Purification and Characterization of the Histones Associated with the Macronucleus of *Tetrahymena*†

Carol A. Johmann* and Martin A. Gorovsky

ABSTRACT: Histone fractions have been isolated from the macronucleus of *Tetrahymena pyriformis*. Five classes of macronuclear histone were purified, using a combination of gel exclusion and ion-exchange chromatography, and were examined with respect to their solubility, electrophoretic, chromatographic, and chemical properties. *Tetrahymena* H4 is very similar to vertebrate H4, except that it exhibits a larger number of acetylated subfractions. In contrast, the other *Tetrahymena* histones vary more extensively from their calf thymus counterparts. *Tetrahymena* H3 resembles calf thymus H3 in its solubility properties and is the only macronuclear histone containing cysteine. However, it differs from vertebrate H3 in composition and has a faster electrophoretic mobility on both urea–acrylamide and sodium dodecyl sulfate–acrylamide gel electrophoresis. *Tetrahymena* H3 also displays a level of acetylation higher than that reported for its vertebrate homologue. Approximately 45% of macronuclear H2B, which resembles calf thymus H2B in composition and solubility, is present in a (mono)acetylated form, not detected in vertebrate somatic H2B. H1, though similar to its calf thymus homologue in solubility, modification (by phosphorylation), and other properties, differs considerably in its content of basic, acidic, and hydrophobic amino acids. *Tetrahymena* does not contain a histone strictly homologous to H2A. Although macronuclear histone X resembles H2A in chromatographic and some solubility properties, it displays compositional and other solubility properties more like H2B than H2A. Fraction X is polymorphic in sodium dodecyl sulfate–acrylamide gels, migrating as two distinct molecular forms. While it is possible that one form is H2A-like and the other more H2B-like, the observation that both forms of X behave identically in solubility fractionation schemes makes this unlikely. Fraction X is both phosphorylated and acetylated which, in addition to two molecular forms, results in a characteristic heterogeneous pattern on urea–acrylamide gels. Characterization of the histone complement of this lower eucaryote should contribute to the understanding of the evolution and biological role of these basic proteins. Moreover, this description represents the most extensive analysis to date of the histones associated with an amitotic, genetically active nucleus. It will serve as a reference to which the histones of the morphologically distinct, mitotically dividing, and genetically inactive micronucleus of this organism can be compared.

Information on the nature of histones in a wide range of organisms is necessary to discern the evolution of these basic proteins and to disclose their biological role in the structure and function of eucaryotic chromatin. Analyses on the histones of vertebrates (Panyim et al., 1971), invertebrates (Cohen and Gotchel, 1971; Oliver and Chalkley, 1972; McMaster-Kaye and Kaye, 1973; Easton and Chalkley, 1972), plants (Nadeau et al., 1974), and unicellular organisms (Iwai, 1964; Iwai et al., 1965; Mohberg and Busch, 1969; Hsiang and Cole, 1973; Franco et al., 1974) have led to suggestions concerning their evolution and their function(s). Yet, despite the abundance of information on the chemical and metabolic properties of histones, their precise function(s) remains unclear.

To extend such studies and to attempt to elucidate further the role of histones, we have been examining these proteins in *Tetrahymena pyriformis*. Many strains of *Tetrahymena*, like most other ciliated protozoans, exhibit nuclear dimorphism. Although both the somatic macronucleus and the germinal micronucleus derive from a common diploid syncaryon during the sexual process of conjugation, they exist in radically different structural and functional states. During the vegetative life cycle of this ciliate, macronuclei divide amitotically and are sites of intense RNA synthesis, while micronuclei divide mitotically and synthesize little or no detectable RNA. It has been suggested (Gorovsky, 1973; this reference also contains a more detailed review of the properties of macro- and micronuclei) that these two nuclei provide a model system in which to study the role of histones in nuclear structure and function. Detailed comparisons of the histones associated with macro- and micronuclei require the complete identification and analysis of the individual histone fractions. In this report, we describe the purification and characterization of the macronuclear histone complement and examine some of the secondary modifications of these histones.

Experimental Procedures

Isolation of Nuclei and Extraction of Histone. Tetrahymena pyriformis, strains[1] WH-6, B-1868-VII (or B VII), and DDH-58-7-5 (referred to as DDH), were cultured axenically in enriched proteose peptone and harvested during early deceleratory growth phase. Macronuclei were isolated, and whole histone was extracted as previously described (see Gorovsky et al., 1975 for description of these methods). Histone H1 was removed from whole histone as described elsewhere (Oliver et al., 1972; modified by Johmann and Gorovsky, 1976).

Calf thymus histone was obtained by homogenizing the

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[1] Strains WH-6 and B VII both belong to syngen 1, while strain DDH belongs to syngen 2. A syngen is defined as a mating group within which mating occurs; no fruitful intersyngenic mating can occur.

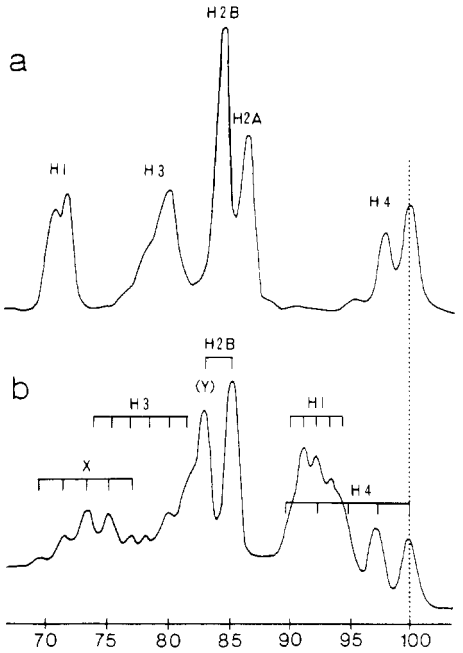

FIGURE 1: Densitometer tracings of urea–acrylamide gels containing whole histone from (a) calf thymus and (b) *Tetrahymena* strain WH-6. The origin of the gels (not shown) is to the left. A scale of the relative electrophoretic mobilities (REM), which were calculated with respect to the leading subspecies of H4, is included.

tissue in 0.14 M NaCl–0.05 M $NaHSO_3$ and, after repeated washings of the homogenate, extracting the histone with 0.4 N H_2SO_4.

