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Title: Active Chromatin Structure of Saccharomyces cerevisiae: High Mobility Group Proteins, Histone Modifications and DNase I Sensitivity.

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Irvin Isenberg

I examined, in three separate studies, the active chromatin structure of the yeast, Saccharomyces cerevisiae.

Yeast contains four proteins having amino acid compositions typical of the high mobility group (HMG) proteins. Three of these are eluted from chromatin by 0.35 M NaCl; one is not, but is eluted by 0.25 N HCl. It follows that HMGs cannot, in general, be defined by extractability criteria. Gel mobilities and amino acid compositions indicate that yeast and animal HMGs have diverged markedly.

In a collaborative study the content of the acetylated histone species associated with the highly transcriptionally active chromatin of yeast was found to contain very high levels of the acetylated species for histones H3, H4 and possibly the H2B variants, H2B-1 and H2B-2. Sixty-three percent of the histone H4 species was represented by the di-, tri- and tetra-acetylated forms. These results show yeast chromatin to be among the most highly acetylated of any chromatins reported thus far.

DNase I digestion rates were measured for yeast chromatin in the presence and absence of these non-histone chromosomal proteins (NHCPs) that are extractable by low salt. This was done with and without butyrate treatment. Removal of the NHCPs from chromatin not treated with butyrate increases the rate of digestion. However, if the chromatin is treated with butyrate, removal of the NHCPs has no discernable effect on the digestion rate.

ACTIVE CHROMATIN STRUCTURE OF *Saccharomyces cerevisiae*: HIGH MOBILITY GROUP PROTEINS, HISTONE MODIFICATIONS AND DNASE I SENSITIVITY

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TABLE OF CONTENTS

	Page
I. Introduction and Background	1
Nucleosomal Structure of Chromatin	1
Active and Inactive Chromatin	4
Chromatin Structure of the Yeast, *Saccharomyces cerevisiae*	8
II. High Mobility Group Proteins of *Saccharomyces cerevisiae*	12
Introduction	12
Materials and Methods	14
Results	17
Discussion	33
III. Histone Modifications of *Saccharomyces cerevisiae*	35
Acknowledgement of Co-Authorship	35
Introduction	38
Materials and Methods	41
Results	45
Discussion	55
IV. Relative Contributions to DNase I Sensitivity of Non-Histone Chromosomal Proteins and Butyrate Protection in the Active Chromatin of the Yeast, *Saccharomyces cerevisiae*	58
Introduction	58
Materials and Methods	62
Results	69
Discussion	93
V. Discussion and Summary	95
Bibliography	98

LIST OF FIGURES

Figure		Page
1.	Phosophorence of yeast 0.35 M NaCl extractable proteins separated on a preparative acid-urea gel . .	19
2.	Acetic acid-urea preparative gel edge strips stained with Amido Black and destained	21
3.	Acetic acid-urea gel of 0.35 M NaCl extractable yeast proteins, HMGa and proteins extracted with acid after the 0.35 M NaCl extraction	25
4.	SDS gels of yeast and calf thymus HMGs	27
5.	Amino acid residue profile of yeast HMGs S_3 and calf thymus HMG 17	29
6.	Acetic acid-urea gels of purified yeast HMGs S_1, S_3 and S_4 and the yeast proteins extracted with 0.35 M NaCl	31
7.	Two-dimensional gels (acid-urea into SDS) of histones from nuclei of calf thymus, chicken erythrocytes, HeLa (grown in the presence of butyrate) and yeast (isolated in the presence and absence of butyrate)	46
8.	Two-dimensional gel (acid-urea into Triton X-100) of yeast histones (isolated in the presence of butyrate)	52
9.	Schematic outline of experiemental procedure for isolation of soluable micrococcal nuclease supernatants from butyrate and non-butyrate protected chromatin and the stripping of non-histone chromosomal proteins	70
10.	Native DNA gel of soluable micrococcal nuclease supernatants from butyrate and non-butyrate protected chromatin	72
11.	SDS protein gels of soluable micrococcal nuclease supernatants from butyrate and non-butyrate protected chromatin	74
12.	Acid-urea gel of (Non-B) Sup 1 and (B) Sup 2	77
13.	Two-dimensional gels (SDS into acid-urea) of (Non-B) Sup 1 and (B) Sup 2	79

14. Acid-urea gels of 0.4 M NaCl extractable proteins from (Non-B) Sup 1 and CM-sephadex stripped proteins from (B) Sup 2 and from (Non-B) Sup 1 82

15. Acid-urea gel of yeast ribosomal proteins and proteins from (Non-B) Sup 1 extracted with 0.4 M NaCl 84

16. Two-dimensional gel (acid-urea into SDS) of the proteins extracted with 0.4 M NaCl from (Non-B) Sup 1 87

17. DNase I digestion time courses 89

18. Schematic summary of DNase I digestion results . . . 91

LIST OF TABLES

Table		Page
I	Amino Acid Composition of Yeast 0.35 M NaCl Extractable Proteins	23
II	Amino Acid Composition of Yeast HMGa and Calf Thymus HMGs	24
III	The Percentage of Acetylated Histone H4 Species in Preparations of Whole Chromatin	50

ACTIVE CHROMATIN STRUCTURE OF *Saccharomyces cerevisiae*: HIGH MOBILITY GROUP PROTEINS, HISTONE MODIFICATIONS AND DNASE I SENSITIVITY

NUCLEOSOMAL STRUCTURE OF CHROMATIN

The regulation of gene activity in eukaryotes is and has been the challenge of contemporary biochemists, biophysicists and molecular biologists since the discovery of DNA as the genetic material (Mirsky & Ris, 1949). Chromatin is the functional complex of the genetic material in the eukaryotic nucleus and consists of the DNA, the histones and other associated proteins and RNA. The discovery of the nucleosome, the fundamental subunit of chromatin, (Hewish & Burgoyne, 1973; Rill & Van Holde, 1973; Sahasrabuddhe & Van Holde, 1974; Noll, 1974; Kornberg & Thomas, 1974; Olins & Olins, 1974) began a revolution in the understanding of the organization of the eukaryotic genome and the relationship of chromatin structure to gene activity.

The nucleosome has been well characterized, and our knowledge of it has been recently reviewed (McGhee & Felsenfeld, 1980; Mirzabekov, 1980).

The nucleosomes released by the initial attack of micrococcal nuclease upon chromatin contain 160-240 base pairs of DNA (Kornberg, 1977). Micrococcal nuclease begins at once to digest the terminal DNA originally derived from the spacer region between adjacent nucleosomes (Sollner-Webb & Felsenfeld, 1975; Axel, 1975; Greil et al., 1976; Noll & Kornberg, 1977). In the course of digestion intermediates of somewhat greater stability are generated. Among

these are the mononucleosomes with 160-170 base pairs of DNA which can be isolated with bound histone H1 (Varsharsky et al., 1976; Rall et. al., 1977; Bakayev et al., 1977; Todd & Garrard, 1977; Simpson, 1978b) and has recently been called the chromatosome (Simpson, 1978b). Other mononucleosome digestion intermediates have been observed as well although at a lower level. These monomucleosome intermediates are due to the association of high mobility group proteins (HMGs) 1, 2, 14 and 17, as well as A24 (an ubiquitin conjugate of H2A) (Albright et al., 1979; Todd & Garrard, 1979; Levinger & Varshavsky, 1980; Albright et al., 1980; Mardian et al., 1980; Sandeen et al., 1980; Annunziato et al., 1981). Further digestion produces a more stable particle containing about 146 base pairs of DNA (Olins et al., 1976; Rall et al., 1977; Joffee et al. 1977; Prunell et al., 1979; Lutter, 1979; Bryan et al., 1979; Simpson & Kunzler, 1979) and an octomer of two each of the four inner histones (H2A, H2B, H3 and H4), but no H1. This structure is the nucleosome core particle and has been the subject of many chemical and physical studies. The inner histones are basic proteins (recently reviewed; Isenberg, 1979) that condense the DNA into the superhelical configuration observed in the nucleosome. Correct renaturation of a histone, H4, and elucidation of conditions that temporarally separated renaturation from aggregation (Li et al., 1972) was the key that led to an understanding of

histone-histone interactions. Histone-histone interactions observed for H3, H2B and H2A, as well, culminated in the proposal of a general scheme for histone interactions: the cross-complexing pattern of histones (D'Anna & Isenberg, 1973, 1974a,b). The nucleosome conformation is maintained by these specific protein-protein interactions and by protein-DNA interactions as well (Shick et al., 1980; Mirzabekov & Rich, 1979; Mirzabekov et al., 1978).

ACTIVE AND INACTIVE CHROMATIN

Gene expression proceeds through several stages, at any of which regulation might occur. Intially a precursor RNA is transcribed; then this molecule is processed, by the removal of some sequences, to form the mature messenger, which is transported to the cytoplasm; finally the RNA is translated into protein. It has been clear for some time that there is transcriptional control in the sense that for most cells only 10% of nuclear RNA sequences are present in the cytoplasm (Getz et al., 1975; Hough et al., 1975) How regulation of gene activity at the transcriptional level is related to the structure of chromatin (recently reviewed, Mathis et al., 1980; Rindt & Nover, 1980) and what chromatin conformational changes may be required for transcription has been a question since the discovery of the nucleosome.

One of the first questions posed by the discovery of the nucleosomal structure of chromatin is whether genes that are actively transcribing are assembled into nucleosomes. The first evidence that genes actively transcribing are assembled into chromatin was the demonstration that nucleoprotein particles produced by micrococcal nuclease or DNase II can be isolated, sedimented with mononucleosomes and contained globin gene sequences in cells expressing this gene (Lacy & Axel, 1975; Axel, 1976), ovalbumin gene sequences in the hen oviduct (Garel & Axel, 1976; Garel et al., 1978), rat liver DNA sequences coding for poly-A polysomal RNA (Lacy & Axel, 1975), sequences specifying cytoplasmic poly-A RNA in human leukocytes (Kuo et al., 1976) and 28S, 18S and

5S ribosomal genes xenopus embryonic cells and erythroid cells (Reeves & Jones, 1976). The general conclusion from these studies is that actively transcribed DNA appears to be complexed with protein to form a compact particulate structure.

Whether these particles of active chromatin are identical to the nucleosomes of chromatin that is inactive transcriptionally has been intensely investigated. The first observation that active chromatin is different from inactive chromatin was an altered, more accessible conformation to digestion by DNase I in active chromatin (Weintraub & Groudine, 1976). Weintraub and Groudine showed that the globin gene sequences in nuclei of cells actively transcribing these genes were hypersensitive to degradation by DNase I. Only those genes coding for adult-specific globin were digested preferentially in the 18-day chick embryo red blood cells (which contain only adult-type globin protein chains), while only embryonic sequences were degraded more rapidly in 5-day chick embryo red blood cells (which contain only embryo-type globin protein chains). Neither set of sequences was hypersensitive to DNase I in fibroblast or brain cells; nor were the genes coding for ovalbumin mRNA degraded rapidly in red blood cell nuclei. A chromatin structure hypersensitive to DNase I has since been detected for other active genes: the ovalbumin gene in hen oviduct (Garel & Axel, 1976; Garel et al., 1978; Bellard et al., 1977), the ribosomal genes in various organisms (Weintraub, 1975; Mathis & Gorovsky, 1978; Stadler et al., 1978), in a number of integrated viral genomes (Frolova & Zalmanzon, 1978; Frolova et al., 1978; Chae et al., 1978; Groudine et al., 1978; Panet & Cedar, 1977; Breindl & Jaenish, 1979), and the induced

heat-shock loci in Drosophila tissue culture cells (Wu et al., 1979). In addition, chromatin sequences complementary to the total population of nuclear RNA (Weintraub & Groudine, 1976), nuclear poly-A RNA (Garel et al., 1977) and the cytoplasmic poly-A RNA (Levy-W. & Dixon, 1977a) are hypersensitive to DNase I digestion.

The property of active chromatin, detected by DNase I as an increased sensitivity to digestion, could be envisioned either as an intrinsic property of individual active nucleosomes or as a property generated by the interaction between subunits of higher order structure. For the globin gene in chick red blood cells and for integrated avian virus genomes, isolated monomer subunits retain DNase I hypersensitive structures, implying that interactions between subunits is not a factor in the enhanced accessibility of these genes (Weintraub & Groudine, 1976; Groudine et al., 1978). However, there appears to be a loss of DNase I hypersensitivity for the ovalbumin gene in isolated subunit monomers from hen oviduct (Garel & Axel, 1976). The discrepancy in these results might be attributed to differences in the procedures for isolating subunit monomers if the property of DNase I sensitivity is a labile one. Recently nucleosomes containing the ovalbumin gene have been prepared with retention of the DNase I hypersensitive state (Senear & Palmiter, 1981).

The factor (or factors) responsible for the DNase I sensitivity has been investigated by analysis of the nucleosomal subunits from active chromatin. Any component that co-solubilizes with active nucleosomes could be involved in the maintenance or generation of an

active chromatin structure. To date, two major biochemical differences between active and inactive chromatin have been observed: active regions are enriched in particular proteins and their histones are more highly acetylated.

The high mobility group proteins 14 and 17 (recently reviewed; Johns, 1982) have been shown to confer DNase I sensitivity to active genes (Weisbrod & Weintraub, 1979; Weisbrod et al., 1980). In the same vein, micrococcal nuclease digestion studies have also shown that HMG 14 and 17 or H6, an HMG 14 and 17-like protein, are associated with nucleosomes that are enriched for active genes (Levy-W. & Dixon, 1978a; Levy-W. et al., 1979a; Goodwin, et al., 1981; Davie & Saunders, 1981a; Egan & Levy-Wilson, 1981). HMG 14 and 17 have been shown to bind to core particles (Mardian et al., 1980; Sandeen et al., 1980; Albright et al., 1980) and specifically are able to recognize amongst HMG-depleted nucleosomes those nucleosomes containing transcribable DNA sequences (Sandeen et al., 1980; Weisbrod et al., 1980; Weisbrod & Weintraub, 1981).

Acetylation of the inner histones (reviewed; Allfrey, 1980), particularly that of H3 and H4, has also been shown to be preferentially associated with nucleosomes from transcriptionally competent chromatin that is sensitive to DNase I (Davie & Candido, 1980; Davie & Saunders, 1981), DNase II (Davie & Candido, 1978) and micrococcal nuclease (Levy-Wilson et al., 1979b; Nelson et al., 1980).

CHROMATIN STRUCTURE OF THE YEAST, S. cerevisiae

Studies of the chromatin structure of S. cerevisiae have a two fold motivation. They are of evident importance for a further understanding of the species itself, but, in addition may shed light on active chromatin in general.

The yeast genome is organized into nucleosomes with a 160 base pair repeat (Lohr & Van Holde, 1975; Lohr et al., 1977a,b; Nelson et al., 1977) and at least part of the genome contains nucleosomes that are phased in relation to one another (Lohr et al., 1977c; Lohr & Van Holde, 1979c).

Yeast chromatin contains the four inner histones H2A, H2B, H3 and H4 (Wintersberger et al., 1973; Franco et al., 1974; Brandt & von Holt, 1976; Moll & Wintersberger, 1976; Thomas & Furber, 1976; Nelson et al., 1977; Franco & Lopez-Brana, 1978; Mardian & Isenberg, 1978a). H4 has an amino acid composition close to that of calf and pea, but the other yeast inner histones have diverged from those of other eukaryotes (Mardian & Isenberg, 1978a). However, calf-yeast and yeast-calf H2A-H2B interactions suggest that the interacting surfaces of H2A and H2B have been highly conserved. The H3 histone of yeast is the most divergent of the yeast histone from those of pea and calf. H1 has not been demonstrated (Marian & Wintersberger, 1980; Moll & Wintersberger, 1976; Wintersberger et al., 1973).

