Studies on the Heterogeneity of Lysine-rich Histones in Dividing Cells*

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SUMMARY

The heterogeneity of the lysine-rich histone from several mammalian cells has been studied. The molecules were cleaved with N-bromosuccinimide and separated into three fractions by exclusion chromatography. The three fractions were Fraction 1, the NH₂-terminal peptide of molecular weight ~6000 which proved to be highly heterogeneous upon electrophoretic analysis; Fraction 2, the COOH-terminal fragment, which consists of two molecular weight groups (14,500 and 15,500), both of which are additionally heterogeneous as was deduced from tryptic fingerprinting and analysis of arginine-containing peptides; and Fraction 3, a protein which had the same molecular weight as the intact, unreacted lysine-rich histone. However, it did contain a spirolactone and we deduce that it is the product of an abnormal N-bromosuccinimide cleavage reaction. It shows the same type of electrophoretic heterogeneity as is seen for unreacted histone.

There are five major classes of histone in mammalian cells. Two of these histone classes, the arginine-rich histones F₁a and F₁b, have shown a remarkable stability against evolutionary changes in primary sequence (1–3). The moderately lysine-rich F₃b and F₃c histones have likewise shown a resistance to evolutionary primary sequence changes (3), although not to the same degree as the arginine-rich histones. In any given creature there is but a single type of parent Fₛ, L, F₂b, or F₁a histone molecule, although microheterogeneity may be introduced either by methylation (4) or by changing the overall change in primary sequence (14). In dividing cells an additional level of complexity is introduced by the phosphorylation of the lysine-rich histones (10), with an attendant decrease in over-all positive charge and a resultant decrease in electrophoretic mobility (16).

We have recently shown that at least part of the sequence heterogeneity arises from the presence of slight differences in molecular weight among some of the lysine-rich histones (17). We have studied the nature of lysine-rich histone heterogeneity further using the NBS1 cleavage approach developed by Cole and his coworkers (18), coupled with a highly sensitive electrophoretic analysis.

MATERIALS AND METHODS

Preparation of F₁ Histones—All histones were prepared from fresh tissue and F₁ histone was isolated by solubilization in 5.0% perchloric acid according to methods described previously (19, 20).

Chromatography—F₁ histone was fractionated by gradient elution chromatography on Amberlite CG-60. A linear gradient of sodium chloride (5% to 8%, 450 ml each) containing 0.1 M Tris buffer, pH 8.2, was used. Chromatography was performed at 4° on a column (2.5 × 40 cm) at a flow rate of 8 to 10 ml per hour maintained by a polystatic pump. This column accommodated 40 to 50 mg of F₁ histone. Protein concentration in the column effluent was monitored by measuring ultraviolet absorbance at 230 nm. Sodium chloride concentration of the effluent was determined from the refractive index.

Electrophoresis—Electrophoresis in 25 cm, 15% polyacrylamide gels was performed according to the method of Panyim and Chalkley (21) except that improved band sharpness was obtained by conducting electrophoresis at 4°. Preliminary results were at 250 volts for 24 hours. F₁ histone samples (10 to 15 μ) were applied in 0.9% acetic acid, 15% sucrose. Electrophoresis was at 250 volts.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out following the method of Panyim and Chalkley (17). N-Bromosuccinimide Cleavage—Initially the cleavage reaction was performed according to the method of Bustin et al. (18). However, improved results were obtained by conducting the reaction in 0.9% acetic acid for 3 min at 27° using 10 moles of NBS per mole of F₁ histone. The reaction was stopped by adding 10 moles of tyrosine for every mole of NBS in the original reaction mixture.

Separation of N-Bromosuccinimide Cleavage Products—A Sephadex G-100 column (5.0 × 150 cm) was utilized. NBS-treated F₁ histone (40 to 50 mg), still in the reaction mixture, was applied to the column. Elution was with 0.05 N HCl at a flow rate of 30 ml per hour. Protein concentration of the effluent was monitored by ultraviolet absorption at 230 nm.

