Release of Free F1 Histone During Nuclease Digestion of Rat Liver Chromatin†

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ABSTRACT: During the extensive digestion of rat liver chromatin with DNase I probably most of the F1 histone is released in a free form and binds to other protein–DNA complexes. The bound F1 histone can be selectively removed by 0.3 M NaCl. When an excess of free histones is mixed with chromatin, the extra histones bound to chromatin are in an amount roughly equal to that of DNA in chromatin. Most of the extra F1 histone can be selectively removed from the chromatin with 0.3 M NaCl. Rat liver chromatin appears to have 10% of the total DNA as free DNA zones available for tight binding of extra F1 histone which is resistant to extraction by 0.3 M NaCl.

Chromatin, isolated from eukaryotic cells, consists of DNA, histones, nonhistone proteins, and a small amount of RNA (Bonner *et al.*, 1968). It is not clear how the chromosomal proteins are arranged on DNA and what the exact role of these proteins is in the function of chromatin (DeLange and Smith, 1971; Elgin *et al.*, 1971; Hnilica, 1972). Therefore, the identification of the sites on DNA to which various types of chromosomal proteins are bound may help in understanding the role of the proteins. Recently Clark and Felsenfeld (1971) have reported that when chromatin is treated with nucleases, about 50% of the chromosomal DNA is digested and the remainder precipitates in the form of DNA–protein complexes. Mirsky and his group reported that although the degree of digestion of chromosomal DNA by nuclease is quite variable depending of the reaction conditions and chromatin preparations (Mirsky, 1971), among histone fractions the lysine-rich (F1 or I) histone most effectively protects DNA against DNase (Mirsky and Silverman, 1972; Mirsky *et al.*, 1972). If the insoluble products of the nuclease digestion of chromatin are indeed protein–DNA complexes, at least the complex of F1 histone and DNA might be specifically dissociated into a soluble form in the presence of 0.5–0.6 M NaCl as in the case of the selective dissociation of F1 histone from chromatin in the same salt concentrations (Ohlenbusch *et al.*, 1967; Fambrough and Bonner, 1968). The dissociation of F1 histone and DNA would offer an opportunity to study the DNA fragments originally associated with F1 histone in chromatin. However, it was found that F1 histone was quantitatively extracted by 0.3 M NaCl from the DNase-treated rat liver chromatin, and the 0.3 M NaCl extract contained a substantial amount of DNA as well as a small amount of other histones and nonhistone proteins. Therefore, it was of interest to study the possibility of the extraction of the F1 histone–DNA complex by 0.3 M NaCl or the selective dissociation of F1 histone and the DNA fragments originally associated with the histone in the presence of 0.3 M NaCl. This report, however, presents evidence that F1 histone is most likely released in a free form and binds to other protein–DNA complexes during the DNase digestion of chromatin.

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Materials and Methods

Materials. Male Sprague-Dawley rats, 200–250 g body weight, were used for the isolation of chromatin from liver. Electrophoretically pure DNase I and *Escherichia coli* alkaline phosphatase were purchased from Worthington Biochemical Corp., Freehold, N. J. Bio-Rex 70 and DNA-grade hydroxylapatite were from Bio-Rad Laboratories, Richmond, Calif.

Bio-Rex 70 was washed with 1 N HCl, water, 1 N NaOH, and water and suspended in an appropriate buffer. The suspension was titrated to the pH of the buffer used for chromatography. Calf thymus histones enriched by either F1, F2b, and F2a2 or F3, F2a1, and F2a2 were prepared as described before (Chae *et al.*, 1972).

Preparation of Chromatin. Chromatin was prepared from pure rat liver nuclei (Fisher *et al.*, 1963) by successive washings in the following solutions: 1% Triton X-100 containing 0.25 M sucrose, 3 mM $MgCl_2$, and 10 mM phosphate (pH 5.8), 0.075 M NaCl–0.024 M EDTA (pH 7.0), and 0.3 M NaCl and 5 mM phosphate (pH 6.5). The details are described in an earlier report (Smith and Chae, 1973). The final chromatin was suspended in cold water at a concentration of 1 mg of DNA/ml and sheared in a VirTis homogenizer at 55 V for 90 sec. This chromatin preparation contained 0.5 mg of nonhistone proteins, 1.1 mg of histones/mg of DNA, and all principal histone fractions.

Digestion of Chromatin by DNase I. Chromatin was treated with DNase I under conditions very similar to those described by Clark and Felsenfeld (1971) as follows. The chromatin was adjusted to a concentration of 250 μg/ml in 5 mM sodium phosphate (pH 6.5) and 1 mM $MgCl_2$, and the reaction was started by adding freshly dissolved DNase I at a concentration of 4 μg/ml. The mixture was stirred for 60 min at 25°. After the reaction the insoluble product was recovered by centrifugation at 30,000*g* for 15 min and washed three times with cold 1 mM EDTA–5 mM sodium phosphate (pH 6.5), each time centrifuging at 30,000*g* for 20 min. The final washed material was used in this study.