The yeast H3 and H4 have only one amino acid sequence variant each, as determined from the gene sequences (personal communication of Smith, M. in Bohm et al., 1981) and from the isolated proteins (Brandt et al., 1980). They have been compared for homology to calf and pea. The amino acid sequence of H3 determined from the protein itself (Brandt & von Holt, 1982) differs in the assignment at positions 31, 42, 94 and 97 from the sequence determined from the gene (personal communication of Smith, M. in Bohm et al., 1981). The yeast histones H4 and H3 and possibly H2B have been shown to be highly acetylated (Davie et al., 1981b; Nelson, 1982). Yeast histones H2B and H2A, however do have amino acid sequence variants. H2B was observed to have two variants by acid-urea gel electrophoresis of purified whole H2B (Mardian & Isenbereg, 1978a; Weber & Isenberg, 1980; Davie et al., 1981b) and amino acid analysis of the isolated variants (Brandt et al., 1980) confirms the two H2B variants. The yeast histone genes, H2A and H2B, have been isolated (Hereford et al., 1979) and there are two copies of each. The amino acid sequences for the two H2Bs have been determined from the genes (Wallis et al., 1980) and differ by four residues at the amino terminus at positions 2, 3, 27 and 35. The amino acid sequences for the two H2As have also been determined from the genes (Choe et al., 1982) and differ by two conservative substitutions in the carboxy terminus at positions 124 and 125. The amino acid sequences for the H2As from the genes confirms the existence of H2A variants (Hereford et al., 1979; Brandt et al., 1980) but however the homology is much closer than indicated by the amino acid analysis of the H2A variants (Brandt et al., 1980). The H2B subtypes are dispensible during the

yeast cell cycle as mutant strains in either of the variants are viable (Rykowski et al., 1981); demonstrating that the H2B variants are interchangeable in yeast chromatin structure.

The presence of a high mobility group protein has been observed in yeast (Spiker et al., 1978) and at least four putative HMGs are present (Weber & Isenberg, 1980; Petersen & Sheridan, 1978).

Approximately 20% of the yeast single-copy DNA is complementary to mRNA, indicating that at least 40% of the genome is transcribed (Hereford & Rosbash, 1977). The DNase I sensitivity of the transcribed chromatin has been determined and is comparable to that of the total yeast chromatin. That the DNase I sensitivity is the same for the transcribed and total yeast chromatin indicates that the entire yeast genome is equally DNase I sensitive (Lohr & Hereford, 1979a) and is entirely in the transcriptionally competent state. The chromatin structure of yeast has been further analyzed by electrophoretic transfer of high-resolution micrococcal nuclease and DNase I digestion DNA gels to DBM paper and hybridized to either cDNA to poly A mRNA, nascent in vitro elongated RNA or sonicated total genome DNA. The hybridization patterns resemble the DNA patterns produced by the total yeast nucleosomes. This indicates that the transcribed and total yeast chromatin are likely organized into the same nucleosomal structure (Lohr, 1981), confirms that the entire yeast genome is equally DNase I sensitive, and supports the

idea that the entire yeast genome is in the active state. The DNase I sensitivity of the yeast genome observed in the nucleus is maintained upon micrococcal nuclease digestion and is present in isolated nucleosomal oligomers and monomers (Lohr & Ide, 1979b), demonstrating that it is possible with yeast to isolate without fractionation active chromatin for biochemical and biophysical analysis.

HIGH MOBILITY GROUP PROTEINS OF *Saccharomyces cerevisiae*

Introduction

The high mobility group (HMG) proteins occur in a variety of animal species (Goodwin et al., 1973, 1975; Watson et al., 1977; Wigle & Dixon, 1971; Smith & Stocken, 1973; Elgin & Bonner, 1972; Franco et al., 1977; Sterner et al., 1978; Johns et al., 1975) and in plants and yeast as well (Spiker et al., 1978). A number of studies have concluded that the HMGs are preferentially located in active regions of the genome (Vidali et al., 1977; Weisbrod & Weintraub, 1979; Levy et al., 1977a,b; Levy & Dixon, 1978a,b), although a recent report (Goodwin and Johns, 1978) has questioned this. In any case, the function or functions of the HMG proteins are not yet known.

The HMGs have been extracted from nuclei or chromatin by either 0.35 M NaCl (Goodwin et al., 1973, 1975) or 5% PCA (Goodwin et al., 1977a,b). They have unusual amino acid compositions. A large number of residues are basic, and a large number are acidic.

Of the many calf thymus HMGs, only four have been shown to be unique proteins (Goodwin et al., 1978a). The rest either are breakdown products or appear in such small amounts that it is difficult to say if they are breakdown products or not. Ubiquitin

(Goldstein et al., 1975; Schlesinger et al., 1975) may be eluted from chromatin by 0.35 M salt (Watson et al., 1978; Walker et al., 1978), but it has an amino acid composition unlike that of the HMG proteins (Schlesinger et al., 1975) and it is doubtful if it should be included in that group.

In the present paper we report a study of the HMGs of the yeast Saccharomyces cerevisiae. The fungi in general constitute a separate kingdom in the five kingdom classification of Wittaker (1969) and are considered a separate major line of evolution alongside plants and animals. It is known that yeast contains at least one HMG (Spiker et al., 1978). Yet, before my work, no one had attempted to characterize how many HMGs are in yeast (Petersen & Sheridan, 1978) or any other fungus or, for that matter, in any plant.

We find that S. cerevisiae contains four proteins having typical HMG amino acid compositions. Three of these may be eluted by 0.35 M NaCl. The fourth is not extractable by 0.35 M NaCl but may, however, be eluted by 0.25 N HCl. It follows that an HMG protein cannot be defined, in general, by criteria based on extractability. Our results also indicate that the HMGs show significant evolutionary divergence.

Materials and Methods

Yeast chromatin was prepared from pressed baker's yeast by the method of Mardian & Isenberg (1978a,b) which is a scaled up version of the method of Tonino & Rozijn (1966). Proteins were extracted either from freshly prepared chromatin or from chromatin first frozen overnight at -80°C in 2.0 M sucrose, 5 mM Tris-HCl, pH8.0, 1 mM $MgSO_4$, and 0.1 mM PMSF. No differences were observed in the proteins obtained by these two procedures. All steps were carried out at 0-4°C, and 0.1 mM PMSF was added to all solutions to inhibit proteolysis.

HMGs were extracted according to the method of Goodwin et al. (1975). The purified yeast chromatin from 400g of cells was suspended in 100 mL of 0.075 M NaCl, 0.025 M EDTA, 1 mM Tris-HCl, pH 7.5, and 0.1 mM PMSF and then centrifuged in a swinging bucket rotor for 10 min at 9000g. This was then repeated. The chromatin pellet was then suspended by one stroke of a tightly fitting Potter-Elvehjem homogenizer in 100 mL of 0.35 M NaCl, 1 mM Tris-HCl, pH 7.0, and 0.1 mM PMSF. It was stirred gently in the cold (2°C) for 1 h. The suspension was centrifuged in a swinging bucket rotor for 10 min at 9000g to pellet the chromatin. The 0.35 M NaCl supernatant was poured off and maintained at 0°C. The chromatin was extracted a second time for 30 min by using 50 mL of the same solution in the same manner. The combined 0.35 M NaCl extracts were made 2% in Trichloracetic acid (TCA) by the addition of 87.6% TCA while being stirred rapidly. (The concentration of the TCA was determined by measuring the index of refraction). After the

addition of the TCA, the suspension was stirred for 5 min to allow the complete precipitation of the low mobility group (LMG) proteins to occur. The suspension was centrifuged in the swinging bucket rotor for 10 min at 9000g. The supernatant was made 0.01 M in β-mercaptoethanol, and then with rapid stirring 2.25 mL of concentrated (29.2%w/v) NH_4OH was added, followed by the rapid addition of 3 volumes of cold acetone.

Histones were obtained from the chromatin pellet which had been extracted by 0.35 M NaCl. The pellet was suspended in 50 mL of glass-distilled water with one stroke of a Potter-Elvehjem homogenizer, and 50 mL of 0.5 N HCl was then added. It was stirred gently at 2°C for 1 hr and then centrifuged in a swinging bucket rotor for 20 min at 9000g. Eight volumes of cold acetone was added to the supernatant.

The HMG and histone precipitates were collected by centrifugation in a swinging bucket rotor for 10 min at 9000g. The HMG precipitate was washed twice with cold acidified acetone (6 volumes of acetone to 1 volume of 0.1 N HCl) and 3 times with cold acetone and then dried under vacuum. The histone precipitate was washed 3 times with cold acetone, pelleted at 9000g for 10 min in a swinging bucket rotor, and dried under vacuum.

The individual yeast HMGs were isolated by using preparative electrophoresis on acetic acid-2.5 M urea-polyacrylamide slab gels (3x140x102 mm). The bands were visualized by phosphorescence and

were excised and eluted electrophoretically as described elsewhere (Mardian & Isenberg, 1978a,b). After elution the protein solution was desalted on a 1.2x29 cm Sephadex G-25 column equilibrated with 0.01 NHCl and lyophilized. Amino acid analyses were performed as previously described (Spiker & Isenberg, 1977).

Sodium dodecyl sulphate (SDS) gels were run according to Thomas & Kornberg (1975), except that the stacking gel was 6% acrylamide instead of 3%. Acetic acid-2.5 M urea gels were run according to Panyim & Chalkley (1969) and were stained in either 0.1% Coomassie Brilliant Blue R, 45% methanol, and 9% acetic acid or 0.1% Amido Black, 2-% ethanol, and 7% acetic acid. Gels were run on a microslab apparatus (Matsudaira & Burgess, 1978).

Results

The yeast proteins extracted by 0.35 M NaCl were separated by preparative gel electrophoresis and visualized by phosphorescence (Mardian & Isenberg, 1978a,b), Figure 1.

The gel edge strips in Figure 2 show four major bands which are also seen by phosophorescence. Table I presents the amino acid analyses of the four proteins.

With but one exception, all known HMGs have high and nearly equal amounts of acidic and basic residues. The exception, H6 of trout (Watson et al., 1979), is rich in basic residues but contains a lower content of acidic residues than other HMGs.

Proteins S_1, S_3 and S_4 have high and nearly equal amounts of acidic and basic residues. S_2 does not; it is low in basic residues. We therefore identify S_1, S_3 and S_4 as at least putative HMG proteins but place S_2 in another category.

Table II presents the amino acid composition of the yeast protein which had been previously identified as an HMG (Spiker et al. 1978). In the present paper we label this protein HMGa (HMG, acid extracted). Table II also shows the compositions of the calf thymus HMG proteins (Goodwin et al., 1978b): HMGs 1 and 2 (Goodwin et al., 1973), HMG 14 (Goodwin et al., 1977a), and HMG 17 (Goodwin et al., 1975).

Figure 3 shows an acetic acid-urea gel of both the salt-extractable and acid-extractable proteins. HMGa is not extracted by salt but is by 0.25 N HCl. It appears as an extra band in preparations of inner histones and in fact was first observed in such preparations (Mardian & Isenberg, 1978a).

Figure 4 shows SDS gels of the individual yeast HMGs and whole calf thymus HMG. Figure 4 again shows that HMGa is not extracted by 0.35 M salt.

Clearly, the mobilities of the yeast and calf HMGs differ, although the mobility of S_3 does not vary much from that of HMG 17. However, the amino acid composition of S_3 differs from that of HMG 17 (Figure 5). The yeast and calf HMGs have diverged markedly.

Evolutionary divergence is also demonstrated by comparisons of acetic acid-urea gel mobilities. Figure 6 shows acetic acid-urea gels of yeast and calf HMGs. Clearly, the mobilities of the yeast and calf thymus HMGs differ. We note that yeast HMG S_4 is a single band on acetic acid-urea gels, although it is a doublet on SDS gels.

Figure 1 Phosphorescence of yeast 0.35 M NaCl extractable proteins separated on a preparative acid-urea gel

Figure 1

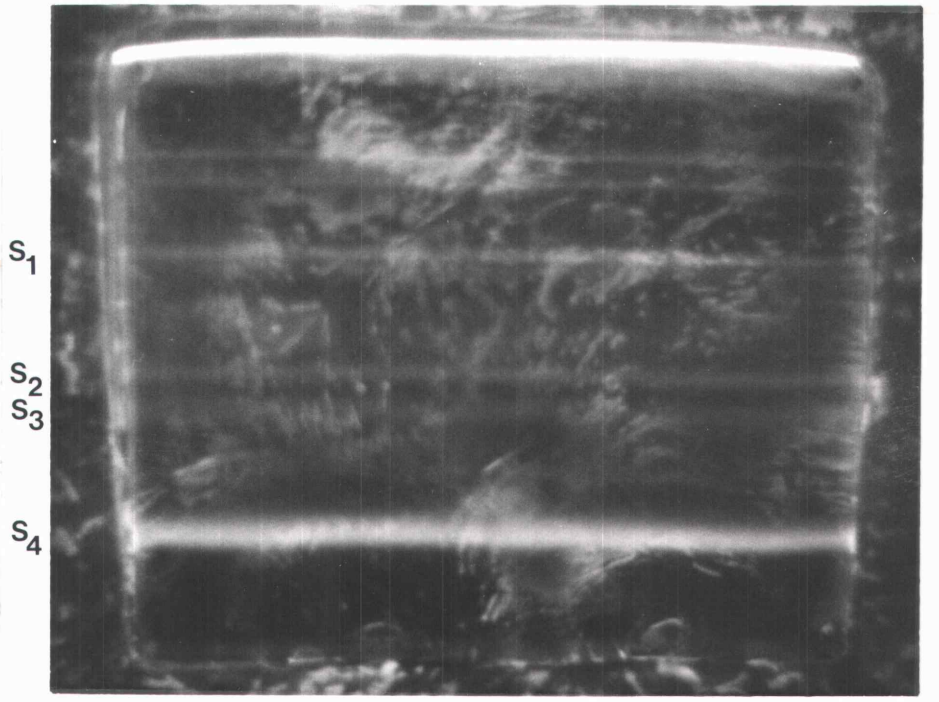


Figure 2: Acetic acid-urea preparative gel edge strips stained with 0.1% Amido Black, 20% ethanol, and 7% acetic acid and electrophoretically destained.

Figure 2

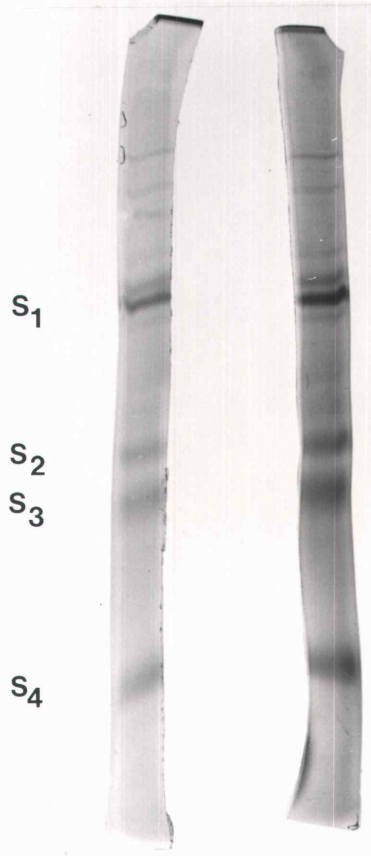


Table I: Amino Acid Composition of Yeast 0.35 M NaCl Extractable Proteins

	mol % in			
	S_1	S_2	S_3	S_4
Asx	7.8	9.9	6.6	9.5
Thr	5.9	5.5	7.3	5.6
Ser	10.4	9.6	9.9	8.0
Glx	9.7	12.5	10.2	11.7
Gly	7.7	13.5	5.6	11.4
Ala	9.9	9.1	10.8	9.5
Cys	0.7	0	0	0
Val	5.5	8.3	5.5	4.8
Met	0	0	0	0
Ile	3.8	0.6	5.7	3.0
Leu	6.5	8.5	5.0	6.7
Tyr	2.0	0.7	4.9	0.7
Phe	2.8	1.7	4.7	5.7
Lys	19.2	8.8	12.2	14.1
His	4.9	7.8	7.2	4.6
Arg	3.5	3.9	4.7	5.1
Asx + Glx	17.5	22.1	16.8	21.2
Lys + Arg	22.7	12.7	16.9	19.2

Table II: Amino Acid Composition of Yeast HMGa and Calf Thymus HMGs

	mol % in				
	yeast HMGa	calf thymus HMG 1	calf thymus HMG 2	calf thymus HMG 14	calf thymus HMG 17
Asx	8.5	10.7	9.3	8.1	12.0
Thr	8.4	2.5	2.7	4.2	1.2
Ser	7.5	5.0	7.4	7.8	2.3
Glx	15.6	18.1	17.5	17.1	10.5
Pro	5.9	7.0	8.9	8.5	12.9
Gly	3.6	5.3	6.5	6.5	11.2
Ala	8.8	9.0	8.1	14.5	18.4
Val	2.3	1.9	2.3	4.2	2.0
Cys	0	trace	trace	0.7	0
Met	0	1.5	0.4	0	0
Ile	6.5	1.8	1.3	0.5	0
Leu	7.5	2.2	2.0	2.0	1.0
Tyr	4.4	2.9	2.0	0.4	0
Phe	2.8	3.6	3.0	0.6	0
Lys	15.9	21.3	19.4	19.0	24.3
His	1.3	1.7	2.0	0.3	0
Arg	5.5	3.9	4.7	5.6	4.1
Asx + Glx	24.1	28.8	26.8	25.2	22.5
Lys + Arg	21.4	25.2	24.1	24.6	28.4

Figure 3: Acetic acid-urea gel of yeast proteins extracted with 0.35 M NaCl (left), HMGa (center), and yeast proteins extracted by 0.25 N HCl from chromatin previously treated with 0.35 M NaCl (right).

