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PARTIAL CHARACTERIZATION OF DINOFLAGELLATE CHROMOSOMAL PROTEINS*

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Summary

Dinoflagellate chromosomal proteins were analyzed by acrylamide gel electrophoresis. The electrophoretic pattern of acid-insoluble chromosomal proteins from *Gyrodinium cohnii* in sodium dodecylsulfate gels is less heterogeneous than that of corn, and is characterized by a paucity of bands representing molecular weights below 43 000. Acrylamide gel electrophoresis of *G. cohnii* and *Peridinium trochoideum* acid-soluble chromosomal proteins in urea at pH 3.2 gives a banding pattern quite different than that of typical histones. Acid-soluble protein from chromatin prepared by the two different methods and from both organisms migrates as one predominant band with a mobility slightly less than that of Histone IV from corn. Its molecular weight, estimated by sodium dodecylsulfate gel electrophoresis, is about 16 000. It is a basic protein (basic/acidic amino acids 1.3) but differs from most histones in that it contains both cysteine and aromatic amino acids and somewhat lower levels of basic amino acids (18 mole % compared with 22 to 30% for histones). In addition, the major acid-soluble component is present in chromatin from log-phase cells but absent in chromatin from stationary-phase cells. For these reasons, the major acid-soluble protein is probably not a histone.

Introduction

In higher organisms, isolated chromatin is composed of DNA, RNA, histones and nonhistone proteins [1,2]. The evidence that the protein component of chromatin plays an important role in regulating RNA synthesis and development is increasing.

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The histones, which generally comprise the bulk of these proteins, are much better understood than the nonhistone chromosomal proteins, yet many questions about them remain unanswered. Histones appear to repress gene activity in higher organisms [1—4]; however, histones may also be responsible for condensation of the eukaryotic chromosome [5,6]. Since the prokaryotes appear to lack histones [1,2], the evolution of these proteins appears to represent a major change in the organization of the DNA in chromosomes and the mode of regulating gene expression. The dinoflagellate algae have nuclei which are in several ways intermediate between the prokaryotes and eukaryotes. Thus a study of their chromosomal proteins should help to elucidate not only the evolution of chromosomal proteins but their functions.

In this study, DNA-associated proteins were found in the dinoflagellate algae *Gyrodinium cohnii* and *Peridinium trochoideum*; however, unlike typical eukaryotes they have only a very small amount of acid-soluble protein, and these proteins differ from the typical eukaryote histones. Moreover, the chromosomal proteins change during the cell cycle.

Materials and Methods

Culture conditions and isolation of nuclei

G. cohnii and *P. trochoideum* nuclei were isolated as described previously [7].

Preparation of chromatin and acid-soluble protein

Chromatin and acid-soluble proteins were prepared according to the 2 M NaCl or the calcium method as described in the preceding paper [8].

Chemical determinations

Extraction of DNA and RNA, and determination of DNA, RNA and protein were all done as described previously [8].

Electrophoresis of acid-soluble proteins

Acid-soluble proteins were prepared as described in the preceding paper [8] and were electrophoresed by the method of Panyim and Chalkley [9]. The gels (diameter 6 mm, length about 6 cm) contained 15% (w/w) polyacrylamide and 6.25 M urea at a final pH of 3.2. After pre-electrophoresis, 25—100 μl samples (dissolved in 10 M urea—0.1% (v/v) mercaptoethanol and incubated at 37°C for 2 h) were loaded on the gels. Since difficulty was encountered in dissolving trichloroacetic acid precipitates in the 10 M urea, only acetone precipitates of acid-soluble protein were used for urea—gel electrophoresis. The gels were run at 1 mA/gel and room temperature for 10—20 min to allow the sample to enter the gels and then continued at 1.5 mA/gel for 3 h. The gels were stained overnight at room temperature with 0.5% (w/v) Buffalo Black in 7.5% (v/v) acetic acid and 20% (v/v) ethanol and destained by diffusion in 7.5% acetic acid—20% ethanol.

Electrophoresis of acid-insoluble proteins

Acid-insoluble proteins were electrophoresed as sodium dodecylsulfate

derivatives using the method of Shapiro et al. (see ref. 10). The concentration of ammonium persulfate was lowered from 15 to 8 mg/ml to obtain a slower polymerization. 10% (w/w) polyacrylamide gels were polymerized in the dark for 1 h at room temperature. The gels (6 mm by about 6 cm) were preelectrophoresed at 3 mA/gel for 1 h at room temperature using the gel buffer described by Weber and Osborn [10].