Fractionation of Histones. 1. Differential Solubilization. The fractionation scheme used was essentially Johns' Method I (1964) with several fractions being extensively recycled through either Johns' Method I or II (1964). The modified technique of Oliver et al. (1972) was also employed.

2. Bio-Gel Exclusion Chromatography. Exclusion chromatography of whole histone was performed on both Bio-Gel P-60 (Böhm et al., 1973) and Bio-Gel P-100 (Sommer and Chalkley, 1974). Appropriate fractions were pooled, concentrated by dialyzing against solid sucrose or Carbowax PEG-6000 (Fisher Scientific Co.), and then dialyzed against 0.4 N H_2SO_4 and precipitated with 4 volumes of ethanol.

3. Carboxymethyl-cellulose Chromatography. Cation-exchange chromatography on Cellex-CM (Bio-Rad Corp., 0.675 mequiv) was used to purify crude (nonlysine-rich) histone fractions obtained from exclusion chromatography and to separate histones Y[2] and H2B. Carboxymethyl-cellulose chromatography was performed on 0.8 × 25 cm columns, at 22 °C, with 0.01 to 0.025 N HCl gradients (100, 200, or 400 ml total volume). Samples (<3 mg) were dissolved in 0.01 N HCl and eluted at an average flow rate of 8 ml/h. Fractions were pooled and subsequently treated as above.

In Vivo Acetylation of Histones. Strain DDH cells, growing logarithmically at 28 °C, were collected at 2250*g* for 3 min, washed once in 10 mM Tris, pH 7.4, and resuspended in Tris. ^{3}H-Labeled sodium acetate (New England Nuclear Corp.; 10 μCi/ml) was added. After incubating for 30 min at 36 °C, macronuclei were isolated and histones extracted as usual. Labeled histones were analyzed directly or oxidized by dissolving in 0.9 N acetic acid–2.5 M urea–15% sucrose and stirring at 22 °C for several days, and/or dephosphorylated by treatment with bacterial alkaline phosphatase, following the procedure of Sherod et al. (1970), prior to electrophoretic analysis.

Gel Electrophoresis. The gel electrophoretic technique of Panyim and Chalkley (1969) was used. Samples were dissolved in 0.9 N acetic acid–2.5 M urea–15% sucrose with or without 0.1 M mercaptoethanol and electrophoresed at 250 V for 25 h at 4 °C on 25-cm long, 15% acrylamide–2.5 M urea gels. Staining of gels was accomplished in 1% fast green in 7% acetic acid. After destaining in 7% acetic acid, the gels were scanned at 630 nm with a Gilford 2400 spectrophotometer. Gels containing isotopically labeled histone were sliced into 1-mm segments, which were solubilized in NCS (Amersham/Searle Corp.). Counting was performed in a Nuclear Chicago Isocap liquid scintillation counter, using Spectrafluor (Amersham/Searle Corp.) as a scintillant. Histone fractions were also analyzed by sodium dodecyl sulfate–acrylamide gel electrophoresis (Panyim and Chalkley, 1971) on 12% polyacrylamide–0.05% sodium dodecyl sulfate gels, pH 7.6, at 80 V for 6 h at 22 °C.

Amino Acid Analysis. Amino acid analyses of purified histone fractions were performed by the AAA Laboratory (14351 22nd Ave. N.E., Seattle, Washington 98125). All samples were dialyzed extensively against deionized H_2O, evaporated to dryness, and subjected to a 24-h acid hydrolysis prior to analysis.

Results

Fractionation and Identification of Macronuclear Histones. Histones[3] from strain WH-6 (Figure 1b) have been partially fractionated according to the differential solubility methods of Johns (1964, 1967) and by the modified technique of Oliver et al. (1972). The results from these fractionation schemes do not provide unambiguous assignments to the electrophoretically separable histone groups. As previously reported (Gorovsky et al., 1973, 1974), macronuclear H1 and H4 exhibit solubility (and other) properties identical with those of their calf thymus counterparts and do not contaminate any of the other fractions. Histones Y[2] and H2B (labeled as such in Figure 1b) have solubility properties similar to calf thymus H2B and only slightly contaminate the H3 fraction. H3, identifiable by its ability to oxidize and also to react with [^{14}C]-*N*-ethylmaleimide (Gorovsky and Keevert, 1975b), behaves like calf thymus H3, but also appears in the H4 fraction. Histone X, on the other hand, does not act like any calf thymus histone. Although it isolates with Y/H2B, it can be removed from that fraction by extensive recycling of the "H2B" sample through Johns' Method I (1964). It should also be noted that the identification of *Tetrahymena* histone classes cannot be deduced from their electrophoretic mobilities alone since, as Figure 1 shows, these are (with the exception of H4; Gorovsky et al., 1973) quite different from those of calf thymus histones.

[2] In earlier publications (Gorovsky et al., 1973, 1974; Gorovsky, 1973), fraction Y was not separated from the fastest subfraction of F3 (H3) and therefore went undetected. In these publications, it constitutes a considerable portion of the peak labeled F3 (H3). In later publications, this peak is labeled as Y if separated from F3 (Gorovsky and Keevert, 1975b) or as F3 + Y if not resolved (Gorovsky and Keevert, 1975a).

[3] The histone nomenclature used here corresponds to the nomenclature used in our previous work as follows: H1 = F1, H2A = F2A2, H2B = F2B, H3 = F3, and H4 = F2A1. Superscripts indicate electrophoretic mobility from faster (0) to slower on urea–acrylamide gels and also usually imply parental (0) and modified forms.