Figure 3

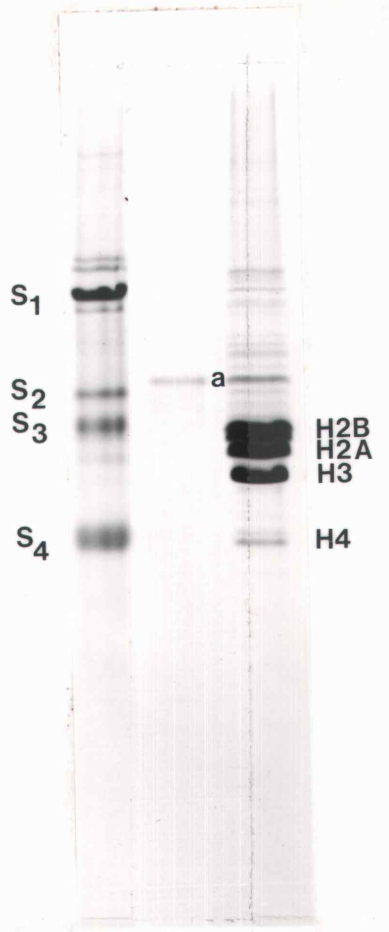


Figure 4: SDS gels of yeast and calf thymus HMGs: (A, C, F, H) calf thymus whole HMG; (B) S_1; (D, J) a; (E) S_3; (G) S_4; (I) yeast proteins extracted by 0.25 N HCl from chromatin previously treated with 0.35 M NaCl.

Figure 4

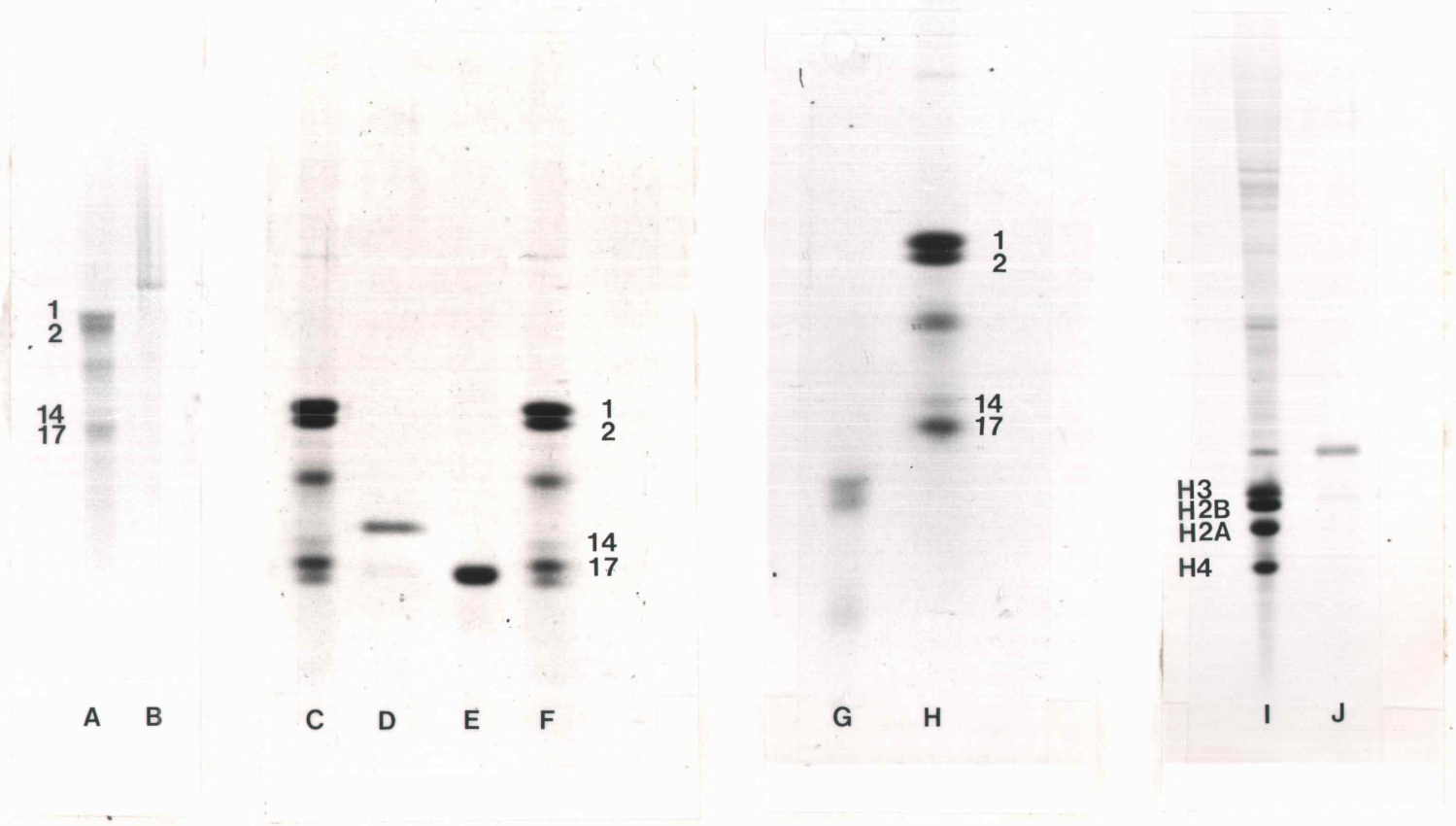


Figure 5: Residue profiles of yeast S_3, and calf thymus HMG 17.

Figure 5

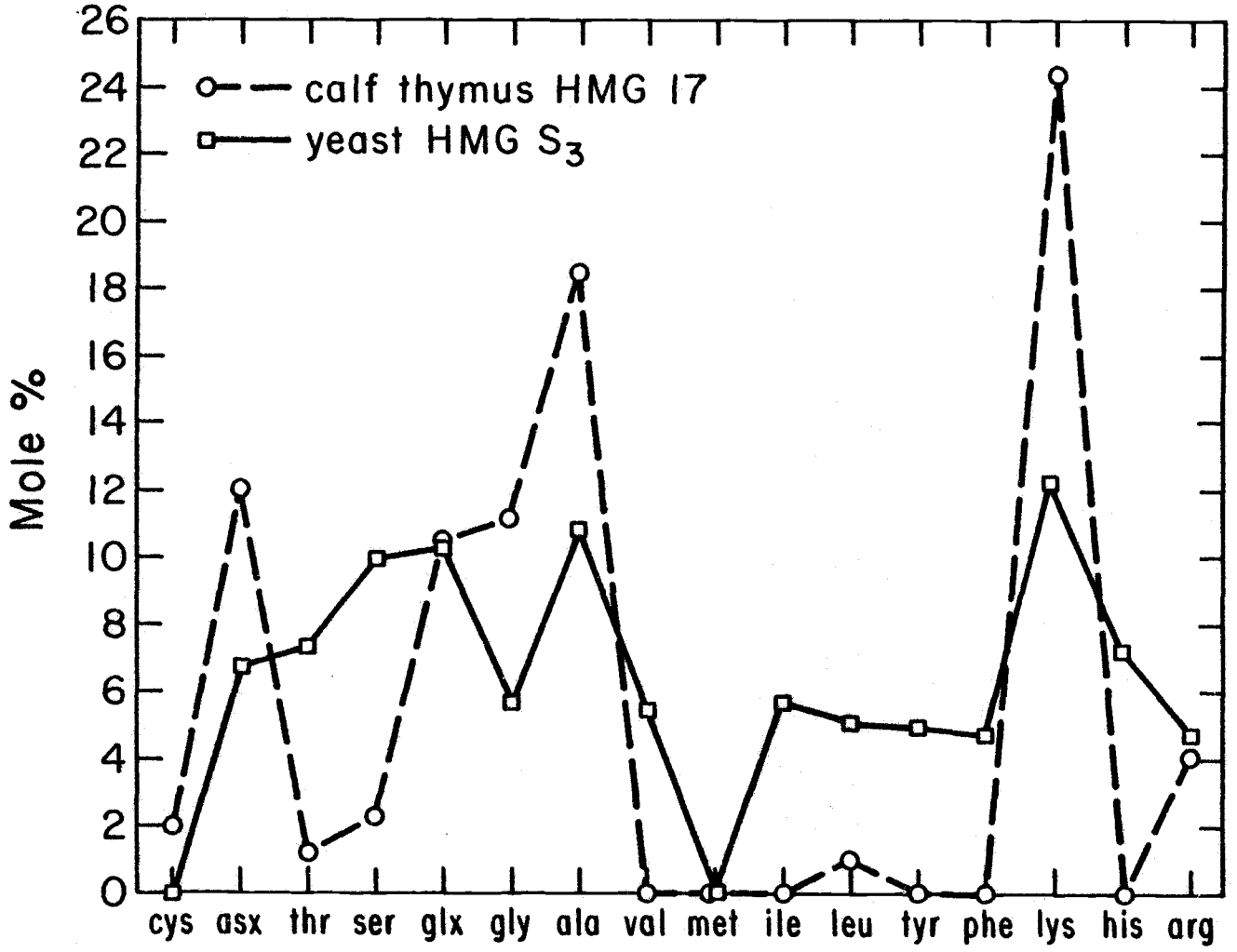

calf thymus HMG 17
yeast HMG S_3
Mole %
0
2
4
6
8
10
12
14
16
18
20
22
24
26
cys asx thr ser glx gly ala val met ile leu tyr phe lys his arg

Figure 6: Acetic acid-urea gels. (Panel A) Yeast proteins extracted with 0.35 M NaCl (A), HMG S_1 (B), HMGs S_3 (C), calf thymus whole HMG with the mobility of HMG 14 indicated (D) and yeast proteins extracted by 0.25 N HCl from chromatin previously treated with 0.35 M NaCl (E). (Panel B) Yeast proteins extracted with 0.35 M NaCl (left) and S_4 (right).

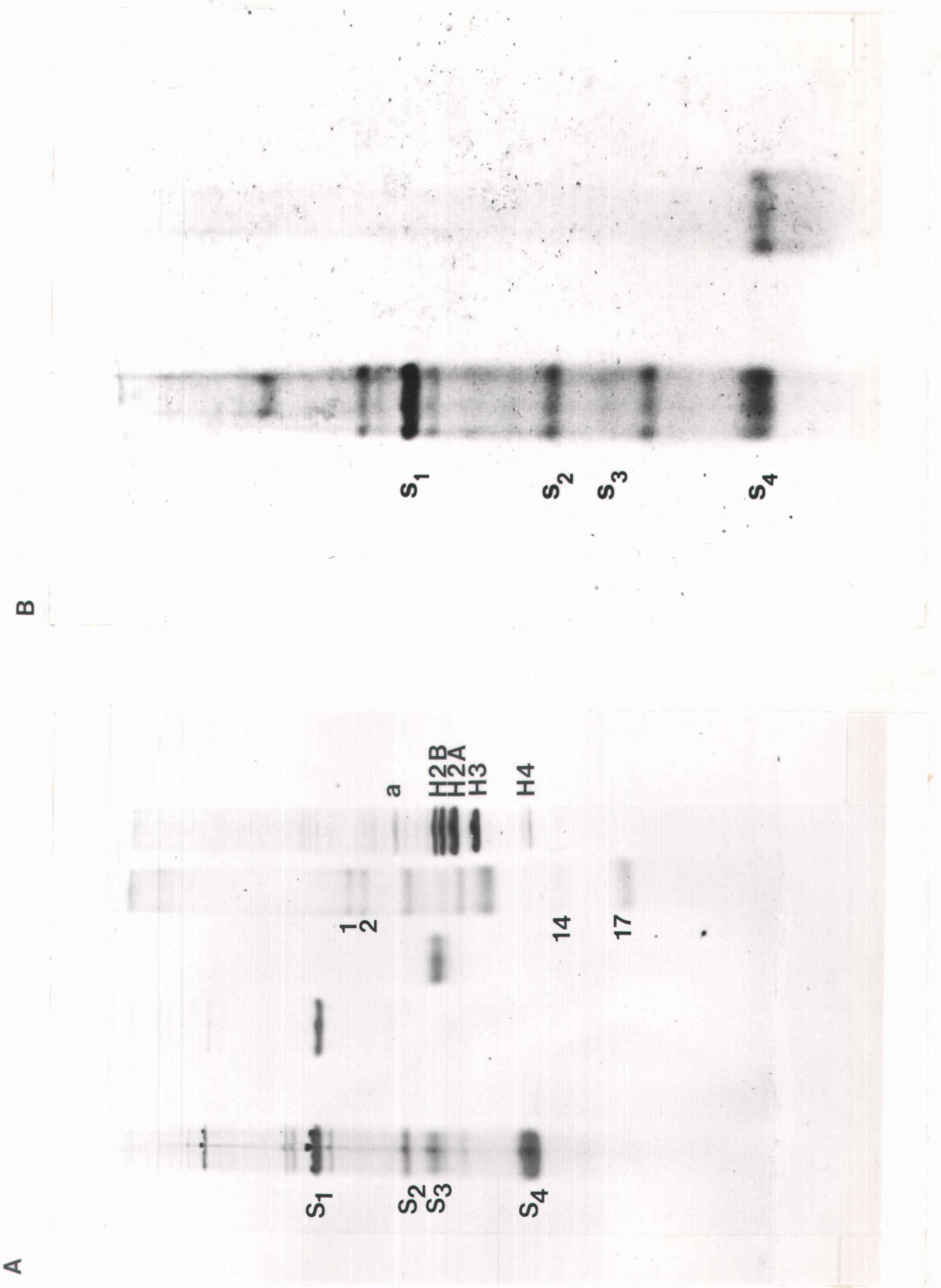


Figure 6

Discussion

Until we understand the function or functions of the HMG proteins, any definition must have an element of arbitrariness to it. One could attempt to define them as those proteins which are (1) extractable from chromatin by 0.35 M NaCl and (2) soluble in 2% trichloroacetic acid. However, we have now shown that at least one protein exists which has a typical HMG composition but which is not extractable from yeast chromatin by 0.35 M NaCl.

Alternately, we could attempt to define HMGs as lysine-rich chromosomal proteins which have high and nearly equal contents of basic and acidic residues. This definition also fails. Watson et al. (1979) have sequenced protein H6 of trout testis. It is clearly homologous to HMG 17 and yet only 6 of the 69 residues are acidic.

There is thus no definition of an HMG protein which does not have an exception. Nevertheless, thus far it has always been possible to know if a protein belongs to the HMG class.

For the yeast proteins, we suggest that a characteristic chemical composition might prove to be more fundamental than a characteristic extractability. For this reason, we designate as HMGs the yeast proteins that we have labeled S_1, S_3, S_4 and a.

Sommer (1978) has isolated a protein from S. cerevisiae by extraction with 0.2 N HCl. This protein has the same amino acid composition as HMGa (Mardian & Isenberg, 1978a,b) and at least similar mobilities in SDS and acetic acid-urea gels. It appears

therefore as if Sommer's protein is the same as HMGa. Sommer has called his protein an H1. We prefer to call it an HMG, since it has a characteristic HMG amino acid composition. Further clarification must evidently await a future understanding of the role of this protein in chromatin.

Since both calf and yeast have four HMGs, the possibility arises that all eukaryotes might have four HMGs. We emphasize, however, that we cannot regard this as known. As seen, a strict definition does not yet exist. In addition, we note that the minor protein components of the 0.35 M NaCl extractable, 2% TCA soluble, protein class have not yet been investigated. Some of these may turn out to be new HMG proteins and not merely breaakdown products or impurities. In this connection, it should be mentioned that while only two HMGs have thus far been reported in trout (Watson et al., 1977), others may exist (G. H. Dixon, private communication). It should also be noted that, despite efforts to block proteolysis, one or more of our HMGs could possibly be breakdown products of other proteins, just as HMG 3 of calf appears to be a breakdown product of HMG 1 (Goodwin et al., 1978a,b). Doubts of this type will most likely not be resolved until we understand at least some aspects of the function of HMGs.