Acid-extracted or whole chromatin pellets were dispersed in 1 ml of 0.01 M sodium phosphate buffer, pH 7.0, 1% (w/v) in sodium dodecylsulfate and 1% (v/v) in mercaptoethanol, allowed to stand overnight at room temperature and then incubated at 37°C for 1—2 h. These solutions were centrifuged at 25000 × g for 5 min, and the supernatant was dialyzed at room temperature against 200 vol. of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecylsulfate (v/v) and 0.1% mercaptoethanol, with three changes. After dialysis, solid sucrose was added to give 10% (w/v) for density, and a drop of 0.05% (w/v) bromphenol blue in water was added as a tracking dye.

Samples of 50—100 μl were applied to the gels to give 50 μg protein per gel. Electrophoresis was carried out at 2 mA/gel for 20—30 min to allow the sample to enter the gel and then at 6 mA/gel for about 4.5 h. Gels which ran slower (judged by the tracking dye) were allowed to run longer. The gels were next fixed overnight at room temperature in 20% (w/v) sulfosalycylic acid [11], then rinsed several times in 10% (v/v) acetic acid. The gels were stained for 2—6 h in Coomassie brilliant blue solution and destained by diffusion in 7.5% acetic acid and 5% methanol [10].

Molecular weight determination

Molecular weights were estimated by sodium dodecylsulfate—polyacrylamide gel electrophoresis as described by Weber and Osborn [10]. The precipitated acid-soluble protein was dissolved directly in 0.01 M sodium phosphate buffer (pH 7.0) with 0.1% sodium dodecylsulfate and mercaptoethanol instead of the procedure described above for the preparation of acid-extracted or whole chromatin pellets. Molecular weight markers were obtained from Mann Research Laboratories (Orangeberg, N.Y.) except for RNAase, which was purchased from Sigma, St. Louis, Mo. All mobilities were measured to the front of the protein band, and the tracer dye front was marked by inserting a small piece of wire (see Weber and Osborn [10]). The mobility of the sample was plotted against a standard curve made from the known molecular weights expressed on a semi-logarithmic scale.

Amino acid analysis

Amido Schwarz-stained bands were cut out of the urea—acrylamide gels, hydrolyzed and the amino acids analyzed according to Houston [12].

Results

In this study, DNA-associated proteins were found in the dinoflagellate algae *G. cohnii* and *P. trochoideum*; however, unlike typical eukaryotes, they have only a very small amount of acid-soluble protein, and these proteins differ

from the typical eukaryote histones. Moreover, the chromosomal proteins change during the cell cycle.

Analysis of chromosomal proteins by sodium dodecylsulfate-acrylamide gel electrophoresis

Fig. 1 shows the patterns for the total chromosomal proteins (A), the acid-insoluble chromosomal proteins (B) and the acid-soluble chromosomal proteins (C), of *G. cohnii* as compared by sodium dodecylsulfate—acrylamide gel electrophoresis. The largest band present in Gel A (lower arrow), as well as a smaller and relatively diffuse band (upper arrow), are removed by acid extraction (Gel B). Because of differences in the banding pattern and degree of staining in sodium dodecylsulfate gels from one run to the next, precise comparisons of these gels can be made only if the gels are run together and the