Table I: Amino Acid Analysis of Fractions from Calf Thymus[a] and *Tetrahymena*[b] Strain B VII.

	Total Amino Acid Content (Mol %)								
Amino Acid	CT H1	B VII H1	CT H2A	B VII X	CT H2B	B VII Y	B VII H2B	CT H3	B VII H3
Lysine	27.3	28.7	11.4	13.0	14.3	15.4	15.2	8.7	11.7
Histidine	0	2.0	3.0	2.7	2.3	1.3	1.3	1.8	2.7
Arginine	2.1	2.3	10.3	5.7	8.5	5.3	5.4	12.7	9.5
Aspartic acid	2.6	8.1	5.6	9.4	5.4	8.0	8.2	4.4	6.8
Threonine[c]	6.0	8.8	4.3	5.9	6.1	5.6	5.5	6.6	5.7
Serine[c]	6.9	7.5	3.5	7.8	7.5	10.4	9.9	4.3	6.7
Glutamic acid	4.7	7.4	9.6	10.1	10.3	10.0	9.4	12.6	12.1
Proline	8.4	6.8	3.9	4.5	4.9	4.7	4.5	4.6	3.8
Glycine	6.7	3.4	10.8	8.7	7.1	7.7	7.3	5.5	6.1
Alanine	23.4	14.8	12.0	9.3	10.2	9.3	9.5	12.6	10.0
Cysteine[d]	0	ND	0	ND	0	ND	ND	0.7	1.1
Valine	6.0	3.8	6.6	5.2	6.4	5.7	5.3	4.7	4.0
Methionine	Tr	0.7	0.4	1.0	1.2	1.3	1.6	1.3	1.3
Isoleucine	0.8	3.0	4.1	4.8	4.2	4.4	4.6	5.0	4.9
Leucine	3.9	1.9	11.0	9.2	6.9	7.3	7.5	9.3	8.4
Tyrosine	0.5	0	2.6	1.1	3.2	1.7	1.5	2.1	1.3
Phenylalanine	0.4	0.6	0.9	2.1	1.9	2.9	3.3	3.0	4.0
Lys–Arg	13.0	12.5	1.1	2.3	1.7	2.9	2.8	0.7	1.2
Basic–Acidic	4.0	2.1	1.6	1.1	1.6	1.2	1.2	1.4	1.3

[a] Calf thymus (CT) data from Panyim et al., 1971. [b] B VII histones: H1, Bio-Gel P-100 fraction further purified by stepwise elution from Bio-Rex 70 chromatography (unpublished data); X, Bio-Gel P-100 fraction (average of two analyses); Y and H2B, Bio-Gel P-100 sample separated by carboxymethyl-cellulose chromatography; H3, Bio-Gel P-100 fraction. [c] Serine increased by 10% and threonine by 5% to compensate for losses during acid hydrolysis. [d] Performic acid oxidized prior to acid hydrolysis. ND: not determined. Tr: Trace.

Table II: Amino Acid Analysis of B VII H4 Preparations.

	Total Amino Acid Content (Mol %)			
	B VII H4's[a]			Calf thymus[b]
Amino Acid	A	B	C	H4
Lysine	9.2	10.2	10.8	10.8
Histidine	3.3	2.3	1.9	2.0
Arginine	7.7	10.8	14.9	13.7
Aspartic acid	13.0	9.2	5.5	4.9
Threonine[c]	5.0	6.2	5.6	6.9
Serine[c]	8.7	6.3	3.5	2.0
Glutamic acid	7.8	8.2	8.4	5.9
Proline	3.6	2.8	1.4	1.0
Glycine	9.8	12.1	16.4	16.7
Alanine	6.0	6.9	7.5	6.9
Cysteine	ND[d]	ND	ND	0
Valine	6.1	6.1	6.5	8.8
Methionine	1.0	1.7	1.5	1.0
Isoleucine	4.2	5.1	4.9	5.9
Leucine	7.5	7.8	7.9	7.8
Tyrosine	2.6	1.8	2.8	3.9
Phenylalanine	4.3	3.6	2.5	2.0
Lys–Arg	1.2	0.9	0.7	0.8
Basic–Acidic	1.0	1.3	2.0	2.5

[a] Preparation A: Bio-Gel P-100 sample, 85–90% pure. Preparation B: Bio-Gel P-60 sample, 90% pure. Preparation C: Bio-Gel P-60, followed by carboxymethyl-cellulose chromatography, >95%. [b] Amino acid composition calculated from H4 sequence (DeLange et al., 1969a). Two samples of calf thymus H4 isolated by Bio-Gel P-100 were independently analyzed; both resembled the published composition of H4. [c] Serine increased by 10% and threonine by 5% to compensate for losses during acid hydrolysis. [d] ND: not determined.

It was thus necessary to utilize different methods to further purify *Tetrahymena* histones. By combining gel exclusion chromatographic techniques with ion-exchange chromatography, the major histone fractions from strain BVII[4] have been completely fractionated and purified. These fractions were subjected to amino acid analysis (Tables I and II) and identifications with respect to calf thymus histones were made. In all cases, the tentative identification of a particular fraction by its solubility properties and/or its electrophoretic mobility agreed with the final identification based solely on its amino acid composition.

Exclusion chromatography on Bio-Gel P-60 (Figure 2a) separates macronuclear histone into five fractions, while that on Bio-Gel P-100 (Figure 3) yields only four. In both cases electrophoretic analysis indicates that the elution order is the same, viz., H1, X, Y/H2B, H3, and H4, even though the patterns of elution differ (compare Figures 2a and 3). Both chromatographic methods yield relatively pure fractions (in most cases, greater than 95% purity), although fractions X, Y/H2B, and H3 from the Bio-Gel P-60 column are slightly cross-contaminated.