We have shown that S_4 runs as a singlet on acid-urea gels and as a doublet on SDS gels. This behavior is unusual, and we note that other HMG proteins, HMG 2 of chicken thymus and HMG 2 of chicken erythrocytes, also have the same properties (Mathews et al., 1979).

HISTONE MODIFICATIONS OF *Saccharomyces cerevisiae*

Acknowledgment of Co-Authorship

I am a co-author of this publication, Histone Modifications of *Saccharomyces cerevisiae*, which was the result of a collaborative research project initiated and directed by Dr. Jim R. Davie. This paper was published under the authorship: J. R. Davie, C. A. Saunders, J. M. Walsh and S. C. Weber. In accordance with graduate school requirements I will designate my contribution to this publication.

At the beginning, Dr. Jim Davie and I discussed the possibility of the yeast, *Saccharomyces cerevisiae*, responding to butyrate in a similar manner to that of multicellular eukaryotes. An examination of acid-urea gels of yeast histones (for example, those in Section II of this thesis) determined that yeast H4 consisted of two bands. I showed that these two yeast H4s were likely to be the zero and mono-acetylated forms of yeast H4 as shown by their identical mobility with calf thymus zero and mono-acetylated H4 on an acid-urea gel (data not shown). This observation suggested that H4 in yeast perhaps would be similar in response to butyrate as multicellular eukaryotes; as at least one putative acetylated yeast H4 species could be detected in the absence of butyrate.

I provided the purified yeast HMGa, whole yeast histone, as well as, the individually purified yeast histones (a gift of Dr. J. K. W. Mardian, formerly a student in the laboratory of Dr. Irvin

Isenberg) that were used as standards to determine the quality of the yeast nuclear isolation proceedures and the identity of the various proteins observed in the two-dimensional gels, Fig. 7 and 8, (by co-migration in two-dimensional gels, data not shown).

I prepared yeast nuclei protected with butyrate by two procedures: Ide & Saunders (1981) and Lohr & Van Holde (1975), Lohr and Van Holde (1977a,b,c). This resulted in the determination that the level of H4 acetylation was not only dependent upon the time of isolation but the method of nuclear isolation as well.

The H2Bs of yeast were shown to be undergoing oxidation of methionine in the stacking gel itself during electrophoretic separation due to the catalyst ammonium persulfate as a result of reducing the proteins with thioglycolic acid and running a second-dimension gel without an ammonium persulfate catalyzed stacking gel.

I suggested that as a control that yeast histones be directly isolated from yeast cells in a very rapid procedure and the level of acetylation be compared to that observed in nuclei isolated in the presence of butyrate to determine if the high levels of acetylation observed were induced hyper-acetylation or rather might possibly reflect the <u>in vivo</u> level of histone acetylation as a result of butyrate protection. I prepared chromatin from yeast cells broken with glass beads and nuclei (by the procedure of Ide & Saunders, 1981) in the presence and absence of butyrate. After isolation of the yeast histones and two-dimensional electrophoresis of the proteins by Dr. Jim Davie the acetylation level of H4 from the

rapidly broken yeast cells appeared almost identical to that of the butyrate protected nuclei. The nuclei isolated in the absence of butyrate showed only zero and mono-acetylated H4. This suggests that the high level of yeast histone acetylation observed with butyrate protection is the in vivo level but, of course, I do not know this to be true.

Introduction

The yeast histones have been isolated and identified (Nelson et al., 1977; Mardian & Isenberg, 1978; Brandt et al., 1980). The four core histones of yeast chromatin include histones H3, H4 and two variant forms each of the histones H2A and H2B (Brandt et al., 1980). A protein which was suggested to be histone H1 (Sommer, 1978) has been found to correspond to HMGa (Weber & Isenberg, 1980).

The histones of multicellular eukaryotes can be enzymatically acetylated in two distinct ways (Dixon et al., 1975). In the first type, acetylation occurs at the 2-amino group of the amino-terminal serine of histones H1, H2A and H4. This type of acetylation follows soon after synthesis and is essentially irreversible. The second type occurs at the ϵ-amino groups of the lysyl residues at the amino-terminal portion of the core histones. This type of acetylation is a reversible modification (Dixon et al., 1975, Morre et al., 1979; Jackson et al., 1975).

It has been suggested that histone acetylation is involved in chromatin assembly. Shortly after their synthesis, histones are sequentially modified by acetylation and then by deacetylation (Dixon et al., 1975, Louie & Dixon, 1972; Ruiz-Carrillo, 1975). This process may mediate the correct interaction of the histone with DNA.

Histone acetylation has also been correlated with increased transcriptional activity in various systems (Allfrey, 1977; Gorovsky, 1977; Chestier & Yaniv, 1979). Several groups have reported evidence to support the idea that increased histone

acetylation may alter the structure of a chromatin region in such a fashion as to be favorable for transcription. DNase I has been demonstrated to preferentially digest transcriptionally competent regions of chromatin (Garel & Axel, 1976; Weintraub & Groudine, 1976) and, interestingly, chromatin that contains hyperacetylated histones is more readily attacked by DNase I (Mathis et al., 1978; Simpson, 1978a; Vidali, 1978a; Davie & Candido, 1980) than that which does not contain hyperacetylated histones. Also, a chromatin fraction containing high levels of acetylated histone species can be obtained by using procedures that selectively solubilize the transcriptionally competent regions of chromatin (Nelson et al., 1978; Sealy & Chalkley, 1978b; Kuehl et al., 1980; Davie & Candido, 1978). Recently, Dobson and Ingram, 1980, have reported that the rate of elongation and, possibly, the rate of initiation for transcription is greater for chromatin containing hyperacetylated histones than that of chromatin containing low levels of acetylated histone species.

Multiple acetylations of the N-terminal portion of the molecule may reduce nucleosome-nucleosome interations which would allow a region of chromatin to become extended (Mathis, et al., 1978; Simpson, 1978a; Whitlock & Stein, 1978; Pospelov et al., 1979; Chahal et al., 1980). Recent studies have reported that histone acetylation may also modulate the structure of the nucleosome (Simpson, 1978a; Bode et al., 1980). These structural alterations may be a prerequisite for a region of chromatin to be transcribed at an increased rate (Chahal et al., 1980).

Yeast provides an excellent system for studying the structure of transcriptionally active chromatin as the majority of the chromatin is in a transcriptionally competent conformation (Lohr & Hereford, 1979a). This makes it especially interesting to examine the level of histone acetylation in this organism. We have examined the content of the acetylated histone species in various yeast strains and compared the modified histone content of yeast to that associated with both chromatins of low transcriptional activity (calf thymus) and chromatins that are inactive (chick erythrocyte).

Materials and Methods

Strains and Growth Conditions

The strains employed in these studies were Y-55, a wild type diploid; A364A haploid; D5, a diploid of A364A; 20B-12α, a haploid carrying the pep_{4-3} mutation which reduced the levels of proteases A, B and C ~95% (Jones, 1977).

Strains were grown on YEPD (yeast extract 1%, Bacto peptone 2%, supplemented with 40 μg/ml each of adenine and uracil) at 30°C on a New Brunswick rotary platform shaker to the appropriate growth stage.

Isolation of Nuclei

I. Yeast: Cells in the vegetative phase were harvested by centrifugation at 2,500 x g for 2 min in a Sorvall GS3 rotor and washed once with distilled water containing the indicated amount of sodium butyrate. From this point on, sodium butyrate was added in the indicated concentrations. Cells were repelleted and resuspended in S buffer (1.1 M Sorbitol, 20 mM KH_2PO_4 (pH 6.5), 0.5 mM $CaCl_2$, 0.5% β-mercaptoethanol) 4 ml/gm wet weight and Zymolyase 5000 (Kirin Breweries) 1.5 mg/ml and spheroplasted at 35°C. The spheroplasts were then harvested by centrifugation at 4,000 x g for 5 min in a Sorvall SS-34 rotor. All further manipulations were at 4°C using buffers treated with 1 mM PMSF. The pelleted spheroplasts were homogenized in 0.1 ml/gm of 18% Ficoll 4000 (Pharmacia) using a loose fitting Teflon homogenizer. This step lyses the cytoplasmic membrane and relase of the nuclei is monitored microscopically. The

mixture was then diluted with an equal volume of 1.0 M Sorbitol, 0.5 mM $CaCl_2$ and loaded onto a preformed Percoll gradient (the gradients were formed by mixing the appropriate volumes of 1.0 M Sorbitol, 0.5 mM $CaCl_2$, pH 6.5, and 100% Percoll 1.0 M Sorbbitol (prepared using Percoll as the solvent), 0.5 mM $CaCl_2$, pH 6.5). Haploid nuclei were loaded onto 32.5% gradients, diploids 35% gradients. The gradients were preformed by centrifugation at 27,000 x g for 50 min in a Sorvall SS-34 rotor. The nuclei were recovered from the middle of the gradient after centrifugation for 15 min at 9,500 x g in a Sorvall HB-4 rotor. The nuclei were then diluted with 2 volumes of 1.0 M Sorbitol, 0.5 mM $CaCl_2$, pH 6.5, and pelleted by centrifugation at 4,000 x g for 5 min in a Sorvall SS-34 rotor. This nuclear isolation procedure is described elsewhere in more detail (Ide & Saunders, 1981).

II. Calf thymus and chicken erythrocytes: Calf thymus tissue or packed erythrocytes from chicken were prepared essentially as described by Weischet et al., 1979 and by Shaw et al., 1976, respectively, except the Wray annd Stubblefield buffer (Wray & Stubblefield, 1970) was modified to include the following: 1 M hexylene glycol, 0.1 mM PIPES (pH 7.0), 1mM $CaCl_2$, 2 mM $MgCl_2$, 15 mM β-mercaptoethanol and 30 mM sodium butyrate. In addition, the cells were homogenized in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle. All isolation buffers contained 30 mM sodium butyrate.

Preparation of Histones

Histones were extracted from calf thymus or chicken erythrocyte by resuspending the nuclei with H_2O and adding 4 N H_2SO_4 to a concentration of 0.4 N. After incubation on ice for 30 min, the insoluble material was removed by centrifugation. The supernatant fraction was dialyzed against 0.1 N acetic acid and lyophilized.

To prepare histones from yeast, the nuclei were resuspended in Buffer A (10 mM Tris-HCl, pH 8.0, 0.5% NP-40, 75 mM NaCl and, in some cases, 30 or 100 mM sodium butyrate was added). After 20 to 30 min incubation on ice; the nuclei were collected by cenntrifugation. The above step was repeated two more times. Nuclei were then resuspended in Buffer B (10 mM Tris-HCl, pH 8.0, 0.4 M NaCl and 30 or 100 mM sodium butyrate was added when necessary). After 30 min incubation on ice, the nuclei were collected by centrifugation and resuspended in Buffer B. After centrifugation, the pellet was acid extracted four times as described above.

Polyacrylamide Gel Electrophoresis

All electrophoresis was done by using a microslab apparatus (Matsudaira & Burgess, 1978) (Idea Scientific, Corvallis, Oregon). Fifteen percent polyacrylamide-sodium dodecyl sulfate slab gels were made as described by Laemmli (Laemmli, 1971). Acid-urea slabs were made according to Panyim and Chalkley (Panyim & Chalkley, 1969) (2.5 M urea was used) with modifications as described by Spiker, 1980. Acid-urea Triton X-100 gels were made according to Zweidler, 1978, (6 mM Triton X-100, 6.7 M urea) with modification as described by Spiker, 1980. Two dimensional gels with acid-urea in the first

dimension and polyacrylamide-SDS in the second dimension were run essentially as described by Davie and Candido, 1978, but using the polyacrylamide-SDS slab gel system of Laemmli, 1971. Two-dimensional gels with acid-urea in the first dimension and acid-urea Triton X-100 in the second dimension were prepared as follows: gel slices from the first dimension were briefly equilibrated (10 min) in 6 mM Triton X-100 and then applied horizontally to the top of the acid-urea Triton X-100 slab gel. The slice was sealed with melted 1% agarose in sample buffer, Spiker, 1980. Gels were stained with 0.25% Coomassie blue G-250 in 45% methanol and 9% acetic acid. Gels were destained briefly (30 to 60 min) by diffusion in methanol/acetic acid/water (2:1:5 vol/vol) and then destained further in 7.5% acetic acid and 5% methanol.

Quantification of Modified Histone Species

The spot of interest was cut out from the two-dimensional gel and placed with 70 µl of 25% pyridine as described by Fenner et al., 1975. After 12 hr, the absorbance at 595 nm of the resulting solution was determined by using a 1.5 mm silanized microcuvette and a microcuvette holder designed for the Cary 14.

Results

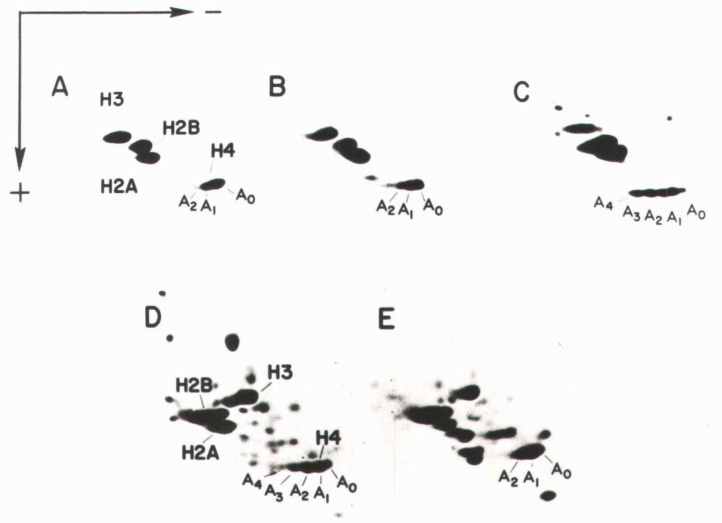
The content of the modified histone forms from different strains of yeast, Saccharomyces cerevisiae, has been examined. The strains utilized in this study were Y55, a wild-type diploid from H.O. Halvorson; 20B-12α, a haploid carrying the pep_{4-3} mutation which reduces the level of proteases A, B and C roughly 95%, (Jones, 1977); A364A, a haploid; and the diploid of the same strain, D5.

Nuclei were isolated in sodium butyrate containing buffers from the above yeast strains, chicken erythrocytes, calf thymus and HeLa cells. Sodium butyrate has been reported to inhibit the histone deacetylating enzymes of multicellular eukaryotes (Boffa et al., 1978; Sealy & Chalkley, 1978a; Candido et al., 1978). Preliminary studies suggest sodium butyrate will also inhibit the yeast histone deacetylases. If cells are grown in the presence of sodium butyrate, the acetylated histone species will accumulate (Riggs et al., 1977). To prepare hyperacetylated histones, HeLa cells were grown for 24 hr in the presence of 5 mM sodium butyrate.

Acid soluble nuclear proteins from the yeast strain Y55, calf thymus, chicken erythrocyte and HeLa nuclei were resolved by two-dimensional electrophoresis, acid-urea gel electrophoresis in the first dimension followed by SDS-polyacrylamide gel electrophoresis in the second. Figures 7a, b and c demonstrate the position of the core histones (H3, H2A, H2B, H4) from calf thymus, chicken erythrocyte and HeLa as well as their associated modified

Figure 7. Two-dimensional electrophoretic patterns of histones extracted with H_2SO_4 from nuclei of calf thymus, chicken erythrocytes, HeLa and yeast. Acid soluble nuclear proteins were resolved by acid-urea gel electrophoresis in the first dimension and by 15% polyacrylamide-SDS gel electrophoresis in the second dimension. A, chicken erythrocyte; B, calf thymus; C, HeLa (cells grown in the presence of 5 mM sodium butyrate for 21 hours); D and E, yeast strain Y55 (nuclei were isolated in the presence or absence of 100 mM sodium butyrate, respectively). X, Y and Z are uidentified protein(s).

Figure 7


A
H3
H2B
H4
H2A
A2 A1
A0
B
A2 A1
A0
C
A4 A3 A2 A1 A0
D
H2B
H3
H2A
H4
A4 A3 A2 A1
A0
E
A2 A1
A0

forms. The assignment of the yeast core histones (Figure 7d and e) was made possible from their known electrophoretic migration positions on acid-urea gels and on SDS-polyacrylamide gels (Mardian & Isenberg, 1978; Weber & Isenberg, 1980).