Amino acid analysis was performed on protein hydrolysates (6 N

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The abbreviation used is: NBS, N-bromosuccinimide.
HCl at 110° for 24 hours) with a Jeolco U. S. A., Inc., amino acid analyzer (model JLC-6AI). The samples were prepared for hydrolysis by the method of Crenfield et al. (22).

Tryptic Digestion—The procedure of DeLange et al. was modified slightly (1). Histone was dissolved in water at a concentration of 4 to 5 mg per ml. The pH was adjusted to 8.2 with 0.1 n ammonium hydroxide. Trypsin, 1 mg per ml in water, pH 8.2, was added in a ratio of 1 mole of trypsin to 500 moles of histone. This amount was added at zero time and again after 15 min. The pH was continuously monitored and maintained at 8.2 with 0.1 n ammonium hydroxide. Digestion was at 37° for 90 min. On occasion, in order to check for completeness of digestion, the trypsin treatment was extended for 6 hours. The reaction was terminated by adding glacial acetic acid to a pH of 3.0. The reaction mixture was lyophilized and redissolved in pyridine-acetic acid-water, 5:5:390, to give a final concentration of 10 mg of starting protein per ml. Approximately 300 μg of the digest was applied to Whatman No. 3MM chromatographic paper. Solvent chromatography in 1-propanol-ammonium hydroxide-water, 6:3:1, was performed for 19 hours. High voltage electrophoresis on a Gilson (model D) high voltage electrophorator in pyridine-acetic acid-water, 5:5:390, pH 4.7, was carried out at 2000 volts for 1.5 hours. After electrophoresis, the chromatogram was dried at 60° and stained for arginine peptides using phenanthrenequinone solution (23). Arginine-containing peptides were located by observation under an ultraviolet light. The chromatogram was again dried and stained with ninhydrin solution. Ninhydrin spots were developed at room temperature.

RESULTS

Heterogeneity of F1 Histone

The heterogeneity of the calf thymus F1 class of histone has been described previously in electrophoretic (24) and chromatographic terms (13). There is general agreement that there are four major subfractions within this histone class. The electrophoretic analysis resolves three of these subfractions as shown in Fig. 1a and the chromatographic method (Fig. 1b) resolves three fractions together with an indication of the presence of the fourth. We have cross-identified the histone fractions from the two methods and this is also shown in Fig. 1b. The first major peak to be eluted from the ion exchange column (Fractions 2 and 3) has the same electrophoretic mobility as the last fraction (6). The band of lowest electrophoretic mobility is column Fraction 4 and, finally, column Fraction 5 has intermediate electrophoretic mobility when analyzed on high resolution polyacrylamide gels. It is clear that the fractions from the column separation are not completely pure, though the extent of cross-contamination rarely exceeds 10%. The column chromatography-electrophoresis assignments are consistent with those of Kinkade and Cole (25), except that in their electrophoretic system the slowest migrating histone is eluted from the column later than the fraction of intermediate mobility.

The electrophoretic heterogeneity is not attributable to phosphorylation of parent histone in this tissue. This was documented in experiments in which it was shown that alkaline phosphatase does not modify the electrophoretic patterns of calf F1 histone, although it has been shown previously that alkaline phosphatase can hydrolyze efficiently the histone phosphate found in the F1 histone of rapidly dividing tissues, and in so doing dramatically modifies the electrophoretic heterogeneity (10).