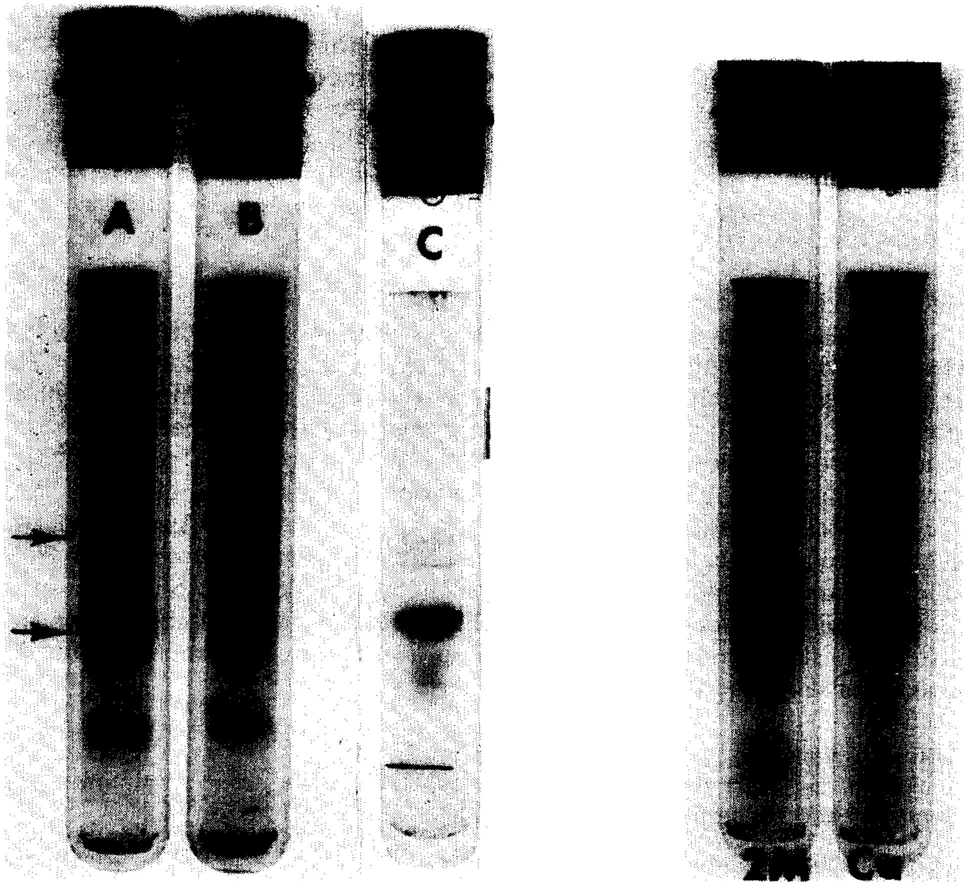


Fig. 1. Comparison of *G. cohnii* (log phase) total and acid-insoluble proteins from calcium chromatin by sodium dodecylsulfate electrophoresis. Gel loads: 50 μg each for total (A), and acid-insoluble (B) protein; 10 μg for acid-soluble protein (C). Gels A and B were run together but separate from C.

Fig. 2. Comparison of total chromosomal protein from 2 M NaCl and calcium chromatin (2 M and Ca, respectively) from log-phase *G. cohnii* by sodium dodecylsulfate electrophoresis. Gel loads: 50 μg each.

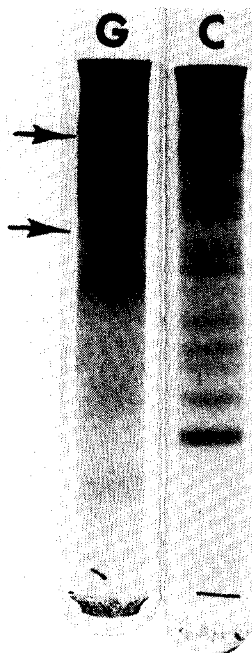


Fig. 3. Comparison of acid-insoluble protein from calcium chromatin of log-phase. *G. cohnii* and corn by sodium dodecylsulfate electrophoresis. Gel loads: 50 μg each for *G. cohnii* (G) and corn (C) acid-insoluble protein. The upper arrow corresponds to a molecular weight of about 74 000 and the lower 43 000. Gels G and C were run together.

marker dye allowed to run the same distance in all gels. The acid-soluble protein from *G. cohnii* calcium chromatin migrates as one major component plus several minor components (Gel C in Fig. 1). The largest band in Gel A represents the major component in Gel C.

In sodium dodecylsulfate gels the electrophoretic pattern of total chromosomal protein from *G. cohnii* as prepared by the 2 M NaCl method is very similar to that prepared by the calcium method (Fig. 2). Thus chromatin prepared by the two different methods contains for the most part the same or very similar chromosomal proteins.

The electrophoretic pattern of acid-insoluble protein from *G. cohnii* chromatin was compared to that of a higher plant, corn, and appears to be less heterogeneous as estimated from the number of bands (Fig. 3). Furthermore, the dinoflagellate acid-insoluble proteins tend to be restricted to a higher molecular weight range (over 43 000) than those of corn.