The yeast histone H4 was composed of five species which presumably corresponds to the unacetylated (A_0), mono-(A_1), di-(A_2), tri-(A_3) and tetraacetylated (A_4) species. The histone H4 heterogeneity was attributed to acetylation because 1) the yeast histone H4 can be separated into five species and resolved in a similar position on the two dimensional gels as the known H4 acetylated species (Figure 7c); and 2) if sodium butyrate was not present during the nuclear isolation, the distribution of the histone H4 species would shift to the right (compare Figure 7d and e) which was probably due to the accumulation of the histone H4 A_0 and A_1 species at the expense of the A_2, A_3 and A_4 species.

The yeast histone H3 was likely acetylated as well because "tailing" was observed when the nuclei were isolated in the presence of sodium butyrate, but not in its absence (compare Figure 7d and e).

The histone H3 and H4 heterogeneity cannot be due to variant forms that differ by one charge, as Brandt et al., 1980, have recently reported that there was only one variant form for histones H3 and H4.

The heavy bands that were present in Figure 7(e) but not in Figure 7(d) could be removed if the nuclei were washed more extensively with Buffer A (see Materials and Methods).

These studies demonstrate that to observe the normal levels of acetylated histone species, it was essential to have sodium butyrate present in the isolation medium. During the course of these studies, we observed that the sodium butyrate concentration required to fully protect the existing acetylated species from deacetylation was dependent on the spheroplasting time and the method of nuclear isolation (e.g., long spheroplasting times required higher concentrations of sodium butyrate.

The content of the acetylated histone H4 species isolated from the nuclei of calf thymus, chicken erythrocyte, yeast (Y55, butyrate present) and yeast (Y55, butyrate absent) was quantified (Table III). The histone H4 A_2 species of yeast were most abundant if sodium butyrate was present during the nuclear isolation, but the histone H4 A_1 species were predominant if sodium buyttyrate was not present. A similar distribution of acetylated histone H4 species was observed for the different strains examined. In all cases when butyrate was present in the isolation buffers, the diacetyl H4 species predominated (see Figure 7)(Y55, Table III, and A364A and D5, not shown). Similar levels of acetylated histone H4 species were observed if the histones were extracted from either nucleosomes (20B-12α strain was used with 30 mM sodium butyrate present during the isolation) or from chromatin rapidly prepared by breaking cells with glass beads (Y55, 100 mM sodium butyrate present during the isolation).

The extent of yeast histone H4 acetylation was much higher than that of chicken erythrocyte or calf thymus. Chicken erythrocyte contained the lowest proportions of the acetylated species. The

Table III. The percentage of acetylated histone H4 species in preparations of whole chromatin.

Cells	A_0	A_1	A_2	A_3	A_4
Calf Thymus	44 ± 4	43 ± 3	13 ± 6	-	-
Chicken erythrocytes	59 ± 1	30 ± 4	11 ± 3	-	-
Yeast					
Y55 (control)	17 ± 3	52 ± 2	31 ± 3	-	-
Y55 (+100 mM sodium butyrate)	10 ± 2	26 ± 3	32 ± 3	21 ± 3	11 ± 4
20B-12α (+30 mM sodium butyrate)	14 ± 2	27 ± 1	31 ± 3	17 ± 4	11 ± 2

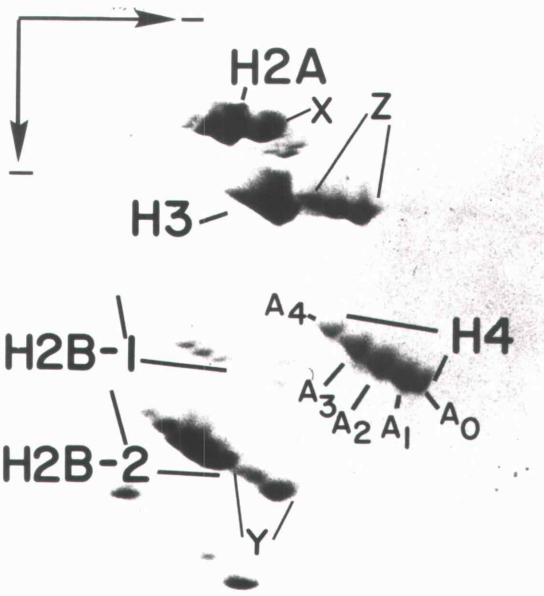
yeast strains contained a 4 to 5 fold increase in the content of A_2, A_3 and A_4 histone H4 species compared to the distribution of these acetylated species from calf thymus or chicken erythrocyte. Even with butyrate absent from the isolation medium, yeast had a three-fold increase in the content of the above species over those associated with calf thymus or chicken erythrocyte.

The histones from logarithmically growing yeast (20B-12α) cells were examined further by resolving the nuclear proteins by a two-dimensional gel electrophoresis system which consisted of an acid-urea gel in the first dimension followed by an acid-urea Triton X-100 gel in the second. We chose to use the conditions of Zweidler, 1978, for preparation of the acid-urea Triton X-100 gels (6 mM Triton X-100, 6.7 M urea). Figure 8 presents the electrophoretic pattern of the nuclear proteins resolved by this system. The assignment of the histones was made by dissecting out the bands and identifying the histone on SDS-polyacrylamide gels. In addition, the positions of an unidentified protein, X, and of a group of unidentified proteins, Y and Z, shown in the electrophoretic pattern (Figure 7e) were determined for the electrophoretic pattern (Figure 8).

In agreement with the results of Brandt et al., 1980, we have identified two variant forms of yeast histone H2B. Both variant forms of H2B contained a number of modified species (7 species for H2B-2 and 5 species for H2B-1) similar to those observed for histone H4.

Figure 8. Two-dimensional electrophoretic patterns of acid soluble nuclear proteins from yeast strain 20B-12α. Acid soluble nuclear proteins were resolved by acid-urea gel electrophoresis in the first diemnsion and by acid-urea Triton X-100 gel electrophoresis in the second dimension. Nuclei isolated in the presence of 30 mM sodium butyrate were obtained from vegetative phase cells. X, Y and Z are unidentified protein(s).

Figure 8



The gel systems that were used contained ammonium persulfate in the stacking gel. This was found to oxidize the H2B methionine (Zweidler, 1978). If the stacking gel was omitted from both gels and the sample reduced prior to electrophoresis (40% thiglycolic acid, 12hr, 50°C), the H2B-2 migrated slower than H2B-1 on the two-dimensional gels. In addition, there were no changes in their content of modified forms or the relative amounts of each variant.

The modified species of histones H2B-1 and H2B-2 may be due to acetylation, phosphorylation or a combination of both. However, extensive digestion of the histones with acid phosphatase did not alter the observed content of either of the major species of the histone H2B variants or the species of histone H4. Thus, the results suggest the major modification of the histone H2B variants may be acetylation.

Discussion

We have examined the content of modified histone species associated with different yeast strains, 20B-12α, Y55, A364a and D5. The yeast strains contained similar levels of modified species each of the histones H3, H4, H2B-1 and H2B-2. For histones H3 and H4 the modification was identified as acetylation (see Results).

The content of modified species for each of the histone H2B variants, H2B-1 and H2B-2 were similar. The major species were probably not phosphorylated as digestion of the histones with acid phosphatase did not alter their patterns on two-dimensional gels. The modification may be acetylation, but we hesitate to make this assignment because the distribution of the modified forms did not alter when sodium butyrate was present or absent during the nuclear isolation. The exact nature of this modification(s) will have to await results from labeling studies.

During the course of this work, we have found that the presence of sodium butyrate in the isolation buffers was essential in order to isolate the highly acetylated histone species. To isolate the highly acetylated histone species, one must select the correct concentration of sodium butyrate by taking into consideration the following: 1) the yeast strain to be used, 2) the time of spheroplasting required, and 3) the nuclei isolation procedure to be used.

Several groups have reported that the histones of multicellular eukaryotes are acetylated and deacetylated at different rates (Moore et al., 1979; Jackson et al., 1975; Nelson et al., 1980; Couvault & Chalkley, 1980). Moore et al., 1979, have reported two types of acetylation: one in which 50% of the acetate was removed with half-life of 3 min and the rest with a much longer half-life of 30 to 40 min. Recently, Nelson et al., 1980, have demonstrated that the transcriptionally competent chromatin regions contain histones that are more rapidly and extensively acetylated than that of histones associated with the bulk of chromatin. Their results would suggest that the very transcriptionally active chromatin of yeast might contain a greater proportion of histones that are rapidly hyperacetylated and deacetylated. It will be interesting to examine the turnover rate(s) of the yeast histone acetyl groups. However, we were concerned that the presence of sodium butyrate during the spheroplasting step during which the cells were still metabolically active would lead to a rapid accumulation of acetylated histone species while the deacetylase(s) were inhibited. This would result in an increased content of the highly acetylated histone species and would not represent the <u>in vivo</u> content of these species. We feel that this was not occurring because: (1) chromatin prepared by rapid disruption of vegetative phase cells by glass beads in the presence of 100 mM sodium butyrate contained high levels of acetylated histone species similar to those extracted from nuclei isolated in the presence of sodium butyrate (30 to 100 mM).

Our results are consistent with the idea that hyperacetylation of histones allows a region of chromatin to be transcribed at an increased rate. We have compared chromatins that vary dramatically in their transcriptional activities. The very transcriptionally active chromatin of yeast contains 4 to 5 times the content of the A_2, A_3 and A_4 histone H4 as that of both the slightly transcriptionally active chromatin of calf thymus and the transcriptionally inactive chromatin of chicken erythrocyte. Furthermore, yeast contains high levels of the acetylated species for histone H3 and, possibly, for the histones H2B-1 and H2B-2 which would make yeast chromatin among the most highly acetylated of any chromatins reported thus far.

RELATIVE CONTRIBUTIONS TO DNase I SENSITIVITY OF NON-HISTONE CHROMOSOMAL PROTEINS AND BUTYRATE PROTECTION IN THE ACTIVE CHROMATIN OF THE YEAST, Saccharomyces cerevisiae

Introduction

The structure of active chromatin and its relationship to gene expression has been extensively studied over the past years (for reviews see Mathis et al., 1980; Rindt and Nover, 1980). To date two major biochemical differences between active and inactive chrommatin have been observed: active regions are enriched in particular proteins and their histones are more highly acetylated.

The high mobility group proteins 14 and 17 (recently reviewed; Johns, 1982), have been shown to confer DNAse I sensitivity to active genes (Weisbrod and Weintraub, 1979; Weisbrod et al., 1980). In the same vein, micrococcal nuclease digestion studies have also shown that HMG 14 and 17 or H6, an HMG 14 and 17-like protein, are associated with nucleosomes that are enriched for active genes (Levy-W. & Dixon, 1978c; Levy-W. et al., 1979a; Goodwin et al., 1981; Davie & Saunders, 1981; Egan & Levy-Wilson, 1981). HMG 14 and 17 have been shown to bind to core particles (Mardian et al., 1980; Sandeen et al., 1980; Albright et al., 1980) and specifically are able to recognize amongst HMG-depleted nucleosomes those nucleosomes containing transcribable DNA sequences (Sandeen et al., 1980; Weisbrod & Weintraub, 1979; Weisbrod et al., 1980; Weisbrod, 1982).

Acetylation of the inner histones (reviewed; Allfrey, 1980; Allfrey, 1977) particularly H3 and H4, has also been shown to be preferentially associated with nucleosomes from transcriptionally competent chromatin that is sensitive to DNase I (Davie & Candido, 1980; Davie & Saunders, 1981, DNase II (Davie & Candido, 1978) and micrococcal nuclease (Levy-Wilson et al., 1979b; Nelson et al., 1980). Acetylation has been correlated with an increase in transcriptional activity in a number of systems (Allfrey, 1977). Many interesting suggestions have been proposed as to how acetylation might function in transcription and replication (for reviews see Allfrey, 1980; Dixon et al., 1975).

Studies of the chromatin of S. cerevisiae have a two-fold motivation. They are of evident importance for a further understanding of the species itself, but, in addition, may shed light on active chromatin in general.

The yeast genome is organized into nucleosomes with a 160 base pair repeat (Lohr & VanHolde, 1975; Lohr et al., 1977a,b; Nelson et al., 1977). Approximately 20% of the yeast single-copy DNA is complementary to mRNA, indicating that at least 40% of the genome is transcribed (Hereford & Robash, 1977). The DNase I sensitivity of the transcribed chromatin has been determined and is comparable to that of the total yeast chromatin. That the DNase I sensitivity is the same for the transcribed and total yeast chromatin indicates that the entire yeast genome is equally DNase I sensitive (Lohr & Hereford, 1979a) and is entirely in the transcriptionally competent state. The chromatin structure of yeast has been further analyzed by electrophoretic transfer of high-resolution DNA gels of

micrococcal nuclease and DNase I digestions to DBM paper and hybridized to cDNA of poly A mRNA, nascent *in vitro* elongated RNA or sonicated total genome DNA. The hybridization patterns resemble the DNA patterns produced by the total yeast nucleosomes. This indicates that the transcribed and total yeast chromatin are likely organized into the same nucleosomal structure (Lohr, 1981), confirms that the entire yeast genome is equally DNase I sensitive, and supports the idea that the entire yeast genome is in the active state. The DNase I sensitivity of the yeast genome observed in the nucleus is maintained upon micrococcal nuclease digestion and is present in isolated nucleosomal oligomers and monomers (Lohr & Ide, 1979b), demonstrating that it is possible with yeast to isolate without fractionation active chromatin for biochemical and biophysical analysis.

Yeast chromatin contains four high mobility group proteins (Weber & Isenberg, 1980; Spiker et al., 1978) and is highly acetylated when isolated in the presence of sodium butyrate (Davie et al., 1981). This highly acetylated chromatin may be the *in vivo* state since the yeast cultures are not grown on sodium butyrate, but rather the butyrate is used only to protect the highly acetylated chromatin during isolation. That this highly acetylated chromatin may be the *in vivo* level in yeast is also suggested by ^{3}H acetate labeling of the inner histones and determining that the turnover rate of acetylation in yeast is on the order of hours rather than as minutes for multicellular eukaryotes (Nelson, 1982). This result supports the idea that the chromatin of logarithmically growing yeast cells is maintained in the acetylated state.

Both the association of high mobility group proteins and acetylation of inner histones have been shown to affect the DNase I sensitivity of transcriptionally competent chromatin. It is not known what the relative importance of these two factors may be in the maintenance of the transcriptionally competent state and to date no one has yet asked this question. In this paper, we have addressed this question by determining the relative DNase I sensitivity of yeast chromatin with the presence or absence of proteins that are extractable by low salt and inner histone acetylation, singularly and in combination.

Materials and Methods

Yeast Growth Conditions

Saccharomyces cerevisiae strain Y55, a wild type diploid was used throughout. Y55 was grown on YEPD (1% yeast extract, 2% Bactopeptone, 2% dextrose) at 30°C on a New Brunswick rotary platform shaker. Yeast cultures were grown to a log phase density of 4-6 x 10^7 cells/ml.

Isolation of Nuclei

Yeast nuclei were isolated by the procedure of Ide and Saunders (1981) with the following modifications. Butyrate protected nuclei were isolated in the presence of 100 mM sodium butyrate. Log phase cultures contained 4-6 x 10^7 cells/ml. All solutions were used with 1.0 mM PMSF. To prepare spheroplasts, cells were suspended at 4 ml/gm in 1.1 M Sorbitol, 0.5% β-mercaptoethanol, 0.5 mM $CaCl_2$, 40 mM HEPES, pH 7.5, 1.0 mM PMSF. The walls were then digested at 30°C with Lyticase (Scott & Schekman, 1980) at 200 U lytic activity per gm of cells. Spheroplasting was judged to be complete when all of the spheroplasts of an aliquot lysed and disintegrated completely when diluted four-fold into water. Spheroplasting took 40-60 min.

Spheroplasts were lysed by 6-8 strokes in a B Dounce homogenizer using the 18% Ficoll solution of Ide and Saunders (1981). One volume of 1.0 M Sorbitol, 0.5 mM $CaCl_2$, 25 mM KPO_4, pH 6.5. 1.0 mM PMSF was added and constant stirring maintained until the lysed spheroplasts were loaded onto preformed 32.5% Percoll gradients. The nuclei were recovered from the middle of the gradient after centrifugation at 9,000 g x 20 min in a Sorvall HS-4.