N-Bromosuccinimide Cleavage of Calf Thymus F1, Histone

Total F1 histone from calf thymus contains a single tyrosine residue per mole of histone and is amenable to cleavage with N-bromosuccinimide. We repeated the method of Bustin et al. (18) and analyzed the products electrophoretically. The data of Fig. 2 indicate that we obtain three fractions from this reaction; however, the bands are very indistinct and poorly defined, indicating that additional modification to the molecules had probably occurred, so that the histone fractions could no longer be adequately resolved in the gel electrophoretic system. We have therefore modified the method of Bustin et al. (18).
...treated with NBS. This time no additional cleavage was noted in the column profile of Fig. 4. The purified material was again treated with NBS as described under "Materials and Methods." The cleavage products were separated on a Sephadex G-100 column (4 × 100 cm) and eluted with 0.05 N HCl. Fraction volume was 2 ml. Both the cleavage products before separation and the separated fractions were subjected to electrophoresis on 15% polyacrylamide gels. Gels A, B, and C correspond to Sephadex G-100 chromatography Fractions A, B, and C.

FIG. 4 (right). NBS treatment of calf thymus Fraction A. Fraction A (8 mg, Gel 1 was treated with NBS following the same procedure for NBS treatment of calf thymus F1 as presented under "Materials and Methods." The NBS reaction mixture was reacted with NBS as described under "Materials and Methods." The reaction mixture was examined electrophoretically on 15% polyacrylamide gels (Gel 2). The NBS reaction products were separated on a Sephadex G-100 column (4 × 100 cm). Fraction volume was 2 ml. Small amounts of B and C fragments were found. The Fraction A from this column separation was collected and again treated with NBS. By electrophoresis on 15% polyacrylamide gels of this A fraction, it was observed that no further B and C NBS products were formed (Gel 3).

Amino acid analysis (Table I) indicated that tyrosine was absent, otherwise, the amino acid composition of Fraction A is identical with that of intact F1 histone except that the F1 molecule has a slightly higher content of arginine. Analysis of the ultraviolet spectrum (Fig. 5) of Fraction A confirmed that tyrosine was absent, however, the alkaline maximum of 310 nm which shifts to an acid maximum of 260 nm is strongly indicative of the presence of a spirolactone, the normal end product of tyrosine after NBS treatment. The F1 histone before NBS treatment shows an absorbance spectrum characteristic of tyrosine in basic and acidic solutions. There is no peak in acid solutions at 310 nm. Normally, the spirolactone is in the COOH-terminal position of the NH-terminal fragment and can be identified as such spectrally. Thus, Fraction C shows a typical spirolactone absorbance which is absent in Fraction B, which shows an ultraviolet spectrum in the 240 to 300 nm region, characteristic of phenylalanine, and exhibits essentially no spectral shifts as the pH is changed. Calculations based on the molar extinction coefficient at 310 nm of the spirolactone (ε310 = 3250 M^-1 cm^-1) contained in each molecule of Fraction C indicate that Fraction A contained 1 ± 0.1 mole of spirolactone per mole of protein (assuming a molecular weight of 21,500, see below). High resolution electrophoresis of Fraction A shows that all the subfractions of the original F1 are capable of forming such intact spirolactone derivatives and in approximately equal amounts.

Fraction B-Fraction B was isolated from NBS-treated whole F1 histone by exclusion chromatography. High resolution electrophoretic analysis revealed that this fraction is made up of two electrophoretic components which differ in mobility by 2%. An analysis of Fraction B in a sodium dodecyl sulfate...
TABLE I
Amino acid composition of F₁ and A, B, and C fragments from Ehrlich ascites tumor cells and calf thymus