The elution profiles from Bio-Gel P-60 chromatography of both calf thymus and *Tetrahymena* histones are shown in Figure 2. The results of the electrophoretic analysis of the peaks are indicated by the labeling. The elution pattern obtained for calf thymus histone (Figure 2b) is very similar to that reported by Böhm and his coworkers (1973). It is obvious, however, that under the same conditions, *Tetrahymena* histones behave differently (Figure 2a). Although H1 and H4 elute in the general regions of their calf thymus homologues, only Y/H2B aligns exactly with its calf thymus correspondent. Fraction X elutes similarly to calf thymus H2A, while B VII H3 is shifted toward a higher elution volume with respect to calf thymus H3.

In contrast, the elution profile of *Tetrahymena* histone obtained from Bio-Gel P-100 chromatography (Figure 3) resembles the one reported for calf thymus histone (Sommer and Chalkley, 1974).[5] As on Bio-Gel P-60, fraction X

[4] The histones of strains WH-6 and BVII have been shown to be electrophoretically identical (Johmann and Gorovsky, 1976).

[5] The significance of this similarity is unclear, since, using the same column and buffer conditions, an exact replica of Sommer and Chalkley's (1974) results with calf thymus histone could not be obtained, although the elution order of the calf histones was the same.

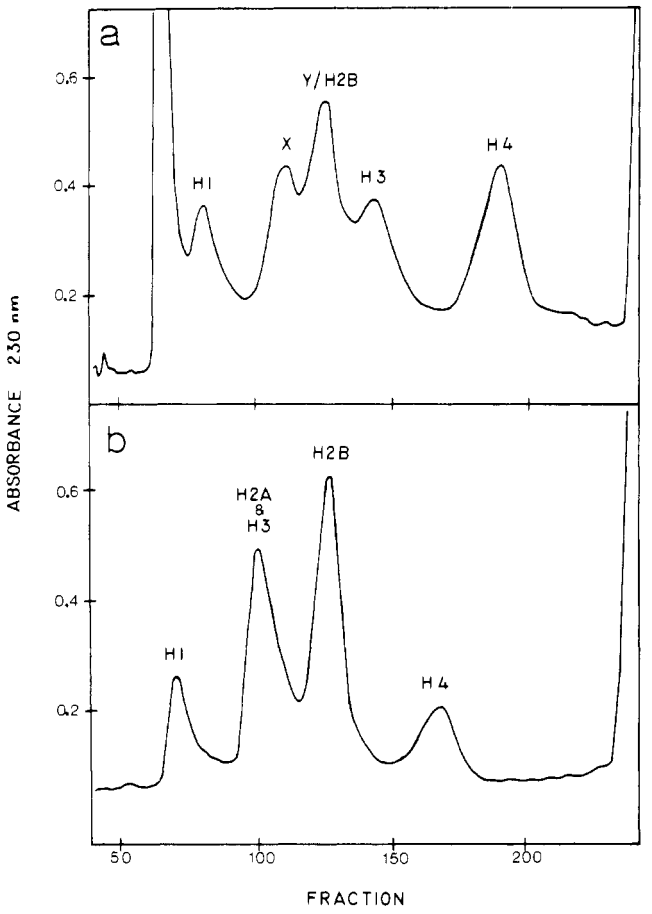

FIGURE 2: Elution profiles of histones separated by Bio-Gel P-60 (100–200 mesh, Bio-Rad Corp.) chromatography. Eighty milligrams of (a) B VII macronuclear histone or 30 mg of (b) calf thymus histone was dissolved in 8 M urea–1% mercaptoethanol, applied to the 2.5 × 165 cm column, and eluted at 22 °C with 0.02 N HCl–0.02% NaN_3–0.05 M NaCl, pH 1.95. Four-milliliter fractions were collected at a flow rate of 25 ml/h. The first peak in column (a) contains nonhistone proteins which contaminate the macronuclear histone preparation. The strong uv absorption toward the end is due to the elution of urea and mercaptoethanol.

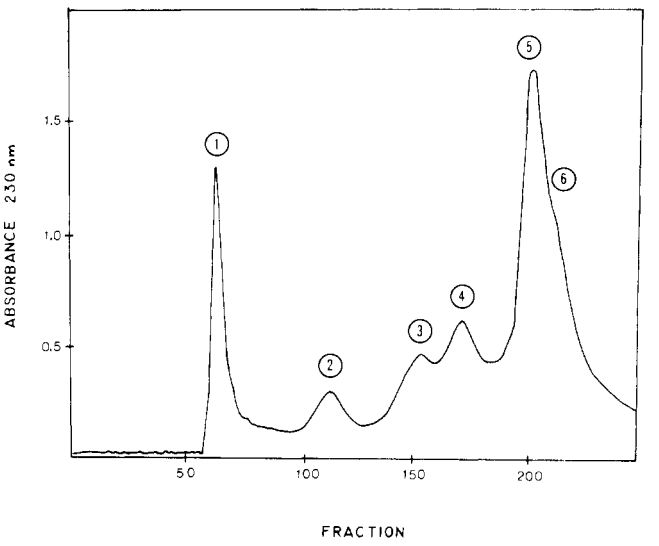

FIGURE 3: Elution profile of B VII macronuclear histones separated on Bio-Gel P-100 (100–200 mesh, Bio-Rad Corp.). Histone (60 mg) was dissolved in 0.01 N HCl–0.02% NaN_3–10% sucrose, applied to a 2.5 × 120 cm column and eluted at 4 °C with 0.01 N HCl–0.02% NaN_3. Fractions (3 ml) were collected at a flow rate of 9 ml/h. The first peak contains contaminating nonhistone protein.

behaves like calf thymus H2A and the Y/H2B complex elutes together in the same region as calf thymus H2B.