Isolation of Chromatin

The isolated nuclei from the Percoll gradients were washed by adding two volumes of 1.1 M Sorbitol, 0.5 mM $CaCl_2$, 25 mM KPO_4, pH 6.5, 1.0 mM PMSF and stirring on ice for 5 min. The nuclei were lysed and washed three times on ice in 10 mM Tris HCl, pH 8.0, 0.5% (v/v) NP-40, 75 mM NaCl, 2.0 mM EDTA, 1.0 mM PMSF by stirring for 30 min on ice in silanized glassware. The chromatin pellet was then washed twice on ice in the same buffer but without the NP-40. Btyrate protection was at 100 mM as before.

Micrococcal Nuclease Released Chromatin

The washed chromatin pellet was then resuspended in micrococcal nuclease digestion buffer: 10 mM NaCl, 10 mM Tris HCl, pH 7.2, 0.5 mM PMSF at 80 A_{260}/ml on ice by 1-2 gentle strokes of a silanized B Dounce. The chromatin A_{260} concentration was determined by dilution in 1% SDS to eliminate light scattering. The chromatin was equilibrated to 25°C and then digested for 30 min by the addition of $CaCl_2$ to 1 mM and 10 U micrococcal nuclease for each 80 A_{260} per ml of chromatin. The chromatin was then centrifuged at 20,000 g for 30 min in an HB-4. The soluble micrococcal released supernatant, which we call Sup 1, was then made 2.0 mM EDTA from 100 mM EDTA, pH 7.0. The pellet was then digested again under the same conditions and this supernatant we name Sup 2. It was made 2.0 mM in EDTA. The supernatants Sup 1 and Sup 2 were stored on a ice bath at 0°C.

Polyacrylamide Gel Electrophoresis

All electrophoresis was performed on microslab gels (Matsudaira & Burgess, 1978) using Idea Scientific minislab units.

Fifteen percent polyacrylamide sodium dodecyl sulfate (SDS) gels were made using the procedure of Laemmli (1971). The resolving gel was made with an acrylamide to bis-acrylamide ration of 30:1.5. The stacking gel was made 6% with an acrylamide to bis-acrylamide ratio of 30:0.8. One-dimensional SDS gels were either 0.5 mm or 0.8 mm in thickness. Samples were mixed with 0.2 volume of 5X sample buffer to give a final concentration of 0.0625 M Tris HCl, pH 6.8, 1% SDS, 10% glycerol, 1.25% β-mercaptoethanol and 0.001% bromophenol blue and boiled at 100°C for 2 min prior to loading. Gels were electrophoresed for 50 min at 200 volts at 4°C.

Fifteen percent polyacrylamide acid-urea minislab gels were made using the acid-urea gel of Spiker (1980). The stacking gel was made 7.5% polyacrylamide with an acrylamide to bis-acrylamide ratio of 30:0.8 and was polymerized with 0.0004% riboflavin (Davie, 1982).

Samples were prepared in one of two ways. Purified proteins were disolved in 2.5 M urea, 0.375 M potassium acetate, pH 4.0, 0.625% β-mercaptoethanol, 0.625% mercaptoacetic acid and 0.05% pyronin Y. Micrococcal nuclease released supernatants were directly analyzed by adding 0.25 volume of 8 M urea, 2.5% β-mercaptoacetic acid, 2.5% β-mercaptoethanol, 1% protamine sulfate (Sigma, histone-free), 1.5 M K acetate, pH 4.0, and 0.20% pyronin Y. Gels were either 0.5 mm or 0.8 mm in thickness and were electrophoresed at 200 volts for 2 hr for histones and 1.5 hr for HMGs at 4°C using 5% (v/v) acetic acid as the tray buffer.

Two-dimensional minislab gel electrophoresis was according to Davie (1982), but used the SDS and acid-urea gel systems described previously. Acid-urea into SDS two-dimensional gels were used for the analysis of HMG proteins. SDS into acid-urea two-dimensional gels were used for the analysis of inner histone acetylation.

Protein gels were stained with 0.1% Coomassie Blue R (SERVA Blue R) in 40% methanol and 10% acetic acid, destained in 25% methanol and 12.5% acetic acid and photographed using a 5545 Å optical band-pass filter, PTR Optics.

Native DNA polyacrylamide gel electrophoresis was performed according to the procedure of Loening (1967). Four percent polyacrylamide gels were made with an acrylamide to bis-acrylamide ratio of 20:1. Gels were 0.8 mm in thickness and electrophoresis was at 90 volts for 1 hr at 4°C. Samples were prepared by treating micrococcal released supernatants with 0.02 vol RNAse (5 mg/ml bovine pancreatic, Schwarz Mann, and 5,000 U/ml T, Sigma) for 15 min at 37°C. Then 0.1 vol 1.6 N NaCl, 0.083 vol 22% Sarkosyl (Sigma) for 15 min at 37°C. Then 0.1 vol 1.6 N NaCl, 0.083 vol 22% Sarkosyl (Sigma) and 0.1 vol of 10 mg/ml self-digested Pronase (CalBiochem) for 3 hr. at 37°C. One vol of 2X sample buffer was added which consisted of 0.2X concentration tray buffer, 20% glycerol, 0.02% bromophenol blue and 0.02% xylene cyanol FF (BioRad). Gels were stained 20 min in water with 1 μg ethidium bromide/ml and photographed with Kodak Tri-X Pan 4 x 5 inch film on a 254 mm transilluminator (Ultra-Violet Products, Inc.) with a red cutoff filter (Corning CS 2-62).

Removal of Non-histone Chromosomal Proteins

Non-histone chromosomal proteins (NHCPs) were removed after Libertini and Small (1981) at 150 mM NcCl with CM-Sephadex C-25-100 (Sigma). Micrococcal nuclease released chromatin at 8-12 A_{260}/ml was treated with 30 mg of dry CM-Sephadex/ml chromamtin with stirring at 0 for 15 min in 10 mM NaCl, 10 mM Tris HCl, 0.2 mM EDTA, pH 7.4, 1.0 mM PMSF. The ionic strength was then raised to 150 mM NaCl by dropwise addition with stirring of 1 M NaCl, 10 mM Tris HCl, 0.2 mM EDTA, pH 7.4, and stirred for 1 hr at 0°C. The CM-Sephadex chromatin suspension was pipeted onto a 1.5 x 12 cm column of CM-Sephadex equilibrated with 10 mM Tris HCl, 150 mM NaCl, 0.2 mM EDTA, pH 7.4, at 20°C. The sample was allowed to come to 20°C and the CM-Sephadex exchanged chromatin was run through the column at a linear flow rate of 25 cm/hr. Fractions were collected and immediately pipeted into Spectraphor 3500 MW dialysis tubing and dialyzed at 4°C against 10 mM NaCl, 10 mM Tris HCl, 0.2 mM EDTA, pH 7.4. The CM-Sephadex bound NHCPs were released by running 2.0 M NaCl, 10 mM Tris HCl, pH 7.4, 0.2 mM EDTA through the column at a linear flow rate of 25 cm/hr. The NHCPs elute just at the 2.0 M NaCl step. NHCPs were isolated by setting the ionic conductivity equal to that of 400 mM NaCl and precipitating with 2% TCA and then 25% (w/v) trichloroacetic acid (TCA). NHCPs were washed three times with -20°C acetone and vacuum dried. All centrifugations were at 10,000 g x 15 min in a Sorvall HB-4.

DNase I Digestions

Chromatin samples were dialyzed three times for 12 hr x 2 changes against 10 mM NaCl, 10 mM Tris HCl, pH 7.4, 0.2 mM EDTA. The DNA concentration was determined by the DABA assay (Thomas and Farquhar, 1978). Chromatin samples were diluted to 30.0 μg DNA/ml and the DNA concentration checked again. When comparing the various chromatin samples, the ionic conductivity was measured and found to be the same to three significant figures. Chromatin samples were equilibrated to 20 C° in a silanized glass tube containing a small Teflon stir bar. Divalent cations were added to a final concentration of 0.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$. For a zero digestion time sample, a 100 aliquot was taken and mixed with an equal volume of 0.6 M PCA - 0.6 M NaCl. DNase I (Sigma) was added to 5 U/ml with constant stirring and 100 samples were taken at various times and immediately mixed with an equal volume of 0.6 M PCA - 0.6 M NaCl. DNase I digestion time courses were done in duplicate for each sample. The acid soluble parts of the samples were obtained as the supernate following centrifugation at 10,000 g for 10 min. The DNA concentration was determined by the DABA DNA specific assay (Thomas & Farquhar, 1978). DNA standards were run concurrent with experimental samples.

DABA was recrystallized and stored according to Thomas and Farquhar (1978). Fluorescence intensities were measured on a computer interfaced spectrometer (Ayres et al., 1974). Excitation was with the 405.5 nm line of a mercury-xeon arc isolated through two grating monochrometers and polarized with a double Glan-Taylor prism polarizer). Emission was detected at right angles to both the

propagation and polarization directions of the exciting light. Emission at 520 nm was isolated by a grating monochrometer after passing through a double Glan-Taylor prism polarizer oriented either parallel ($F_{\parallel}$) or perpendicular ($F_{\perp}$) to the excitation polarizer. Stray exciting light was removed with a Corning (CS 3-72) cutoff filter. A sensitivity correction (Ayres et al., 1974) was made for differential light transmission in the two polarization modes. After correction, fluorescence intensity was computed as $F = F_{\parallel} + 2F_{\perp}$.

Miscellaneous Procedures

Silanized glassware was used in all steps for preparing nuclei, chromatin, micrococcal nuclease released chromatin, DNase I digestions and isolation of histones and NHCPs.

Micrococcal nuclease was stored at -20°C at 10,000 U/ml in 50 mM Tris HCl, pH 7.5, 20% glycerol. DNase I (Sigma) was stored at -20°C at 2,800 U/ml in glass distilled water.

Yeast NHCPs and histones were isolated as described before, elsewhere (Weber & Isenberg, 1980; Davie et al., 1981).

Lyticase was prepared as Fraction II of Scott & Schekman, (1980).

RESULTS

Successive micrococcal nuclease digestions were done on both the butyrate treated and non-butyrate treated samples, (Figure 9). The samples were centrifuged for 30 min at 20,000 g to release two successive soluble supernatants called Sup 1 and Sup 2 (see Materials and Methods Section). The distribution of various components is outlined in Figure 9. Non-butyrate protected material (Non B) had oligomers and monomers in Sup 1, and monomers and just a trace of dimers in Sup 2. On the other hand, butyrate protected chromatin (B) yielded oligomers only in Sup 2, with none in Sup 1 (Figures 10 and 11).

Acetylation of yeast H4 is one of the consequences of butyrate protection (Davie et al., 1981). Therefore we determined the acetylation levels of (Non B) Sup 1 and (B) Sup 2 by protamine release of the proteins directly from the supernatants onto an acid-urea gel (Figure 12). (B) Sup 2 shows an increased number of bands with decreased mobility in the H4 region of the gel, compared to (Non B) Sup 1 which is what we expected. That these new bands in the (B) Sup 2 are indeed modified forms of H4 was confirmed by two-dimensional SDS-AU gel electrophoresis (Figure 13). This gel shows that (B) Sup 2 has more modified forms of H4 than (Non-B) Sup 1. In addition, the gel shows an increased complexity in modification of the H2Bs, a result similar to that observed for whole chromatin (Davie et al., 1981).

Fig. 9. Schematic outline of experimental procedure.

Figure 9

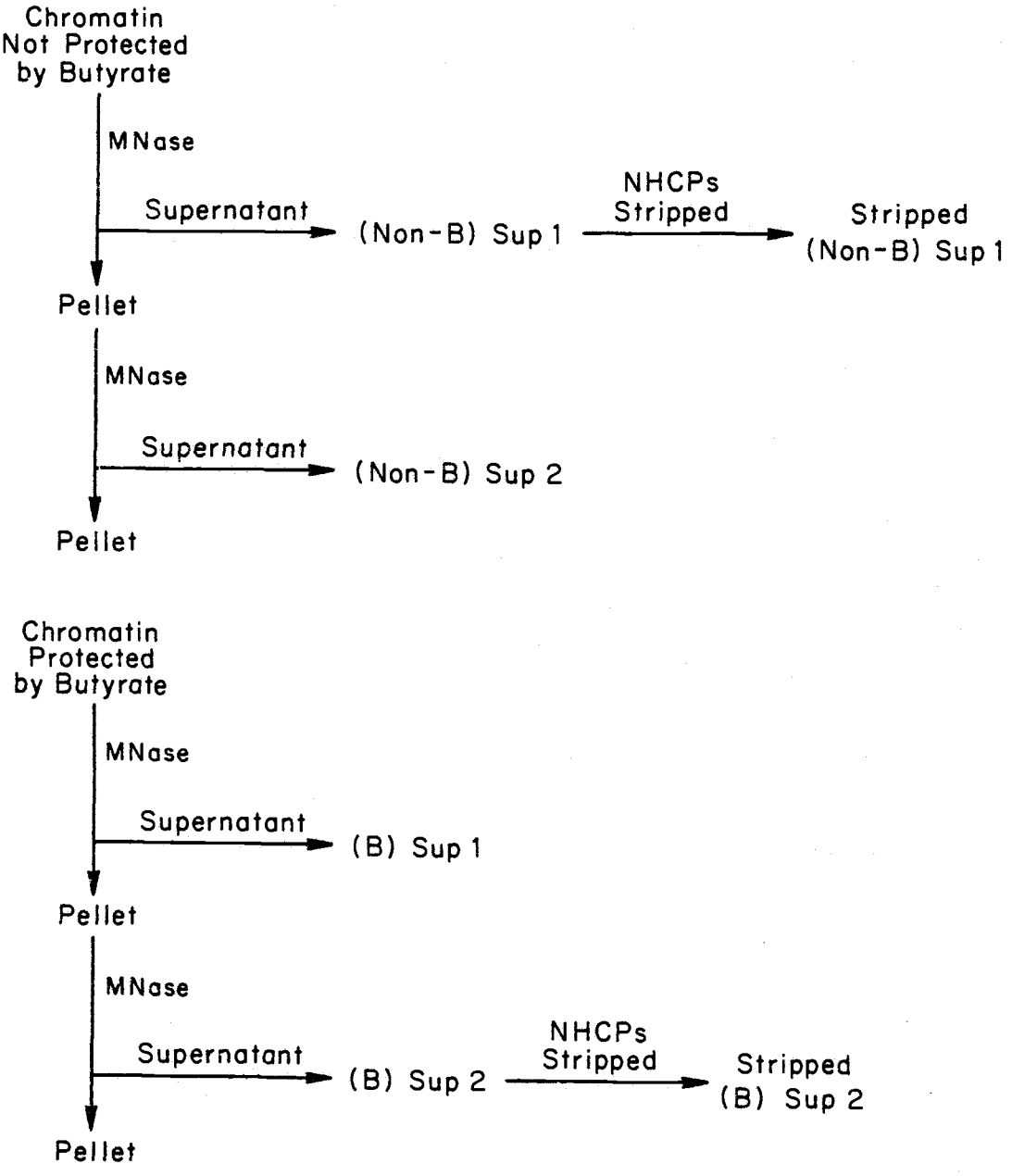

Chromatin
Not Protected
by Butyrate
MNase
Supernatant
(Non-B) Sup 1
NHCPs
Stripped
Stripped
(Non-B) Sup 1
Pellet
MNase
Supernatant
(Non-B) Sup 2
Pellet
Chromatin
Protected
by Butyrate
MNase
Supernatant
(B) Sup 1
Pellet
MNase
Supernatant
(B) Sup 2
NHCPs
Stripped
Stripped
(B) Sup 2
Pellet

Fig. 10. Native DNA gel: (A), (Non-B) Sup 2; (B), (Non-B) Sup 1; (C), (B) Sup 2; (D), (B) Sup 1; (E), Hae III PM2 DNA standard.