F₁ histones and the NBS-cleavage products were prepared under “Materials and Methods.” The purity of the histones were analyzed by polyacrylamide gel electrophoresis. Corrections for hydrolytic loss of serine and threonine were not made. Data is presented in terms of mole per cent of total amino acid content.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>F₁</th>
<th>A</th>
<th>B</th>
<th>B₁</th>
<th>C</th>
<th>Ehrlich Ascites Tumor Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>28.1 ± 0.2</td>
<td>29.3 ± 2.2</td>
<td>36.7 ± 2.5</td>
<td>34.6</td>
<td>34.2</td>
<td>17.8 ± 1.5</td>
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<tr>
<td>Arginine</td>
<td>2.2 ± 0.1</td>
<td>1.3 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td>0.7</td>
<td>0.7</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>2.7 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>2.7</td>
<td>3.1</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.4 ± 0.2</td>
<td>5.5 ± 0.3</td>
<td>5.7 ± 0.3</td>
<td>5.3</td>
<td>6.0</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>Serine</td>
<td>6.1 ± 0.7</td>
<td>5.7 ± 0.4</td>
<td>5.0 ± 0.2</td>
<td>6.4</td>
<td>5.8</td>
<td>8.0 ± 1.1</td>
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<tr>
<td>Glutamic Acid</td>
<td>4.3 ± 0.2</td>
<td>3.9 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.7</td>
<td>2.6</td>
<td>6.6 ± 0.3</td>
</tr>
<tr>
<td>Proline</td>
<td>8.3 ± 0.2</td>
<td>8.3 ± 1.5</td>
<td>8.3 ± 0.4</td>
<td>8.0</td>
<td>8.5</td>
<td>10.5 ± 1.1</td>
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<tr>
<td>Glycine</td>
<td>7.2 ± 0.4</td>
<td>7.1 ± 0.3</td>
<td>6.2 ± 0.9</td>
<td>7.5</td>
<td>7.4</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>25.4 ± 1.2</td>
<td>25.9 ± 1.7</td>
<td>25.2 ± 0.7</td>
<td>23.0</td>
<td>23.6</td>
<td>27.0 ± 0.5</td>
</tr>
<tr>
<td>Valine</td>
<td>4.1 ± 0.4</td>
<td>4.9 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td>4.2</td>
<td>4.9</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.0</td>
<td>0.7 ± 0.1</td>
<td>0.7</td>
<td>0.7</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.2 ± 0.2</td>
<td>4.4 ± 0.1</td>
<td>3.3 ± 0.1</td>
<td>3.1</td>
<td>3.4</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6</td>
<td>0.6</td>
<td>trace</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6</td>
<td>0.6</td>
<td>trace</td>
</tr>
</tbody>
</table>

Fig. 5. Ultraviolet spectra at pH 7 and pH 12 of calf thymus F₁ and its NBS fragments A, B, and C. Spectra were recorded at pH 7 (----); at pH 12 (--.--).

The difference in molecular weight of 1,000 ± 300 is very similar to the difference in molecular weights of the intact F₁ histones reported previously (17), and confirmed in Fig. 6. The small difference in molecular weight was exploited to achieve a separation and partial purification of the B subfractions as documented in Fig. 7. Amino acid analysis indicates considerable similarity between the two B subfractions (Table I) and compares well with the data obtained by Bustin and Cole (15) for a single B fragment from one of the F₁ subfractions. Both B fractions contain a single mole of arginine and phenylalanine per mole of peptide and the positive charge density in this end of the molecule is much greater than in the NH₂-terminal end of the F₁ histone. Tryptic fingerprint analyses (Fig. 8) showed that the two B fractions are indeed very similar and that most peptides are in common, although, as expected, the larger subfraction does show two additional spots. The larger fraction, B₁, shows the presence of three major arginine-containing peptides in the tryptic fingerprint. The smaller fraction, Bₛ, contains only two major arginine peptides. Retreatment with extra trypsin and more extended incubations excluded the possibility that these spots were the result of incomplete hydrolysis of the molecule.

It is possible to identify the B fractions with their parental histone of origin by cleaving the column-purified F₁ subfractions with NBS and analyzing the products on long gels. The analytical patterns are presented in Fig. 9. We conclude that
FIG. 6 (left). Molecular weight determination of the two B fragments on SDS polyacrylamide gels. The B fragment from calf thymus F1 was isolated on a Sephadex G-100 column. This fragment was electrophoresed on SDS gels as described under "Materials and Methods." The standard histone mobility curve was taken from published data of Panyim.