Analysis of chromosomal proteins by urea—acrylamide gel electrophoresis

The acid-soluble protein extracted from either *G. cohnii* or *P. trochoideum* chromatin prepared by either of the two methods migrates as one major component in urea-acylamide gels at pH 3.2. Fig. 4 compares this acid-soluble protein from log-phase *G. cohnii* calcium chromatin (GL) with corn epicotyl (C) histones. While the acid-soluble protein from corn chromatin shows the characteristic histone banding pattern, that protein from dinoflagellate chromatin shows a strikingly different pattern [13]. Careful comparison of

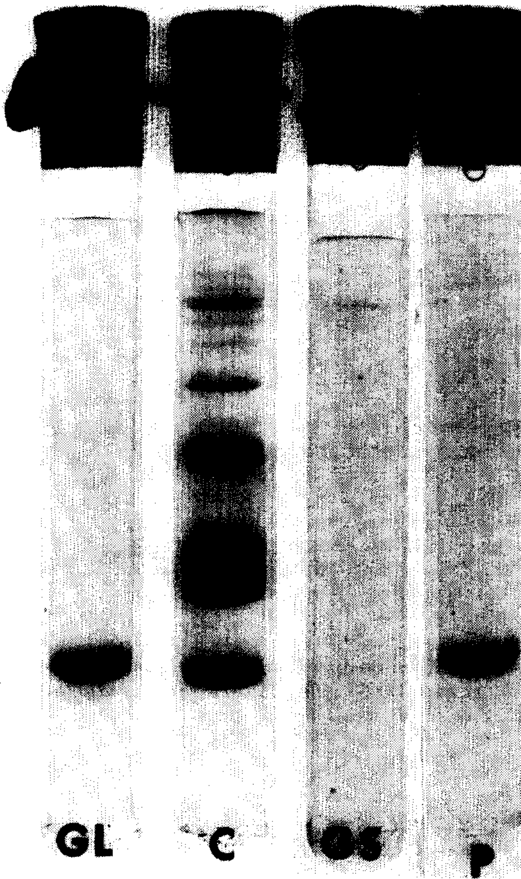


Fig. 4. Comparison of acid-soluble protein from dinoflagellate chromatin with higher plant histones by electrophoresis in acidic urea—polyacrylamide gels. Left to right: log-phase *G. cohnii* (GL), 20 μg; corn epicotyl histone (C), 25 μg; stationary-phase *G. cohnii* (GS), 10 μg; log-phase *P. trochoidium* (P), 11 μg. All samples were incubated in the presence of 0.1% mercaptoethanol. GL, P and C were prepared from calcium chromatin, but GS is from 2 M NaCl chromatin.

many runs shows that the major component runs slightly slower than the fastest moving histone fraction of higher plants and animals (Histone IV).

Taken together, the sodium dodecylsulfate and acidic urea—gel electrophoresis indicates that the major band from acid-soluble protein is a single protein.

When heavier loads were applied to the gels, it became apparent that there was also a second significant but less prominent band (Fig. 5). A slower moving major band became apparent, and many minor bands became visible in gels with heavier protein loads. The minor bands may represent acid-soluble chromatin proteins present in very small amounts, although the possibility that some are ribosomal or other contaminants has not been ruled out.

The reason for two major bands when heavier loads are applied is apparent in Fig. 6. Addition of mercaptoethanol produced a different protein band with