Figure 10

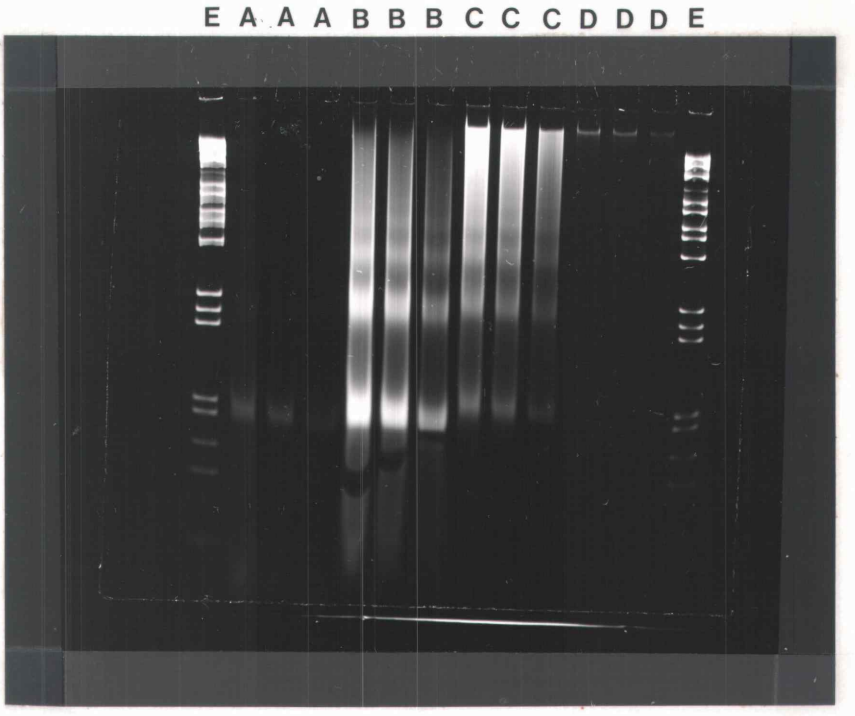


Fig. 11. SDS protein gels: (A), (Non-B) Sup 1; (B), (Non-B) Sup 2; (C), (B) Sup 1; (D), (B) Sup 2.

Figure 11

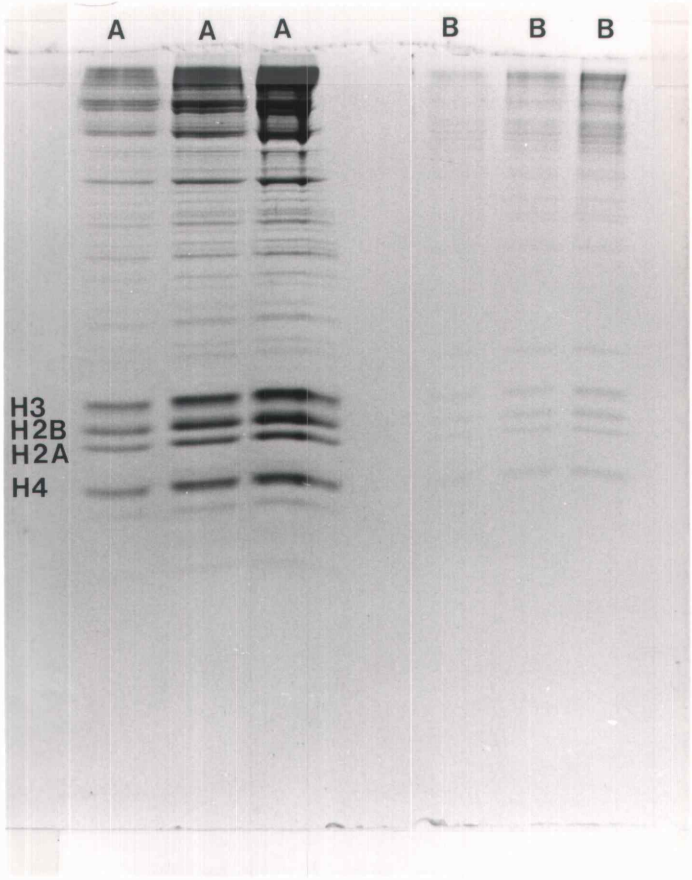


Figure 11

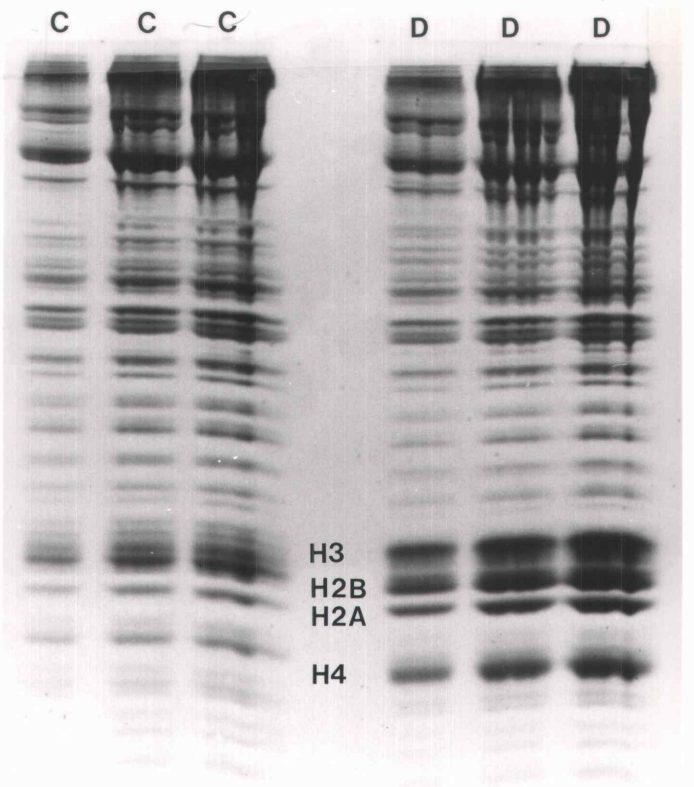


Fig. 12. Acid-urea gel: (A), (Non-B) Sup 1; (B), (B) Sup 2.

Figure 12

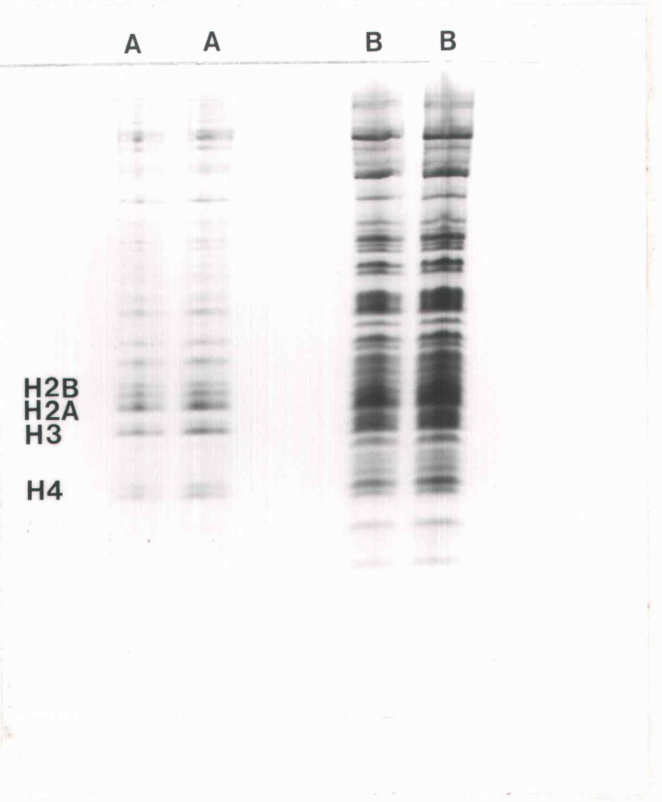
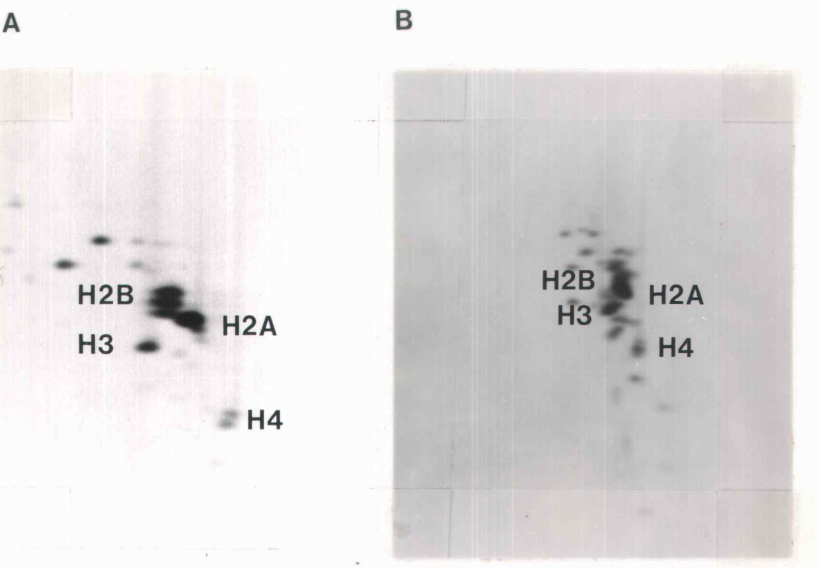


Fig. 13. Two dimensional gel with resolution in the first-dimension in a SDS gel and the second dimension in an acid-urea gel: (A), (Non-B) Sup 1; (B), (B) Sup 2.

Figure 13



To prepare oligosomes stripped of non-histone chromosomal proteins, while minimizing the danger of nucleosomal sliding, a low ionic strength stripping procedure was used. We kept well below the 400 mM NaCl ionic strength known to induce sliding (Beard, P., 1978; Steinmetz et al., 1979; Spadafora et al., 1979; Weischet & Van Holde, 1980).

Non-histone chromosomal proteins were isolated both by 400 mM NaCl extraction of (Non B) Sup 1, and by exchange onto CM-Sephadex at 150 MM NaCl from (Non B) Sup 1 and (B) Sup 2. Analysis of 400 mM NaCl extractable NHCPs from (Non B) Sup 1 on an acid-urea gel (Figure 14A) shows the presence of yeast HMGs S1, S3, S4 but not HMGa, confirming the previous result that HMGa is not removed by salt (Weber & Isenberg, 1980). We note, however, that proteins other than HMGs are removed as well. The proteins removed by CM-Sephadex at 150 mM NaCl from (Non B) Sup 1 and (B) Sup 2 were compared (Figure 14B). Both showed almost identical gel patterns. Figure 14B, for given amounts of chromatin, shows that yeast HMGs S3 and S4 are present in both types of samples in approximately equal amounts. However, HMG S1 is much reduced in the (B) Sup 2 compared to (Non B) Sup 1.

To see if we were possibly observing ribosomal proteins, rather than chromosomal ones, we compared our released non-histone chromosomal proteins with yeast ribosomal proteins (Figure 15). Not only do the HMGs migrate differently from all ribosomal proteins, but all of the salt extractable proteins do also.

Fig. 14. Acid-urea gels. (Panel A): (A) Proteins extracted with 0.4 M NaCl from (Non-B) Sup 1; (B) calf thymus whole HMG protein; (C) Proteins extracted with 0.4 N H_2SO_4. (Panel B): (A) calf thymus whole HMG protein; CM-Sephadex stripped proteins from (B) [(B) Sup 2]; and from (C) [(Non-B) Sup 1]; (D) yeast HMGa.

Figure 14

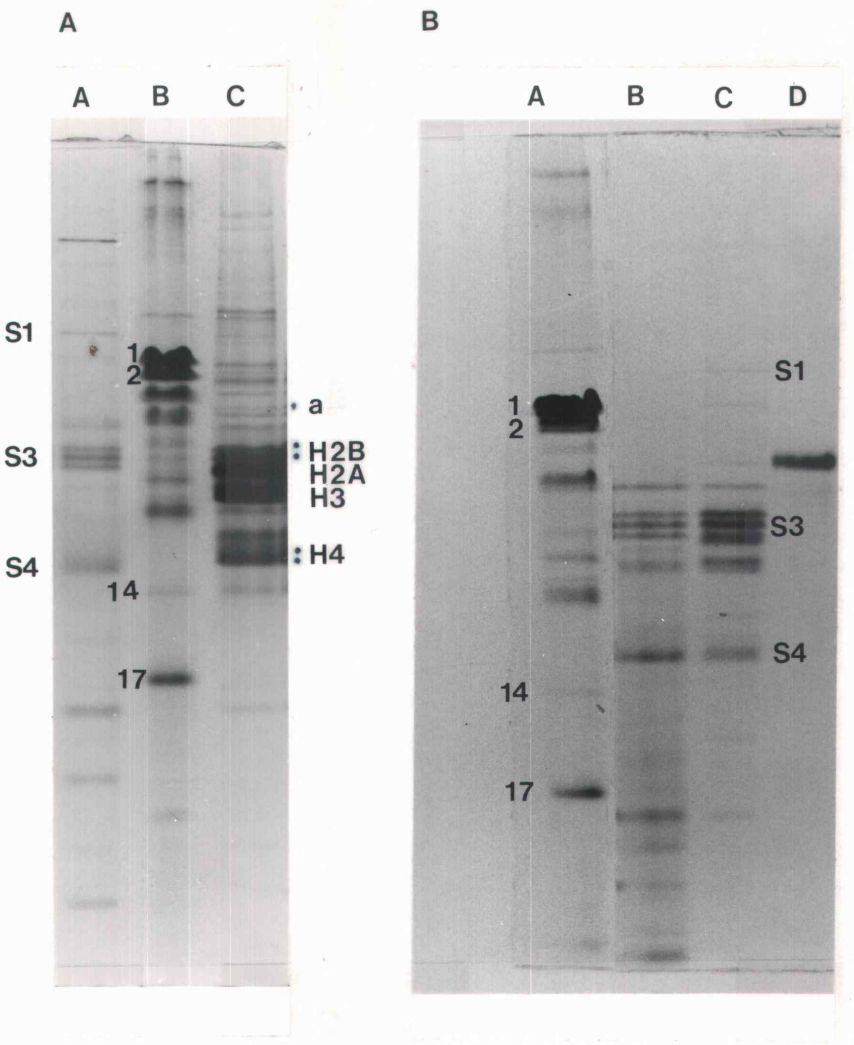
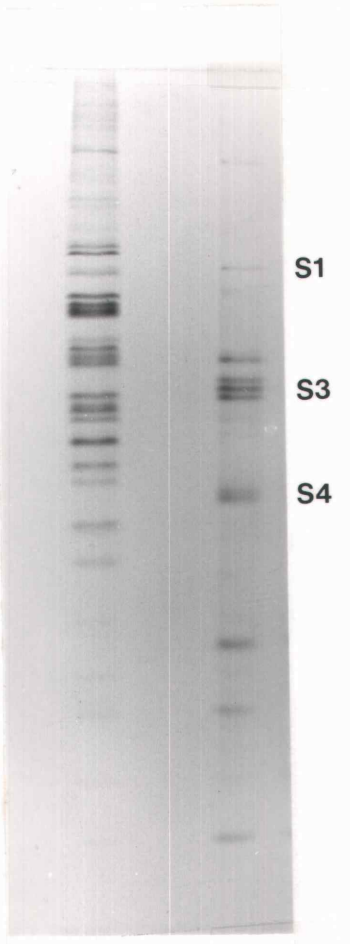


Fig. 15. Acid-urea gel: (left) yeast ribosomal proteins and (right) proteins extracted with 0.4 M NaCl from (Non-B) Sup 1.

Figure 15



The NHCP proteins were analyzed on two-dimensional acid-urea-SDS gel (Fig. 16). Yeast HMG S3 runs as a triplet in the acid-urea dimension, but as a single molecular weight species in the SDS dimension. This heterogeneity in HMG S3 was not observed in studies of pressed block yeast HMGs (Weber & Isenberg, 1980). HMG S4 runs as a single species in the acid-urea dimension, and as a doublet in the SDS dimension, which is the same as found in pressed block studies (Weber & Isenberg, 1980).

DNase I digestion rates were measured for (Non B) Sup 1 and (B) Sup 2, both before and after stripping NHC proteins (Figure 17). Butyrate protection increases the digestion rate (Figure 17). In fact, not only does the rate increase, but the total digestion reaches a higher level. However, the most striking result stems from a comparison of the rate changes of the different oligomers when the NHCPs are removed. Removal of the NHCPs from the (Non B) Sup 1 increases the rate (Figure 17). On the other hand, for the butyrate protected oligomers, removal of the NHCPs had no discernible effect whatsoever on the rate (Figure 17). We repeated the experiment on the butyrate protected oligomers with four different samples, independently prepared, and obtained the same result each time. The digestions were repeated on five independent samples for (Non B) Sup 1 again with reproducible results. Figure 18 conveniently summarizes our major result that the rate of DNase I digestion, and changes of rate, depends upon whether or not protein modifications are protected by butyrate during isolation.

Fig. 16. Two dimensional gel with resolution in the first dimension, an acid-urea gel, and in the second dimension, a SDS gel, of the proteins extracted with 0.4 M NaCl from (Non-B) Sup 1.