FIG. 7 (center). Preparation of B\textsuperscript{B} and B\textsuperscript{S} fragments by Sephadex G-100 column chromatography. Calf thymus F1 (40 mg) was reacted with NBS as described under "Materials and Methods." The reaction mixture was applied to a Sephadex G-100 column (7 X 150 cm). Fraction volume was 5 ml. The regions indicated were collected and B\textsuperscript{B} and B\textsuperscript{S} were subjected to electrophoresis on polyacrylamide gels. The amount of cross contamination of one B fraction in the other was determined by curve resolution of microdensitometer scans.

FIG. 8 (right). Tryptic fingerprints of the B fragments from NBS-treated calf thymus F1. Fingerprinting was performed as described under "Materials and Methods." B\textsuperscript{B} and B\textsuperscript{S} were prepared as shown in Figure 7. Arginine containing peptides are indicated by cross-hatched spots. The intensity of the arginine staining spots is indicated by frequency of cross-hatching. The arrow indicates the peak present in B\textsuperscript{B} and almost absent in B\textsuperscript{S}.

Fig. 9. Location of the B\textsuperscript{B} and B\textsuperscript{S} fragment with respect to the four calf thymus IRC-50 fractions. IRC-50 fractions were prepared as described under "Materials and Methods." This fractionation is illustrated in Fig. 1b. Each of the IRC-50 fractions was treated with NBS. The cleavage products were then subjected to electrophoresis on long polyacrylamide gels. Control, NBS cleavage products from unfractionated calf thymus F1. The numbers at the top left of each scan refer to IRC-50 fractions shown in Fig. 1b. The vertical broken lines indicate that a portion of the gel photograph has been omitted.

one of the first and also the last F1 subfractions which are eluted from the ion exchange column (3 and 6) and which migrate in an identical fashion when intact, both contain the smaller (more rapidly migrating) B subfraction (B\textsuperscript{B}). The two intermediate F1 subfractions from the column (4 and 5) which have different (and slower) mobilities when intact, both contain the larger B\textsuperscript{B} fraction. Since the COOH-terminal fragments of these latter two F1 histones have identical mobilities (i.e. the apparently homogeneous B\textsuperscript{B}), the observation that the intact F1 subfractions have slightly different mobilities must reflect the effect of differing NH\textsubscript{2}-terminal fragments upon the electrophoretic characteristics of the intact molecules. This evidence in favor of the existence of two components of the electrophoretically homogeneous B\textsuperscript{B} fraction is consistent with the tryptic fingerprints shown above in which three arginine-containing peptides were obtained even though amino acid analytical data suggested that arginine was present at a concentration of only 1 mole per mole of protein. We conclude that the B\textsuperscript{B} subfraction contains two or three major peptides of identical electrophoretic mobility, of very similar molecular weight, but with some difference in the region of the single arginine residue.

Fraction C—The smallest fraction was isolated by exclusion chromatography and shown to be free from other reaction products electrophoretically. Fraction C behaves in an anomalous manner upon sodium dodecyl sulfate electrophoresis, giving rise to a series of bands of decreasing intensity as molecular weight increases as shown in Fig. 10. The mobilities of the bands are related logarithmically (Fig. 10) and we suspect that Fraction C has formed a series of aggregates which differ from...
FIG. 10. Molecular weight determination of the C fragment by sodium dodecyl sulfate gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed as described under "Materials and Methods." The number of molecules of C fragment per aggregate was based on a calculation which assumed that the most rapidly migrating band was the monomer form of the C fragment. This assumption is supported by measurement of the molecular weight of this band which indicates a value of ~6000.