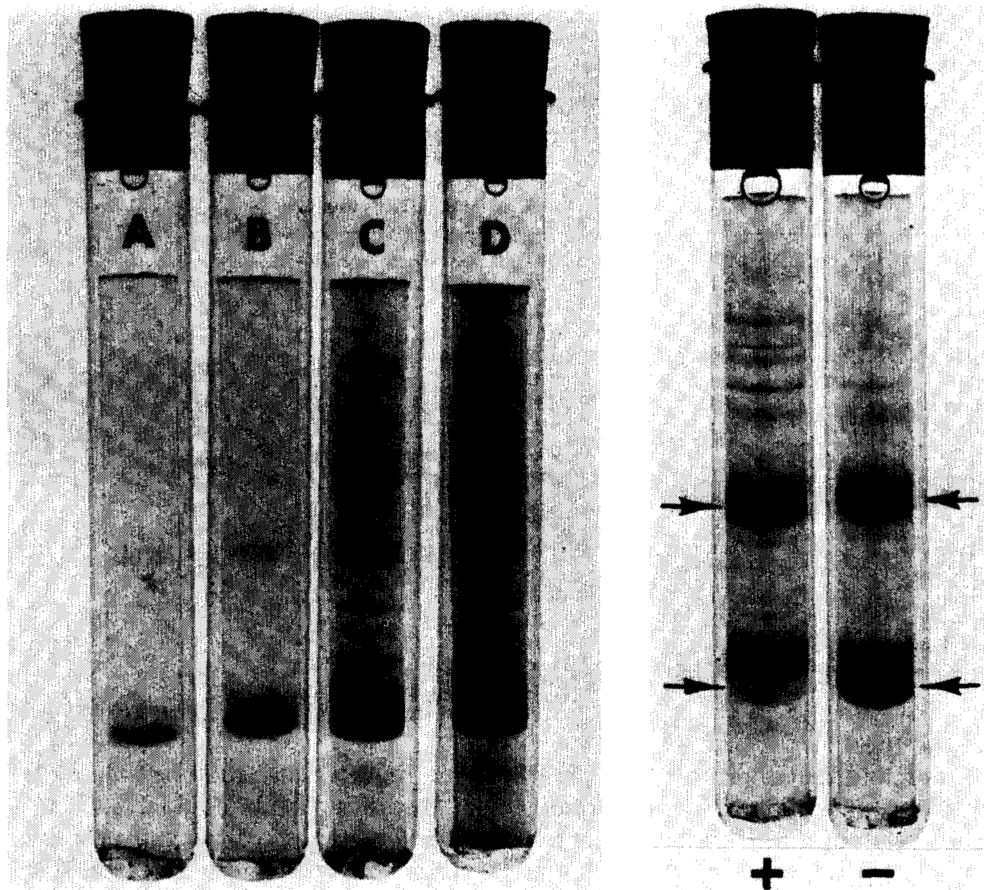


Fig. 5. Load series of acid-soluble protein from log-phase *G. cohnii* calcium chromatin separated electrophoretically on acidic urea-polyacrylamide gels. Approximately 8 μg protein were applied to Gel A, and the loads were successively doubled in Gels B to D.

Fig. 6. Effect of mercaptoethanol on the electrophoretic mobility of acid-soluble protein from log-phase *G. cohnii* calcium chromatin in acidic urea polyacrylamide gels. Upper arrows indicate the position of the RNAase in each gel. Lower arrows indicate the mobility of the major acid-soluble component pretreated for 2 h at 37°C in 10 M urea with (+), and without (—) 0.1% (v/v) mercaptoethanol. Each gel received 20 μg of sample protein and 25 μg RNAase.

about 5% less mobility when electrophoresed in urea. The lack of effect on the mobility of the RNAase standard suggests that the mercaptoethanol in the sample does not change the performance of the gels. The RNAase contained a small amount of mercaptoethanol which may have produced a small amount of reduction in the sample without 0.5 M mercaptoethanol. These results suggest that the reduction of intramolecular cysteine residues could be responsible. This would imply that at least two cysteine residues are present in each molecule. The amino acid analysis (Table I) suggests only one such residue; however,

TABLE I

AMINO ACID COMPOSITION OF THE MAJOR PROTEIN IN THE ACID-SOLUBLE FRACTION FROM *G. COHNII* CHROMATIN

The figures for amino acid composition are not corrected for hydrolytic losses.

Amino acid	Mole %	Approximate number of residues (based on a mol. wt of about 16 000)
Lysine	14.8	24
Histidine	1.1	2
Arginine	2.2	3
Aspartic acid	5.3	8
Threonine	7.6	12
Serine	7.6	12
Glutamic acid	8.8	14
Proline	5.7	9
Glycine	13.0	21
Alanine	17.1	29
Half cystine	0.8	1
Valine	4.6	7
Methionine	0.2	0.3
Isoleucine	1.7	3
Leucine	7.1	11
Tyrosine	1.1	2
Phenylalanine	1.6	3

it is possible that the mole % of half cysteine represents an underestimate due to losses. The reduction of intramolecular disulfide bonds could result in a more open or more extended conformation which may alter the protein's mobility. Although this particular electrophoresis system separates proteins mainly on the basis of charge, it can also separate according to size and conformation [14].