The results of the gel electrophoretic analysis of the Bio-Gel P-100 column fractions are given in Figure 4. None of the fractions show evidence, on either urea–acrylamide or sodium dodecyl sulfate–acrylamide gels, of significant cross-contamination. All fractions, except H4, are at least 95% pure. H4 appears to be contaminated by approximately 10–15% nonhistone protein (section of gel not shown). Rechromatography of the H4 sample on carboxymethyl-cellulose removes this contaminant, as demonstrated by analytical electrophoresis. Figure 4 also indicates that purified X migrates as two peaks on sodium dodecyl sulfate–acrylamide gels.

Ion-exchange (carboxymethyl-cellulose) chromatography of PCA-insoluble histone (whole histone from which H1 has been extracted with 5% perchloric acid) partially separates the histones in the general order of X, Y, H2B, H4, and H3. In addition, it resolves contaminating nonhistone protein from the histones. It is thus useful as a final purification step after exclusion chromatography.

Histones Y and H2B, which isolate together in the solubility fractionation schemes and by exclusion chromatography, are separable by carboxymethyl-cellulose chromatography. The carboxymethyl-cellulose column profile of the fractionation of the Y/H2B complex (obtained from Bio-Gel chromatography) is shown in Figure 5, along with the electrophoretic analyses of the pooled fractions. The middle portion of the column peak contains both Y and H2B in varying proportions.

The amino acid composition of the purified B VII histones were analyzed; the results are given in Tables I and II. As previously reported for WH-6 H1 (Gorovsky et al., 1974), B VII H1, though similar to calf thymus H1, differs significantly in its ratio of basic to acidic amino acids, primarily due to a higher (>twofold) content of acidic residues. Moreover, histidine is present (which is not the case for vertebrate H1), while tyrosine (the basis for the *N*-bromosuccinimide cleavage reaction; Bustin and Cole, 1969) is probably absent. The hydrophobic residue content of B VII H1 is only 70% that of calf thymus H1.

Histone X displays compositional similarities to both calf thymus H2B and H2A, yet differs from both in several ways. In comparison with both H2B and H2A, fraction X is arginine-poor, aspartic-acid-rich, and slightly valine-poor. Moreover, while its serine content more closely resembles that of H2B, its high leucine content is representative of H2A. Clearly, X has no strict chemical homologue among calf thymus histones. However, it is possible that, since X migrates as a doublet on sodium dodecyl sulfate gels (Figure 4), fraction X consists of two proteins, one H2A-like and the other H2B-like, rather than two proteins of intermediate characteristics (see Discussion).

Histone H3 resembles calf thymus H3 in composition, although it does differ significantly in its content of lysine (higher), arginine (lower), aspartic acid (higher), serine (higher), and alanine (lower). It is the only *Tetrahymena* histone which contains cysteine, as shown by its ability to react with *N*-ethylmaleimide and to undergo oxidation.

B VII fractions Y and H2B are identical with each other and similar to calf thymus H2B, although their ratios of basic to acidic amino acids are slightly lower (due to lower content of arginine and higher content of aspartic acid).

Table II presents the amino acid compositions of several

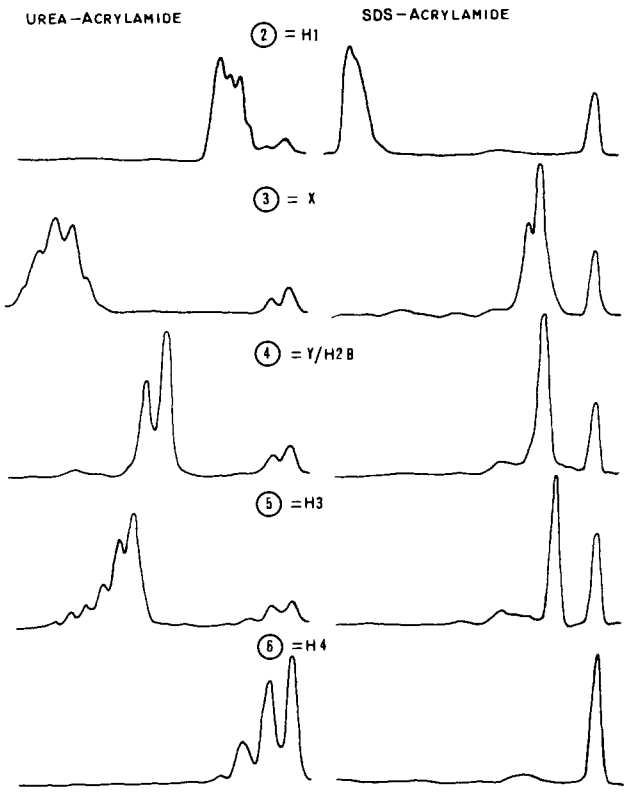

FIGURE 4: Densitometer tracings of urea-acrylamide (left column) and sodium dodecyl sulfate-acrylamide (right column) gels containing the Bio-Gel P-100 fractions from the column profile shown in Figure 3. Calf thymus H4 was used as a mobility marker in all but the H4 gels. Each sample was also run without calf thymus H4, to verify the absence of H4. The apparent molecular weights of the B VII histones (calculated with respect to calf thymus histone standard sodium dodecyl sulfate gels) are: H1, 24 500 and 23 500; X, 14 200 and 13 600; H2B, 13 600; H3, 13 300; and H4, 11 300. As noted previously (Gorovsky et al., 1974), there is a marked dependence of the mobility (and therefore the estimated molecular weight) of *Tetrahymena* H1 on the pH at which sodium dodecyl sulfate-gel electrophoresis is performed. H1 appears to be much smaller on the pH 10 sodium dodecyl sulfate-gel system of Panyim and Chalkley (1971).