FIG. 11. Electrophoretic resolution of F1 histone from Ehrlich ascites tumor cells (EATC) from calf thymus, mouse liver, and rat liver. F1 histones from these tissues were prepared as described under "Materials and Methods." Low pH-urea and sodium dodecyl sulfate electrophoresis was performed according to the procedure under "Materials and Methods." One another by 1 molecule of Fraction C. The fastest migrating band of this series has a molecular weight of 6000, a value in good agreement with that predicted by Bustin et al. (18) from amino acid analysis data. For polypeptides of this size, diffusion is considerable and it is not possible to obtain sufficient resolution to assay for molecular weight heterogeneity.

Poor resolution also prevented us from studying possible heterogeneity in our standard gel system. However Fraction C does indeed possess considerable heterogeneity in 30% polyacrylamide gels as shown below. At least four subfractions can be identified, although the range of mobilities is very small.

Heterogeneity of F1 Histone in Other Mammalian Systems—The electrophoretic patterns of calf thymus, rat liver, and mouse liver F1 histones are compared in both low pH gels and in sodium dodecyl sulfate gels in Fig. 11. Several points emerge. (a) The electrophoretic heterogeneity in these lysine-rich histones in low pH systems is highly variable. Although both calf and mouse show several bands, the spacing is not the same. Except for mouse liver which possesses additional lysine-rich histones (the F2 subfraction, which has been little studied), the mobility of the fastest F1 band is essentially the same in all cases. This was ascertained by adding an F1 internal standard 6 hours after the beginning of the electrophoresis and also by mixing experiments. (b) The electrophoretic heterogeneity of the rat liver system is much less than the other animals. (c) The mouse Ehrlich ascites tumor cells F1 histone is the most complex, reflecting an additional phosphorylation-induced heterogeneity imposed upon various subfractions of the F1 histone as documented previously (26). Calf thymus, mouse liver, and rat liver do not show detectable levels of F1 phosphorylation (8). (d) All of the F1 samples consist of differing amounts of two molecular weight subfractions as seen upon analysis in the sodium dodecyl sulfate system. The molecular weights of the two histones from calf and mouse F1 are essentially the same, but the smaller rat liver F1 is smaller than the comparable histone from the other two species. (e) That rat liver has two molecular weight species indicates that the single major band of the rat F1 in the urea system must be composed of at least two components.

A comparison of the elution profiles obtained when each F1 histone was treated with NBS as described above and separated on Sephadex G-100 is shown in Fig. 12. The profiles are in general quite similar. All samples have a typical Fraction A which proved to be resistant to further cleavage with NBS, showed a typical spiro lactone ultraviolet absorption spectrum, and had the same electrophoretic mobility as uncleaved F1. Likewise, all samples contained B and C fractions, eluting at their customary positions. However, the mouse ascites tumor is notable in that it contains a fraction which elutes slightly ahead of the main C fraction, emerging as a leading shoulder to the main C peak. We denote this Fraction C'.

**Fraction B in Calf, Mouse, and Rat**—The B fractions from the several tissues were prepared by exclusion chromatography
Fig. 13. The B fragments of NBS-treated F1 histone from calf (C. Thymus), Ehrlich ascites tumor cells (EATC), mouse (M.) liver, and rat (R.) liver resolved on low pH, urea gels, and sodium dodecyl sulfate gels. B fragments of the various tissues were prepared from the Sephadex G-100 column separation shown in Fig. 3. Electrophoresis in low pH, urea, and sodium dodecyl sulfate gels is described under "Materials and Methods."

as described above. They were analyzed on sodium dodecyl sulfate gels and in the urea system and the results are shown in Fig. 13. The B fractions in all cases consist of at least two molecular weight subfractions. The lower molecular weight subfraction in all four systems has the same mobility, but the slower migrating subfraction (sodium dodecyl sulfate gels) is somewhat smaller in rat than the other systems. This is consistent with the observation that rat F1 histone has a molecular weight component somewhat smaller than that found in the mouse or the calf. The analysis in the high resolution urea system indicates that small but significant differences are found in Fraction B, which, as previously seen for calf thymus, do not completely reflect the electrophoretic heterogeneity of the intact F1 histone. Thus rat liver intact F1 consists of 1 major and 3 minor bands, but the B fragments show the presence of 2 major and 3 minor bands, again indicating that the intact F1 from rat liver is heterogeneous. The B fragments from the mouse tumor and mouse liver are quite different. The mouse liver Fraction B has a faster migrating component than the tumor Fraction B; however, as is discussed in an accompanying paper, the phosphorylation of the mouse tumor F1 occurs within the B region and this will serve to reduce the electrophoretic mobility of the tumor bands relative to those from the liver. The B fractions show the presence of additional minor fractions not observed previously in the calf system. The assignments of small differences in mobility were made by mixing various samples and subjecting these materials to co-electrophoresis.