Since mercaptoethanol was not used in the preparation of the samples shown in Fig. 5, other factors must have caused the reduction or oxidation. The mercaptoethanol was included originally to guard against dimer formation (or higher order association) through disulfide bridges, but this type of association was not observed and the mercaptoethanol always caused a small decrease in mobility. A similar decrease in the electrophoretic mobility of calf thymus Histone III by reduction was recently reported by Panyim et al. [15].

The major acid-soluble component is not an artifact of the calcium method, because it also occurs in the acid-soluble protein extracted from *G. cohnii* chromatin prepared by the 2 M NaCl method. As shown in Fig. 4, the acid-soluble protein from calcium chromatin of *P. trochoideum* (P) also migrates as one major component and is compared to a similar gel with corn histone (C).

To see if typical histones are present in the intact nucleus, but lost during preparation of the chromatin, the electrophoretic patterns of acid-soluble protein from nuclei and calcium chromatin from log-phase cells were compared. A preparation of *G. cohnii* nuclei was divided into two unequal parts immediately after the 48000 × *g* centrifugation [7]. Two-thirds of this nuclear pellet were

used to prepare chromatin by the calcium method [8], and one-third was analyzed as nuclei. The pellet for nuclear analysis was washed once with 0.14 M NaCl. The electrophoretic patterns of acid-soluble protein from nuclei and chromatin are essentially the same (both contain one and the same major band) which argues against the possibility that typical histones are actually present in the nuclei but are lost during preparation of the chromatin.

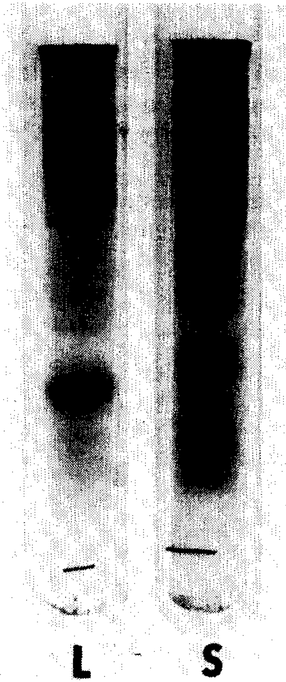
To check the possibility that the 0.14 M NaCl washes of the nuclei, prior to acid or chromatin extraction above, remove histones or significant amounts of the major acid-soluble protein, the proteins extracted with these washes were electrophoresed in the acidic urea—gel system. The electrophoretic pattern thus obtained is completely different from that of the histones. Furthermore, the major acid-soluble protein is not present in the 0.14 M NaCl wash, and the minor bands of the acid-soluble chromatin protein differ from the small number of bands obtained from the 0.14 M salt wash. Thus the 0.14 M NaCl washes of the nuclei do not remove a group of proteins resembling typical histones or even detectable amounts of the major acid-soluble protein.

Since histones of some tissues are subject to degradation [4,15], it was of interest to see if inhibitors of histone degradation had any effect on the acid-soluble protein obtained from dinoflagellate chromatin. To test this possibility, acid-soluble protein was extracted from chromatin of *G. cohnii* as usual except that 5 mM $NaHSO_3$ was included in all solutions used for the preparation of nuclei and chromatin except the 2.2 M sucrose solution. This omission was necessary, because a precipitate formed when both bisulfite and Triton X-100 were present in the 2.2 M sucrose. The electrophoretic pattern of acid-soluble protein from chromatin prepared in this manner was indistinguishable from that of chromatin prepared without the bisulfite. In addition, the acid-soluble protein/DNA ratio of chromatin isolated in the presence of bisulfite did not differ significantly from that of chromatin isolated without bisulfite [8].

An interesting observation concerning the major component of the acid-soluble protein is that it is apparently absent in chromatin from stationary-phase cells (Fig. 4). To see if this absence might be due to proteolysis, nuclei and chromatin were prepared in the presence of 25 μM phenylmethylsulfonyl-fluoride, an inhibitor of proteolysis [8]. This inhibitor did not bring about the reappearance of the banding pattern characteristic of log-phase chromatin. Furthermore, the acid-soluble protein/DNA ratio did not change significantly when this inhibitor was included [8]. Acid-soluble protein from *P. trochoideum* stationary-phase chromatin shows an electrophoretic pattern identical to that of *G. cohnii.*

Another interesting feature in these gels of acid-soluble protein from stationary-phase chromatin is the very thin band near the top of the gel (arrow), which is characteristic of late-log and stationary-phase chromatin but undetectable in that of rapidly growing cultures. Finally, the sodium dodecylsulfate gels in Fig. 7 show that the total chromosomal proteins from dividing and nondividing cells differ in several bands (more than can be accounted for in the acid-soluble proteins alone).