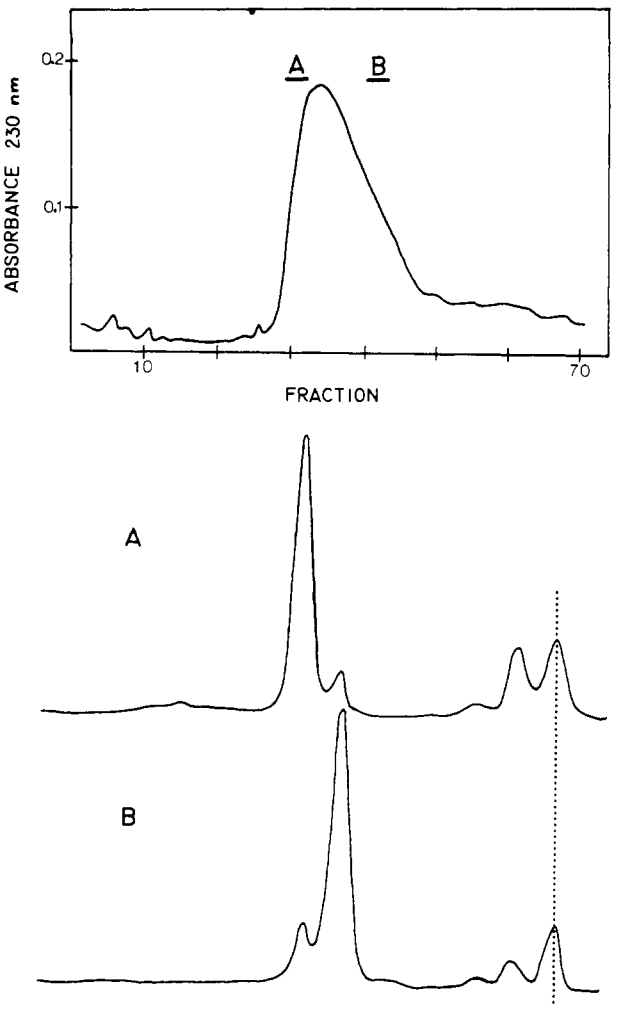

FIGURE 5: Carboxymethyl-cellulose chromatography of the Bio-Gel P-100 Y/H2B fraction (Figure 4) and urea-acrylamide gel tracings of the carboxymethyl-cellulose fractions labeled A and B. The sample in gel A has a REM = 83, while that in B has a REM = 85. Thus, A and B correspond to Y (or $H2B^1$) and $H2B^0$, respectively. Calf thymus H4 was used as a mobility marker for calculation of the REM.

B VII H4 preparations and compares them with the composition of calf thymus H4. Only when Bio-Gel P-60 chromatography is combined with carboxymethyl-cellulose chromatography (Table II, preparation C) does the resulting purified (>95%) H4 closely resemble calf thymus H4. The deviations observed are no greater than differences reported for various calf thymus H4 preparations and some may still be due to the presence of small amounts of contaminating nonhistone protein.

In Vivo Acetylation of Macronuclear Histones. It has been shown that acetylation of histones occurs postsynthetically and results in the formation of N^{ϵ}-acetyllysyl residues (see Allfrey, 1971, for review). The acetylation of *Tetrahymena* histones can be studied by isotopically labeling cells in vivo (Gorovsky et al., 1973). One result of acetylation is an overall reduction in the net positive charge of the protein molecule, which enables differentially acetylated species of the same histone molecule to be separable by polyacrylamide gel electrophoresis (Panyim and Chalkley, 1969; Wangh et al., 1972).

Electrophoretic analyses of strain DDH histones, labeled in vivo with H^3-labeled sodium acetate, are shown in Figure 6. Strain DDH was used for these studies since it has been shown (Johmann and Gorovsky, 1976) that (due to the inconstancy of the electrophoretic mobility of Y/H2B) the histones of strain DDH are more completely resolved on urea-acrylamide gels than those of strains WH-6 or BVII.[6] To increase the clarity of and to assure lack of ambiguity in correlating a gel's optical density pattern with its acetylation profile, H1 was routinely removed (5% PCA) from labeled histone samples prior to gel electrophoresis. (H1 migrates in the H3-Y region in strain DDH, see Johmann and Gorovsky, 1976).

It has been demonstrated (Gorovsky et al., 1973) that the heterogeneity of WH-6 H4, which consists of five electrophoretic subfractions, is the result of differential acetylation. This conclusion is supported by the present observation of acetylated H4 subspecies in strain DDH (Figure 6).

Purified *Tetrahymena* H3 consists of five or six subfractions (Figure 4), several of which migrate in the region of histone X. It is evident from a comparison of Figures 6a and 6b that this microheterogeneity of DDH H3 is caused by differential acetylation of the parent form ($H3^0$) and that the ^3H counts appearing under bands X^0, X^1, and also prob-

[6] Similar studies, with the same results, were also done on BVII histones.