Fraction C from Calf Thymus and Mouse Ascites Tumor—Sodium dodecyl sulfate electrophoresis is in general not satisfactory for analyzing the low molecular weight C fractions. One can do little more than estimate that the molecular weight of the unusual C' fraction obtained from the mouse tumor is significantly higher than the rest of the C fraction.

Analysis on 30% polyacrylamide gels in urea is more revealing, and, as is shown in Fig. 14, significant differences in mobility are detected between the various C fractions from the different systems; C' is seen to move more slowly than the bulk of the C fractions. The gels reveal that calf thymus may perhaps also contain a small quantity of C'. In all cases the C fraction is heterogeneous and imperfectly resolved.

The mobility variations among the C fractions are reflected in the amino acid analyses of these materials (Table I). The differences are especially pronounced in the C' fraction which shows a much higher content of basic amino acids, serine, and glutamic acid and a dramatically decreased amount of alanine.

Fig. 14. Electrophoretic resolution of C fragments on 30% polyacrylamide gels. EATC, Ehrlich ascites tumor cells.

**DISCUSSION**

The cleavage by NBS of the F1 histone complement from several sources has been studied using electrophoretic techniques. The cleavage reaction can be conducted under much more gentle conditions than those in common use. These gentler reaction conditions preserved the integrity of the products as was evidenced by the high resolution of the products upon electrophoresis. Three products are obtained after complete reaction. These are the expected NH2-terminal and COOH-terminal peptides together with a product which has a molecular weight and electrophoretic mobility identical with that of unreacted F1 histone; however, it is resistant to further cleavage with NBS. Thus, we conclude that tyrosine has reacted with NBS in the expected fashion, but the subsequent cleavage was abnormal. Several possible reaction schemes are shown in Table II. Scheme I is the normal cleavage reaction; Scheme II is abnormal in that attack at the positive charge center (produced by NBS) is through the NH2-proximal carboxyl group. In general, the involvement of such a 6-membered ring intermediate has been ruled out by work in model systems. However, if Scheme II were involved, we would obtain a molecule of the same molecular weight as the original F1 histone, but it would contain an ester linkage. Attempts to cleave such an ester bond with dilute alkali or hydroxylamine were unsuccessful and we feel that Scheme II is improbable. The COOH-proximal amino acid to the tyrosine residue is aspartic acid (asparagine) (14) and Schemes III and IV detail a possible involvement of such an amino acid in generating the NBS cleavage-resistant Fraction A that we have described. These latter two reaction schemes seem plausible, but the problem has not been investigated further.

The major products of the NBS cleavage of F1 histone as described by Cole and his co-workers (18), are a small NH2-terminal fragment (Fraction C) and a large COOH-terminal fragment (Fraction B). The NH2-terminal fragment has a molecular weight of approximately 6000; however, in the sodium dodecyl sulfate electrophoretic system, aggregation occurs and this makes a precise determination difficult. This value is in good agreement with that calculated by Rall and Cole from amino acid composition (14). This fraction is heterogeneous
**TABLE II**