Figure 16

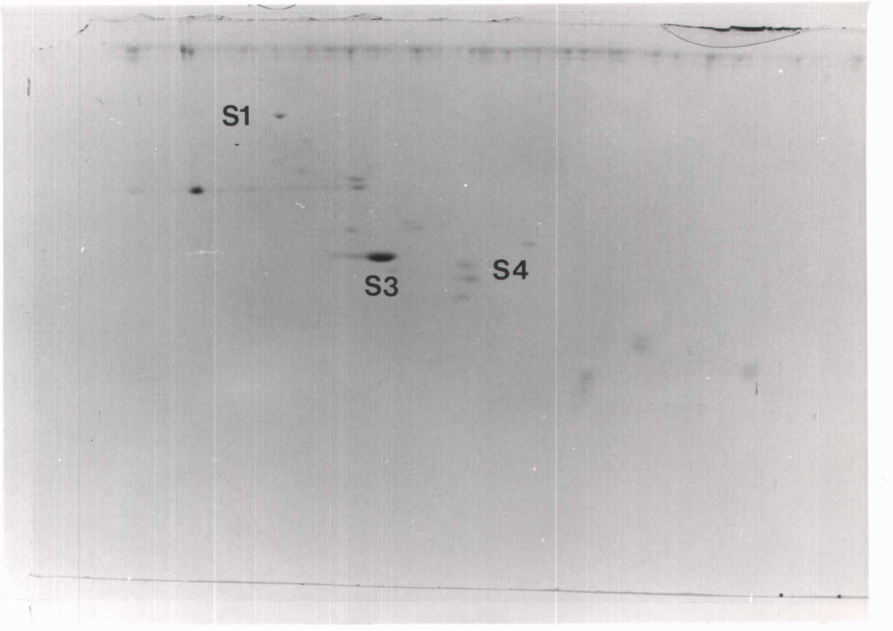


Fig. 17. DNase I digestion time courses: (Panel A): (Non-B) Sup 1 and (B) Sup 2; (Panel B): (Non-B) Sup 1 and CM-sephadex stripped (Non-B) Sup 1; (Panel C): (B) Sup 2 and CM-sephaadex stripped (B) Sup 2.

Figure 17

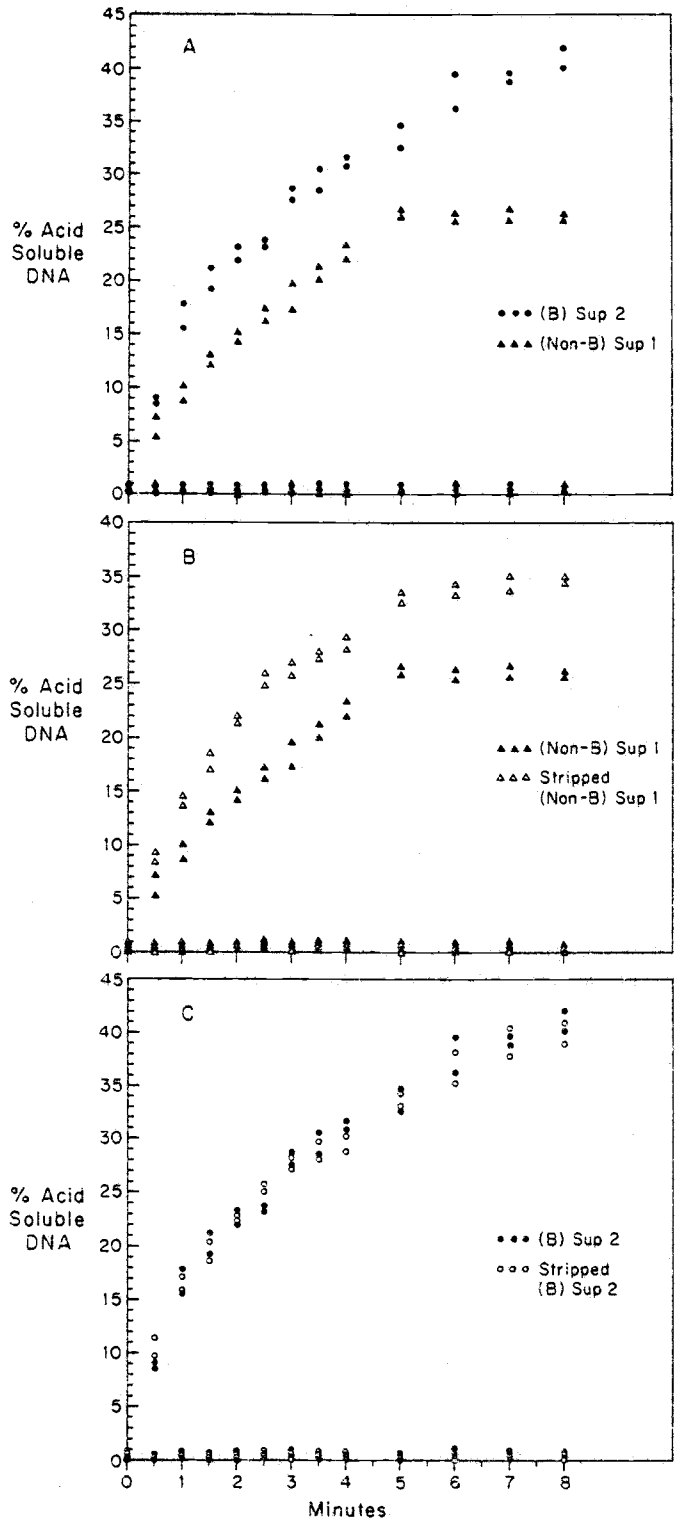
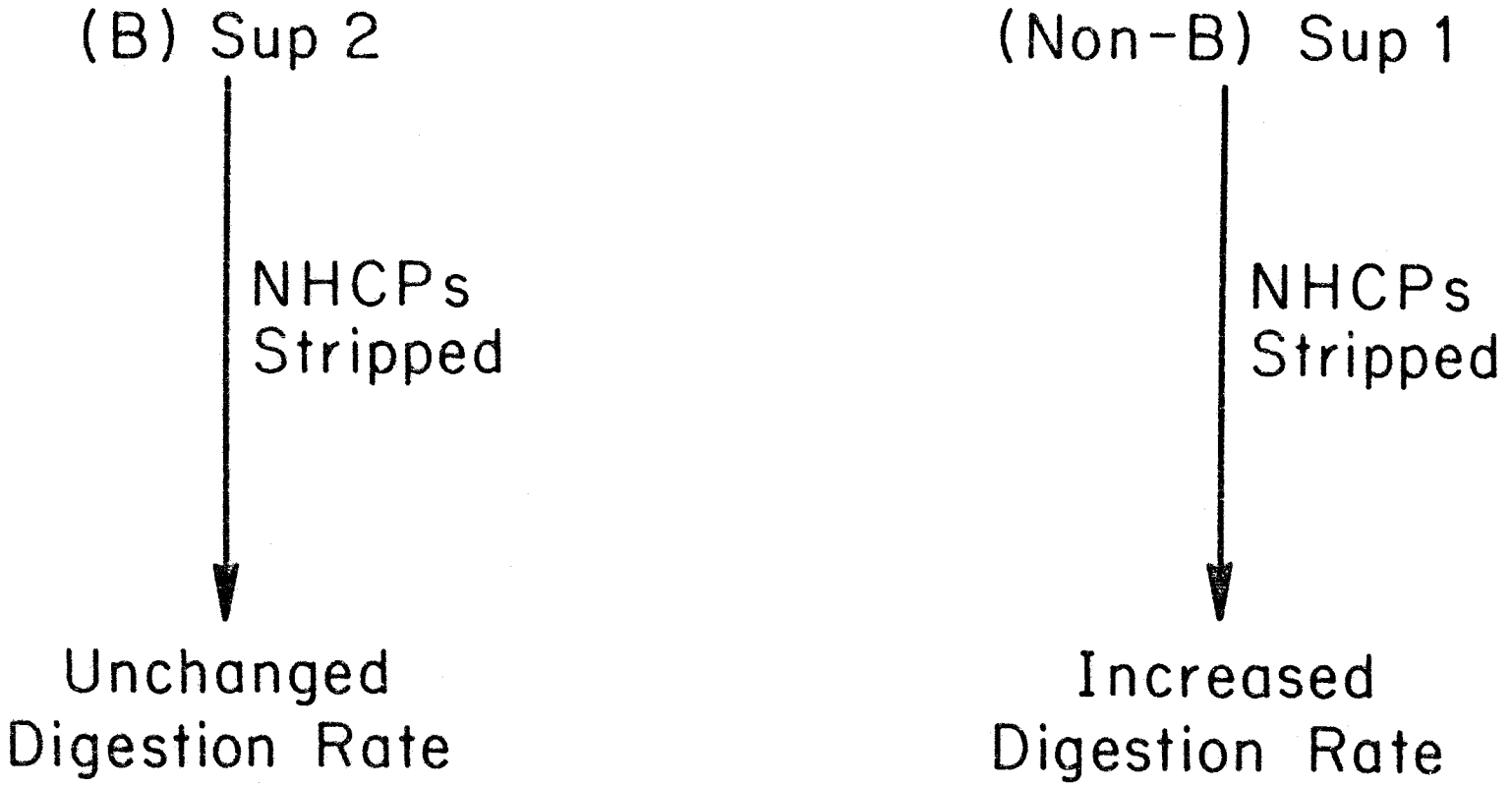

A
% Acid Soluble DNA
(B) Sup 2
(Non-B) Sup 1
B
% Acid Soluble DNA
(Non-B) Sup 1
Stripped (Non-B) Sup 1
C
% Acid Soluble DNA
(B) Sup 2
Stripped (B) Sup 2
Minutes

Fig. 18. The rate of DNase I digestion, and changes of rate, depends upon whether or not protein modifications are protected by butyrate during isolation.

Figure 18


(B) Sup 2
NHCPs
Stripped
Unchanged
Digestion Rate
(Non-B) Sup 1
NHCPs
Stripped
Increased
Digestion Rate

Discussion

The most striking result we observe is that the rate of DNase I digestion, and the changes of that rate, depend upon butyrate protection. It is known that butyrate inhibits deacetylase activity (Vidali et al., 1978a,b; Candido et al., 1978; Sealy & Chalkley, 1978a; Boffa et al., 1978; Cousens et al., 1979) and leads to hyperacetylation in many species (Riggs et al., 1977; Hagopian et al., 1977; Mathis et al., 1978; Simpson, 1978a; Nelson et al., 1980; D'Anna et al., 1980; Bode et al., 1980; Jackson & Chalkley, 1981; Perry & Chalkley, 1981; Egan & Levy-Wilson et al., 1981; Boffa et al., 1981; Pantazis & Bonner, 1981; Reczek et al., 1982; Cousens & Alberts, 1982).

D'Anna et al. (1980) reported that butyrate protection resulted in modifications other than acetylation. It enhanced the phosphorylation of H2A in Chinese hamster cell cultures, although Pantazis and Bonner (1981) reported no effect of butyrate protection on the phosphorylation of H2A in mouse L1210 cells. In addition, Boffa et al. (1981) stated that in HeLa S3 cells the effect of butyrate is to suppress phosphorylation. The phenomenon has not yet been studied in yeast, and the various reports give no guideline as to what we might even guess the case to be. However, we certainly cannot rule out that changes in phosphorylation are responsible for our phenomena. This complicates any definitive interpretation that we might put forth.

We find that butyrate protection enhances the DNase I digestion rate. Furthermore, the digestion rate is no longer dependent on whether or not the non-histone chromosomal proteins are stripped

from the oligomers. It is known in yeast (Davie et al., 1981a; Nelson, 1982) that butyrate increases the acetylation level. If for the sake of discussion, we assume that it is the acetylation which is responsible for our results, we may conclude the following: With acetylation, the presence or absence of non-histone chromosomal proteins has no discernible effect on DNase I digestion. The structure has been altered so that the acetylation level alone is the determining factor. However, we emphasize that we do not know that acetylation causes this. Other modifications may result from butyrate treatment, either phosphorylation or something else yet unknown. Our conclusion, therefore, is a conditional one only. If our results are due to acetylation, then acetylation leads to an overriding of the protein effects.

Furthermore, we may speculate that, if the above is true, it may imply that in the presence of acetylation, the DNA is generally more accessible to other proteins during physiological activities and, at that time, the non-histone chromosomal proteins that we remove play no role in the accessibility of the DNA. We point out that acetylation and deacetylation appear to be dynamic processes because butyrate enhances the acetylation levels by inhibiting the deacetylase rather than inducing an acetylase. Butyrate dramatically affects the pattern of gene expression in cultured Friend erythroleukemic cells by inducing 38% new RNA transcripts, as well as many new proteins not found in the control cells (Reeves & Cserjesi, 1979).

DISCUSSION AND SUMMARY

The active chromatin structure of the yeast, Saccharomyces cerevisiae, is similar to a subset of the chromatin from metazoan eukaryotes, and is commonly referred to as transcriptionally competent chromatin (Lohr & Ide, 1979b; Lohr & Hereford, 1979a; Lohr, 1981). I have shown in this work that yeast chromatin shares two further characteristics of transcriptionally competent chromatin: the presence of high mobility group proteins (Weber & Isenberg, 1980) and in a collaborative study that the inner histones are highly acetylated (Davie et al., 1981). I have, as well, determined the effect of butyrate protection of histone modifications and of the low salt extractable NHCPs on the chromatin structure of yeast (Weber & Isenberg, submitted 1982).

Spiker et al. (1978) established that HMGs are widely distributed throughout the eukaryotes by identifying an HMG protein in yeast (HMGa) and one in wheat. The isolation, characterization and identification of the HMGs of yeast was the first systematic analysis of HMGs in a separate kingdom (Weber & Isenberg, 1980). A total of four HMG proteins were identified and this supports the idea that HMGs are present in all eukaryotic chromatin, although the function or functions are still unknown. However, recently in rat liver a single-stranded DNA binding protein, HD-25, was shown to be a helix-destabilizing protein and was identified as the same protein as HMG 1 of both calf thymus or rat liver (Boune et al., 1982). This observation is the first possible in vivo function for HMG 1 obtained.

The comparison of the yeast HMGs to the calf thymus HMGs (a kingdom to kingdom comparison) showed that the HMGs had diverged and were not evolutionarily conserved in a manner analagous to the inner histones. To this date this study of the yeast HMGs remains the most complete characterization of HMGs outside the kingdom of animals.

Yeast chromatin contains very high levels of the acetylated species for histones H3, H4 and possibly the H2B variants, H2B-1 and H2B-2, when isolated in the presence of butyrate (Davie et al., 1981). This makes yeast one of the highest acetylated chromatins observed so far. Yeast chromatin in vivo may contain this high level of acetylation as evidenced by two observations: Chromatin prepared by rapid disruption of log phase cells by glass beads in the presence of butyrate contained high levels of acetylated histone species; histones isolated from nuclei prepared in the absence of butyrate from log phase cells (strain 20B-12α) show levels of acetylation similar to that of histones prepared from nuclei isolated in the presence of butyrate. Recently, Nelson (1982) observed that the turnover rate for acetylation and deacetylation for H3 and H4 by acetate labeling in log phase yeast was on the order of hours and has interpreted this as indicating that these histones are maintained in the acetylated state during growth.

Many interesting proposals have been suggested for the function of histone acetylation including a role in transcription (Allfrey, 1980, 1977) and in replication (Dixon et al., 1975). Exactly what the function of histone acetylation might be in yeast chromatin remains unknown.

Butyrate protection of histone modifications enhances the rate of DNase I digestion rate. Removal of the low salt extractable NHCPs from chromatin isolated in the absence of butyrate increases the rate of digestion. The most striking result I have observed is that if the chromatin is isolated in the presence of butyrate, removal of the NHCPs has no discernable effect on the digestion rate (Weber & Isenberg, submitted 1982).

It is known in yeast (Davie et al., 1981; Nelson, 1982) that butyrate increases the acetylation level. If for the sake of discussion, I assume that it is the acetylation which is responsible for my results, I may conclude the following: With acetylation, the presence or absence of these non-histone chromosomal proteins has no discernible effect on DNase I digestion. The structure has been altered so that the acetylation level alone is the determining factor. However, I emphasize that I do not know that acetylation causes this. Other modifications may result from butyrate treatment, either phosphorylation or something else yet unknown. My conclusion, therefore, is a conditional one only. If my results are due to acetylation, then acetylation leads to an overriding of the protein effects.

Furthermore, I speculate that, if the above is true, it may imply that in the presence of acetylation, the DNA is generally more accessible to other proteins during physiological activities such as transcription and replication and, at that time, these low salt-extractable non-histone chromosomal proteins play no role in the accessibility of the DNA.

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