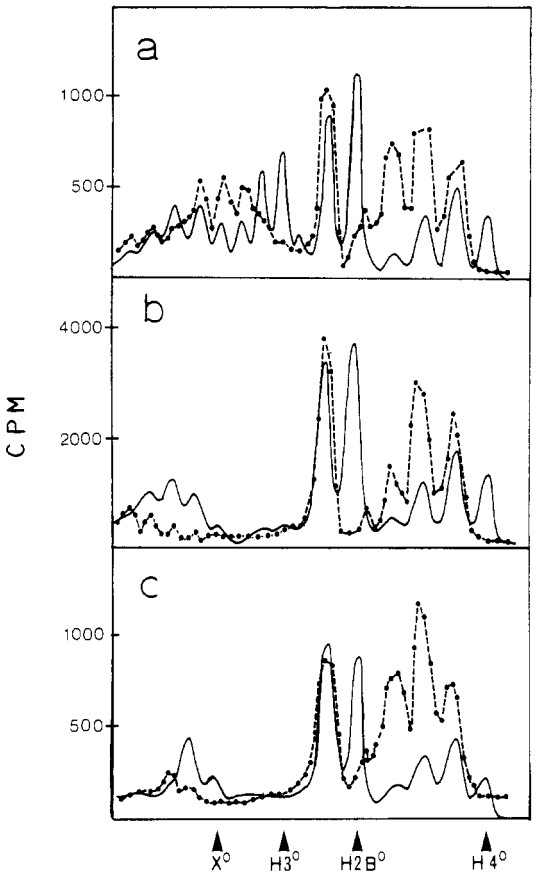

FIGURE 6: Densitometer scans (—) and count profiles (●- - -●) of urea–acrylamide gels containing strain DDH macronuclear PCA-insoluble histone labeled in vivo with ^{3}H-labeled sodium acetate. The histone in (b) has been actively oxidized, while that in (c) has been both dephosphorylated and oxidized. The small OD peak in between $H3^0$ and $H2B^1$ in (a) is thought to be a degradation product of H3, as it appears in varying amounts in different preparations of DDH PCA-insoluble histone and is oxidized (gels b and c). The ^{3}H counts just to the right of $H2B^0$ belong to $H4^4$.

ably X^2 actually belong to the slower moving H3 bands ($H3^3$, $H3^4$, $H3^5$; see footnote 3).

^{3}H-Labeled sodium acetate is incorporated into Y but not H2B (Figure 6). Since Y and H2B are compositionally identical (Table I), it is apparent that Y (or $H2B^1$) is an acetylated form of H2B.

Histone X of *Tetrahymena* is quite unusual in that it has no electrophoretic counterpart among vertebrate histones (Figure 1). It has a slow relative electrophoretic mobility and exhibits extensive microheterogeneity (Figures 1b and 4). That some of the microheterogeneity of X is due to acetylation is shown in Figure 6b, where [^{3}H]acetate-labeled DDH histone has been oxidized to remove any ambiguity arising from the comigration of H3 and X subfractions. It is evident that bands X^3, X^4, and possibly X^2 are acetylated, while X^0 and X^1 are not. However, acetylation cannot be the entire cause of the observed microheterogeneity of X. The results of acetylation studies coupled with dephosphorylation of labeled samples are given in Figure 6c, where H3 has been actively oxidized. It is apparent that, while X^3 and X^4 are phosphorylated, X^0 and X^1 are not. It is also likely that X^2 is phosphorylated and that the small peak to the left of X^1 in Figure 6c is either due to incomplete dephosphorylation or the presence of an acetylated, but not phosphorylated, subspecies of either X^0 or X^1. After dephosphorylation, the acetylated forms of X apparently shift into the X^1 and/or X^2 region(s), indicating that significant amounts of X containing more than two acetate groups per molecule are not present.

Discussion

The histones of *Tetrahymena* (amicronucleate strain GL) have been examined by others (Hamana and Iwai, 1971). By using three different chromatographic techniques, these investigators were able to separate five main histone groups. On the basis of amino acid composition, they established correspondence between *Tetrahymena* and calf thymus histones. However, many of their fractions, including the highly conserved H4 (DeLange et al., 1969a,b), differed significantly in composition from their calf thymus counterparts. Moreover, the gel system employed by these workers was incapable of resolving secondarily modified subfractions, products of proteolysis, or even the five main fractions clearly. Since it is our intention to examine and compare the histones of macro- and micronuclei of *Tetrahymena,* it was necessary to first purify and further characterize the macronuclear histone complement in strains which exhibit nuclear dimorphism. Like Hamana and Iwai (1971), we conclude that *Tetrahymena* macronuclear histone is composed of five main classes, separable by a combination of gel exclusion and ion-exchange chromatography, which can be correlated with the five calf thymus histones. However, correspondence with respect to solubility, electrophoretic, and chromatographic properties, as well as amino acid composition, is not always exact.

Histone H4. In many ways, *Tetrahymena* H4 strongly resembles its calf thymus counterpart (Gorovsky et al., 1973). It comigrates with calf thymus H4 on both gel electrophoretic systems. In addition, its solubility and chromatographic properties are similar. The electrophoretic microheterogeneity of macronuclear H4 is (though more extensive) like calf thymus H4 (DeLange et al., 1969a; Wangh et al., 1972), the direct result of differential acetylation. Moreover, when extensively purified, *Tetrahymena* H4 is compositionally equivalent to vertebrate H4 (Table II). Many of the deviations observed in the composition of the less pure macronuclear H4's (Table II, preparations A and B) are similar to those observed by Hamana and Iwai (1971) for H4 of strain GL and, in fact, by Franco et al. (1974) for yeast H4. Thus, it is necessary, when demonstrating compositional homology (or lack of homology) by amino acid analysis, to be assured of the utmost purity of the sample in question. It is apparent that at least between B VII H4 and calf thymus H4, the compositions have remained fairly conserved.

Histone H3. Although similar to calf thymus H3 in solubility properties and in overall composition (including the presence of cysteine), macronuclear H3 displays significant differences in several amino acid contents which may account for its higher electrophoretic mobility (Figure 1) and its behavior on Bio-Gel P-60 chromatography (Figure 2). Moreover, sodium dodecyl sulfate–gel analysis of purified B VII H3 (Figure 4) indicates that its molecular weight (13 300) may be lower than that of calf thymus H3 (15 324: DeLange et al., 1972). A smaller molecular weight could also partially explain the higher electrophoretic mobility and the faster elution from P-60 exclusion chromatography of *Tetrahymena* H3. In view of the extreme conservation of H3 electrophoretic properties among animals (Panyim et al., 1971) and of H3 structure between plants and animals (Patthy et al., 1973), the differences between

Tetrahymena and calf thymus H3 described in this report are striking.

As is the case for vertebrate H3 (DeLange et al., 1970; Allfrey, 1971; Wangh et al., 1972), B VII H3 displays differential acetylation of a parental form. However, in this lower eucaryote, the acetylation is more extensive—six subfractions are easily demonstrated as opposed to only three (or four) for calf thymus H3.