Possible schemes for N-bromosuccinimide cleavage of histone $F_1$

Upon electrophoresis in 30% polyacrylamide gels, presumably reflecting the types of variation described by Cole in the sequence analyses of one $F_1$ histone fraction from calf thymus and two such fractions from rabbit thymus. A very unusual variant (C' fragment) of this NH$_2$-terminal fraction is found in Ehrlich ascites tumor cells. Not only is the molecular weight of this fragment substantially higher than the normal range found for the C fragment, but the amino acid composition is also quite different from either normal C or B fractions. In fact, the C' fragment does not strongly resemble either of the NH$_2$- or COOH-terminal fragments electrophoretically. As will be described in a subsequent paper, this fragment is phosphorylated in vivo, an observation highly characteristic of the lysine-rich group of histones. This unusual C fraction is not tumor-specific, as other tumor lines have been analyzed similarly and do not show the presence of this fraction.

The COOH-terminal fragments of NBS cleaved $F_1$ histones from several mammalian systems presented in many ways the most interesting results, the more so because it had initially been proposed that very little variation occurred in this end of the molecule (14). However, it is clear that in all of the systems we have studied, the origin of the molecular weight variation of the intact $F_1$ histone lies in the COOH-terminal part of the molecule. Electrophoretic separation in the low pH urea system is a function of both molecular weight and charge density, and it is apparent that the different molecular weight fragments of the COOH-terminal fraction must have very similar charge densities, as we find the COOH-terminal fragments of at least four $F_1$ histones in calf thymus distributed into only two electrophoretic groups. In general, this observation appears to hold for cows, rats, and mice, although it breaks down in rapidly dividing tissues where an additional heterogeneity due to phosphate charge density variation is observed. That each of the two molecular weight groups of Fraction B, B$^A$ and B$^B$, are heterogeneous as expected is shown by trypic digestion and fingerprint analysis of the purified B fractions. Although B$^A$ and B$^B$ contain only a single mole of arginine per mole of peptide, the fingerprint analysis showed three major arginine-containing spots in material from B$^A$ and two arginine-containing peptides in B$^B$. We have excluded incomplete trypic digestion and we suspect that each of the B subfractions is heterogeneous (as expected in view of four parent molecules), and furthermore, that at least part of the heterogeneity is located in the region of an arginine residue. This could be obtained in two ways: (a) the arginine residue is in the same position in a given position in a given B subfraction but a small change in its immediate vicinity gives rise to a heterogeneity of peptides or (b) an arginine could have been replaced by a lysine residue and a different lysine replaced by an arginine residue at another site. Both methods would not change the over-all charge density on the peptide and this would not affect the electrophoretic homogeneity. However, the first proposal perhaps has more merit because of its simplicity.

The lysine-rich histone group provides the exception to the

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general rule that histone primary sequence is highly conserved throughout evolution. Not only have the sequences changed from creature to creature, but there is clear evidence of sequence microheterogeneity within the lysine-rich histone group itself. However there are extensive sequence homologies within the F1 histones from a given animal as is shown by the identity of most of the tryptic peptides of both the small and the large B peptides. Although there may be certain hot spots for mutational change, it is also apparent that substantial portions of the molecule are changing much more slowly during evolutionary time. It may be deduced, because almost all animals examined have multiple F1 fractions and because the pea plant has three F1 histones which differ in molecular weight, that several closely related F1 genes may have arisen from a parental F1 gene at an early point in evolutionary history and that subsequently changes and variation at certain points were permitted (even changes in molecular weight); however, much of the molecule (in particular the COOH-terminal half) maintained a constancy of sequence more reminiscent of the typical histone behavior. It seems unavoidable but that this behavior must reflect the function of the various parts of the molecule which may conceivably involve cross-linking of nucleohistone strands.

Thus, in conclusion, we have achieved two aims: (a) extended the notion that the F1-histone fraction (in contrast to the other histone classes) is highly complex and variable even in closely related animals and (b) developed an electrophoretic system which permitted us to analyze the localization of in vivo phosphorylation within the B or C fragments of the F1 histone.

REFERENCES
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