The molecular weight of the major component of dinoflagellate acid-soluble protein was determined by electrophoresis in sodium dodecylsulfate with mercaptoethanol [10]. As shown in Fig. 8, five proteins of known mole-



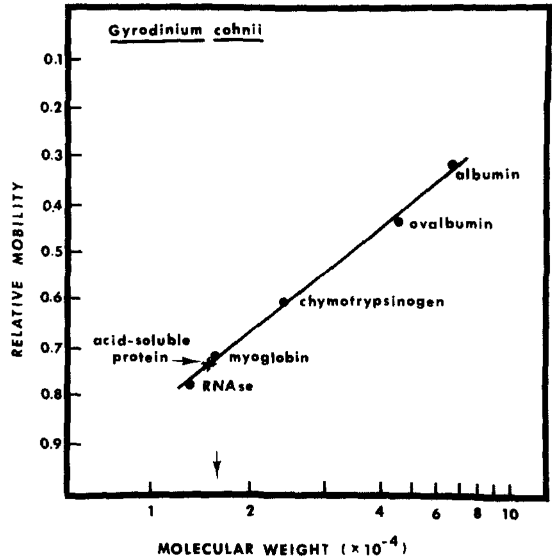


Fig. 7. Sodium dodecylsulfate gel electrophoresis of total chromosomal proteins from chromatin of log-(L), and stationary-phase (S), cells of *G. cohnii*. Approximately 40—50 μg of protein were loaded on each gel. Chromatin was prepared from isolated nuclei by the 2 M NaCl method and 100 μM phenylmethylsulfonyl fluoride was included in all steps. Gels L and S were run together.

Fig. 8, Molecular weight determination of the major acid-soluble protein from *G. cohnii* by sodium dodecylsulfate—acrylamide gel electrophoresis.

cular weight were used to construct a standard curve. The RNAase showed a mobility close to that expected (probably due to reduction of the internal disulfide bridges) [16] and therefore did not distort the standard curve. The molecular weight of the sample protein was extrapolated from its relative mobility in two separate electrophoresis runs (several gels in each). For the first run, the average molecular weight was 16500, but in the second run it was 15500. The approximate molecular weight of the acid-soluble protein was therefore taken as 16000; however, this technique could produce a small overestimate of the molecular weight. Since the anomalous behavior of Histone IIb2 is due to its high basic amino acid content [17,18], this effect should be less in the major acid-soluble chromosomal protein from *G. cohnii*, because it has fewer arginine and lysine residues (ref. 4, Table I). This molecular weight is somewhat larger than the 11000 of the smallest Histone (IV) but smaller than the 21000 of the largest Histone (I) [4].

Amino acid analysis

Table I shows the amino acid analysis of the stained bands in the gels corresponding to the major acid-soluble protein according to the procedure of Houston [12] and indicates that this protein is basic but not strongly basic.

Since this protein is to be compared with histones, it is of interest to note that it contains cysteine and the aromatic amino acids, tyrosine and phenylalanine, and there is at least one cysteine per molecule (molecular weight taken as 16 000) of this protein, but this figure may be low due to destruction during hydrolysis of the sample.

Discussion

Developmental changes in dinoflagellate chromosomal proteins

Since at least some of the proteins associated with DNA seem to be involved in the control of gene expression and therefore development, changes in the amounts or types of these proteins are of special interest. The quantitative differences in the relative amounts of RNA and protein in chromatin isolated from log- and stationary-phase cells shown in the preceeding paper [8] are taken to reflect developmental changes in the chromatin. The gel electrophoresis patterns of chromosomal proteins from these cells also show differences which are most striking in the acid-soluble protein. The major acid-soluble component of log-phase chromatin is completely absent in stationary-phase chromatin. The only visible band in urea—acrylamide gels of acid-soluble protein from stationary-phase chromatin is a thin band of low mobility. This band is not an aggregate due to formation of disulfide bridges, because these samples were incubated with mercaptoethanol.