Histone H2B. Studies on vertebrate histones indicate that H2B is unique among the histones in that it does not appear to be significantly modified in most somatic tissues of these organisms, although Candido and Dixon (1972) have observed acetylated H2B in trout testis cells during spermatogenesis. However, McMaster-Kaye and Kaye (1973) were able to electrophoretically separate cricket H2B into two subfractions. They suggested that the slower H2B subfraction (comprising only about 10% of the total H2B) could be an acetylated form of H2B. In this report, we present proof that a histone, which resembles H2B compositionally, chromatographically, electrophoretically and in solubility properties, is acetylated in the macronucleus of *Tetrahymena.* Moreover, the acetylated H2B (formerly identified as Y) comprises approximately 45% of the total H2B in exponentially growing cells. This is the first unambiguous demonstration of H2B heterogeneity within a single cell type.

Histone H1. In many ways, *Tetrahymena* H1 is similar to the lysine-rich histone of higher organisms. It is soluble in 5% perchloric or trichloroacetic acid, has the highest molecular weight of any *Tetrahymena* histone (Figure 4), exhibits electrophoretic microheterogeneity caused by phosphorylation, is easily dissociated from chromatin by salt, and is highly susceptible to proteolytic degradation (Gorovsky et al., 1974). Unlike H1 in all other organisms studied to date, *Tetrahymena* H1 is not the slowest migrating histone upon low pH, urea–acrylamide gel electrophoresis (Figure 1b). In addition, a comparison of its amino acid composition with that of calf thymus H1 (Table I) indicates that *Tetrahymena* H1 differs dramatically in content of basic, acidic, and hydrophobic residues.

Histone X. Tetrahymena does not contain a histone which exhibits electrophoretic properties (Figure 1) analogous to those of H2. In spite of its low electrophoretic mobility, however, fraction X resembles H2A more than any other *Tetrahymena* histone does. X isolates like H2A (unpublished results) in the ethanol–guanidinium chloride extraction method of Johns (1967) and elutes like H2A in both exclusion chromatographic techniques described in this report. Like H2A (Panyim and Chalkley, 1969; McMaster-Kaye and Kaye, 1973), X is the only *Tetrahymena* histone whose relative mobility changes significantly as the urea concentration is increased from 2.5 to 6.25 M in the urea–acrylamide gel electrophoretic system of Panyim and Chalkley (1969). The relative mobility of X^0 changes from 78 (2.5 M urea) to 74 (6.25 M urea), while that of calf thymus H2A changes from 88 to 85. However, compositionally X is more similar to H2B, although it does share a high leucine content with H2A (Table I). Moreover, X behaves similarly to H2B in the selective extraction procedure of Johns (1964). If X is indeed *Tetrahymena*'s equivalent of H2A, then this histone appears to be much less conserved than previously thought.

Since sodium dodecyl sulfate–gel analysis (Figure 4) indicates the presence of two molecularly distinct forms of X, the possibility exists that one subspecies is H2A-like while the other is more H2B-like. That this possibility is unlikely is evident from the observation that both subspecies of X behave identically in solubility assays. In fact, both subspecies can be removed from the Johns (1964) "H2B" solubility fraction by extensive recycling, without concurrent loss of H2B. Thus, the two forms of X appear to be closely related to each other and distinct from either vertebrate H2A or H2B.

In addition to its low electrophoretic mobility, histone X displays, on urea–acrylamide gels, extensive microheterogeneity, a great deal of which can be explained by overlapping acetylation and phosphorylation. This property of being both phosphorylated and acetylated is unusual. Only the nonlysine-rich histones of trout testis cells have been shown (Candido and Dixon, 1972; Louie and Dixon, 1972) to be significantly modified by both phosphorylation and acetylation.

That fraction X consists of two molecularly distinct forms, namely XL and XS (migrating respectively in positions X^0 and X^1 on urea–acrylamide gels), is supported, not only by the polydispersity of purified X on sodium dodecyl sulfate–gel electrophoresis (Figure 4), but also by the lack of phosphorylation and acetylation in these gel peaks (Figure 6). Moreover, *Tetrahymena* micronuclear histone (see Gorovsky and Keevert, 1975a,b), which consists of only X^0, X^1, $H2B^0$, and $H4^0$, displays no secondarily modified subspecies of H2B or H4. Thus, it is likely that the two X subfractions observed in micronuclear histone (mobilities equal to X^0 and X^1) are two distinct forms of X, rather than parental and modified species. It is, therefore, possible that each macronuclear electrophoretic X subfraction, except X^0, represents multiple species of X. For example, band X^1 may consist of the molecularly distinct XS, plus some monoacetylated and/or monophosphorylated XL, band X^2 mostly of diphosphorylated XL and monophosphorylated XS but also some diacetylated XL, monoacetylated–monophosphorylated XL, and monoacetylated XS, and so on for bands X^3 and X^4. This would explain the lack of a simple progression in the relative levels of isotopic incorporation (Figure 6) in the X fractions, although this could also be caused by a lower rate of acetate turnover in X than in H4.

In summary, four of the five histones (H1, H3, H2B, H4) of the macronucleus of the lower eucaryote *Tetrahymena* are clearly homologous to four of the major histone classes found in calf thymus (and other vertebrates). However, with the exception of H4, all of these *Tetrahymena* histones differ from their calf thymus counterparts in one or more ways: composition, molecular weight, electrophoretic mobility, solubility properties, or in the nature and degree of their secondary modification. The properties observed for fraction X are sufficiently different from any calf thymus histone to make any conclusion about its homology uncertain. It is possible that the differences between *Tetrahymena* and vertebrate histones will be reflected in differences in chromatin structure and function between this unicellular organism and the highly differentiated cells of higher animals. Hopefully, additional comparative studies on the histones and on the chromatins of these and other groups of organisms will aid in elucidating the evolution and biological role of these basic proteins.

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