linker region may be nuclease inaccessible to a degree not seen in most eukaryotes, where nucleosomes may pack face-to-face (55-57). Digestions of erythrocyte nuclei under the same conditions as yeast detect no such dinucleosomal resistance.

The mononucleosome tail may provide further evidence for a different linker arrangement in yeast. This tail, which contains DNA sizes much larger than expected on the basis of a 165 bp repeat, may be formed by asymmetric cleavage of dinucleosomal DNA at a site outside of the linker (or alternatively, within the DNA of an end nucleosome on a longer oligonucleosome).

The nuclease resistance of the dinucleosome does not imply a dinucleosomal repeat in yeast chromatin. It is evident that micrococcal nuclease does not detect a simple $2N, 4N, \dots$ repeat, although dinucleosomes would be the smallest discrete product. However, a yeast arrangement is still possible where single nucleosomes non-equivalently alternate along the DNA path. Such dinucleosomal repeats have been proposed for the chromatin 250 \AA fiber (55,56). DNase I studies of avian erythrocyte and rat liver chromatin detect dinucleosomal repeats, probably of this kind (58,59) (for these chromatins, micrococcal nuclease gave the usual register).

The general excision pattern of the oligonucleosomes is unusual; it is consistent with a closely packaged

nucleosomal structure (but not necessarily higher order), in which nuclease access to the linkers internal to any fiber segment is severely restricted. This study differs from a previous report, where yeast mononucleosomal DNA accumulated rapidly at the expense of larger fragments during the course of digestion (1). The chromatin isolation procedure used in this investigation incorporates at all steps, including spheroplasting, a concentration of Mg^{++} (0.5 mM) sufficient to maintain the condensation of the 250 Å fiber in mammals (56,60-62); exposure to detergents, chelating agents, and polyamines is avoided.

Yeast mononucleosomes do not appear to have a pronounced resistance to further digestion at the core particle DNA size (146 bp), as do those from erythrocyte. This observation must be interpreted in the context of the relatively slow production of yeast mononucleosomes of any size. The core size particle may be more resistant to exonucleolytic trimming than mononucleosomes with longer DNA, but still not accumulate relative to the slowly generated and degraded di-, tri-, and tetranucleosomes.

The single extant physical study of isolated yeast mononucleosomes, which had a very short DNA length (128 ±30 bp), reports that such particles tend to dissociate into H3•H4 and H2A•H2B complexes, under conditions where calf thymus nucleosomes with the same DNA complement are stable (5). Furthermore, yeast histones do not reconstitute with 146 bp of DNA into a nucleosomal complex, in

contrast to reconstitution experiments with calf thymus and chicken erythrocyte histones (5).

Yeast H3 and H4 form complexes in solution without the concomitant increase in α -helix content that is observed upon the formation of the homologous complex in calf or pea (3). The $(H3)_2 \cdot (H4)_2$ homotypic tetramer of complex eukaryotes is by itself capable of directing the self-assembly of a nucleosome-like complex containing 146 bp of DNA (63-66).

These observations suggest that the low yield of mononucleosomes seen here may not solely be the result of the mode of nucleosome packing. Intra-nucleosome histone-histone or histone-DNA interactions in yeast may differ from those of complex eukaryotes, and contribute fundamentally to mononucleosome instability.

In erythrocyte, at physiological ionic strength, sequential reconstitution on long DNA with the $(H3)_2 \cdot (H4)_2$ tetramer, and then H2A·H2B dimers, gives rise to oligonucleosomes organizing a 167 ± 4 bp DNA repeat (67). Inclusion of H1 does not change the repeat, regardless of when it is added. The repeat length, and perhaps also its assembly independent of H1, remarkably parallel the features of yeast. This hints that some aspects of nucleosome-nucleosome interactions in yeast are common to all eukaryotes.

Only a single non-inner histone nucleosomal protein is found in yeast. This is detected only in modest

amount, and in significant quantities only on the trinucleosome. The protein has an SDS mobility corresponding to erythrocyte H1, but this observation offers no basis for drawing a functional analogy. Nor can it be concluded that yeast oligonucleosomes have only a single bound non-core protein in vivo. For one thing, the short linker region of yeast may present a binding site of lower affinity in small oligonucleosomes as compared to intact chromatin, and proteins may be removed during digestion (68). Furthermore, it is known that H1, and HMGs 14 and 17, bind to nucleosomes in equilibrium, and can exchange under conditions similar to those used for isolation and digestion in this work (6,69,70).

It should also be noted that the protocols for isolating yeast nuclei are lengthy, and may provide opportunities for the metabolic alteration of chromatin function and structure. Spheroplasts in a growth medium are capable of nearly normal RNA and protein, but depressed DNA levels of synthesis (71,72). However, it is not known what metabolic stresses occur during spheroplasting in a non-growth environment. In view of these complexities, the failure to observe HMGs or ubiquitin conjugates should be interpreted with caution.

The abundance of the 40 kilodalton peptide, one of a simple spectrum of proteins released from this chromatin preparation by RNase, is striking. It is unknown whether it is self-associated in a complex, or runs as an independ-

dent entity on the nucleoprotein gel.

The solubilization of this peptide by RNase suggests that it may be a constituent of a nuclear ribonucleoprotein (hnRNP). Mammalian hnRNPs are characterized by a conserved group of 6 proteins, ranging in molecular weight from 32,000-40,000 daltons (73); in the simple eukaryote, Physarum polycephalum, a single major protein of 32,000 daltons has been identified (73). HnRNPs may be implicated in the packaging and processing of RNA immediately upon transcription, and as such may be a structural component of chromatin (74,75). In yeast, neither hnRNPs nor the 40 kilodalton peptide have as yet been reported.

Perspective

The strong contrast between yeast and erythrocyte nucleosomal complexes leads one to pose a number of functional questions. Is the dual nature of yeast nucleoproteins correlated with the transcriptionally competent and incompetent chromatin domains of complex eukaryotes? If yeast is primarily transcriptionally competent, does the apparent lack of H1 and the HMGs imply a coordinate, differentiation related role for these proteins? Do the micrococcal nuclease excision patterns reflect real differences in yeast nucleosome-nucleosome interactions, and might these be generalized in some sense to all competent chromatin? Are isolated yeast mononucleosomes inherently unstable, and if so, can this be related to transcrip-

tional function? These and similar problems will require much further investigation.

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