As compared by sodium dodecylsulfate—acrylamide gel electrophoresis, different patterns were also observed for the acid-insoluble protein from log- and stationary-phase chromatin of *G. cohnii*, although the differences were not as striking as in the case of the acid-soluble protein. Aside from the acid-soluble protein band, several minor but definite differences can be seen.

Dinoflagellate acid-soluble protein — histone?

Histones are generally defined as low molecular weight basic proteins which are associated with DNA. Most of the acid-soluble protein of dinoflagellate chromatin migrates as one band in urea—acrylamide gels at pH 3.2 and has a molecular weight that falls within the range of the five most common histones. In addition, amino acid analysis shows it is a basic protein. On this evidence alone, it would seem justifiable to classify this protein as a histone, since it fits the general definition for histones. However, in higher organisms possessing typical eukaryotic histones, the amount of histone generally does not increase with increased template or metabolic activity [19]. With dinoflagellate chromatin, the amount of acid-soluble protein not only changes markedly but is present in rapidly dividing cells (presumably more active cells) and absent (or much less abundant) in stationary-phase cells, which is contrary to what one would expect for a histone. Furthermore, the relative amount of this protein is much smaller than one would expect for a histone. Assuming that in typical eukaryote chromatin most of the acid-soluble protein is histone and the five histone fractions are present in roughly equal amounts [20], the protein/DNA ratio for each histone is about 0.2. This is more than twice that of the highest acid-soluble protein/DNA ratio for calcium chromatin, which is a bit higher than 2 M NaCl chromatin [8].

Like the histones, the major acid-soluble protein is a low molecular weight, basic protein; however, it is slightly less basic than the least basic known histones, the basic (Arg, Lys, His)/acidic (Asp, Glu) amino acids being 1.3 as compared with 1.4 for Histone IIb1, the least basic histone [2,4]. Histone IIb1 differs more distinctly in that it contains quite different amounts of several amino acids and no cysteine. Based on urea—acrylamide gel electrophoresis at pH 3.2, this protein resembles Histone IV; however, its estimated molecular weight is about 45% greater, it stains a different color with Buffalo Black (grey rather than blue) and it contains cysteine. In addition, the proteins associated with DNA in dinoflagellate chromatin do not stabilize the DNA against heat denaturation as do histones [3,4]; however, the amount of acid-soluble protein may be too small to alter the melting profile.

Although the major acid-soluble protein has several properties in common with histones, it clearly differs from all known histones, and it seems unwarranted to group it with those proteins commonly considered to be histones. This conclusion is, however, contrary to the report of Stewart and Beck [21] based on immunofluorescent techniques, which suggested that histones are present in dinoflagellate chromosomes. The validity of their tests rests heavily on the purity of the DNA—histone preparations which were used as antigens, but the method these authors used to isolate DNA—histone actually yields whole chromatin [22] rather than just DNA—histone as they assumed.

Histones and evolution

The appearance of histones in phylogeny is not known, but since prokaryotes seem to lack histones [1,2] this probably occured after the divergence of prokaryotes and eukaryotes. Therefore, information on the occurrence of histones in primitive eukaryotes may provide a means for determining where histones appeared first. The histones of higher animals and plants tend to be very similar and some are nearly identical [4]. The fact that the primary structure of Histone IV is nearly identical in organisms as widely separated on the evolutionary scale as peas and cows [4], suggests that at least this histone arose only once (before the divergence of higher plants and animals) and that its primary structure has since been highly conserved. Several eukaryotic microorganisms, *Tetrahymena* [23,24], *Euglena* [25], and *Physarum* [26,27], possess acid-soluble proteins similar to but perhaps not identical with the histones of higher plants and animals. Although histones have been reported in the green alga *Chlorella* [28], the chromatographic data as well as amino acid analysis suggests that this acid-soluble protein differs considerably from typical histones and should be examined further by gel electrophoresis. The reports on the histones in fungi differ greatly and many conflict each other [29—35], yet it seems certain that if fungi do contain histones, the five major fractions typical of eukaryotes are not present [34,35].

The fact that some eukaryotes such as dinoflagellates (and possibly other microorganisms) lack histones (or at least contain basic proteins which are not only different from the known histones but are present in much smaller amounts) suggests that the nuclear membrane evolved before the histones. Much more study is needed before the evolutionary origin of the histones is

clear; however, our findings suggest that the dinoflagellates are among the most primitive eukaryotes with respect to their histones.

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