Figure 1 shows the amount of acid-soluble nucleotides released from rat liver chromatin during the digestion with DNase I at concentration of 2 and 4 μg/ml at 25°. At the end of the 120-min reaction, about 60 and 45% of chromatin DNA was released into nucleotide form at 4 and 2 μg/ml concentrations of DNase I, respectively. At the DNase I concentration of 4 μg/ml, about 45% of total chromatin DNA was hydrolyzed at 60 min. At this time about the same

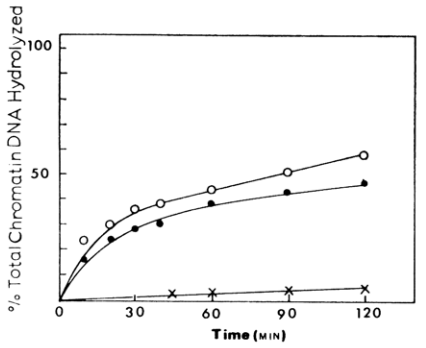

FIGURE 1: Release of acid-soluble nucleotides from rat liver chromatin during the digestion with DNase I. Rat liver chromatin was treated with DNase I at the concentrations of 0 (×), 2 (○), and 4 (●) μg/ml as described in Materials and Methods and aliquots were taken out at the time intervals indicated and mixed with an equal volume of cold 1 N $HClO_4$. After 5 min in ice the samples were centrifuged and the supernatants were read at 260 nm. Each value indicates the per cent of the optical density obtained from the nontreated chromatin hydrolyzed in 0.5 N $HClO_4$ at 90° for 30 min.

amount of DNA was found in the supernatant after the centrifugation of the insoluble reaction product in the absence of acid. No significant degradation of DNA was observed when chromatin alone was incubated under the same reaction condition.

Polyacrylamide Gel Electrophoresis. Acid–Urea Gel System. Histones extracted with cold 0.4 N H_2SO_4 were dialyzed against 7 M urea–0.9 M acetic acid–0.1% β-mercaptoethanol and applied on 0.5 × 10 cm polyacrylamide (15%) gels in 2.5 M urea prepared as described by Panyim and Chalkley (1969). Electrophoresis was carried out for 3–4 hr at 150 V in 0.9 N acetic acid. The gels were stained with Amido Black and destained electrophoretically in 10% ethanol and 10% acetic acid. The gels were scanned at 600 nm in a Gilford spectrophotometer equipped with a gel-scanning device, and the areas under F1 and F2a1 histone peaks were determined with the aid of a planimeter.

Sodium Dodecyl Sulfate Gel Electrophoresis. Free proteins or the protein samples containing DNA were dissociated in 1% sodium dodecyl sulfate–0.01 M sodium phosphate (pH 7.0)–0.1% β-mercaptoethanol overnight at room temperature and applied on sodium dodecyl sulfate polyacrylamide (10%) gels prepared as described by Weber and Osborn (1969) with a stacking gel (Waehneldt and Mandel, 1970). Electrophoresis was carried out in 0.1% sodium dodecyl sulfate–0.1 M sodium phosphate (pH 7.2) as described before (Smith and Chae, 1973). The DNA in the protein samples does not interfere with the separation of proteins (Smith and Chae, 1973). Proteins were stained with Coomassie Blue as described by Fairbanks *et al.* (1971).

Chemical Determinations. Protein and DNA were determined by the method of Lowry *et al.* (1951) and Burton's diphenylamine reaction (Burton, 1956), respectively. Bovine serum albumin and calf thymus DNA were used as standards.

Results

Selective Extraction of F1 Histone with 0.3 M NaCl from the DNase-Treated Chromatin. As shown by Mirsky (1971), the degradation of chromatin DNA was mainly due to the exogenous DNase I rather than any chromatin-bound nuclease (Figure 1). When the insoluble product resulting from the digestion of rat liver chromatin by DNase I was extracted

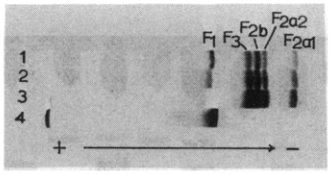

FIGURE 2: Acid–urea gel electrophoresis of histones. From top to bottom: histones isolated from (1) nontreated chromatin, (2) DNase-treated chromatin, (3) the pellet after the extraction of the DNase-treated chromatin with 0.3 M NaCl, and (4) the 0.3 M NaCl extract. Histones are indicated by the nomenclature of Johns (1964) and Phillips and Johns (1965).

with varying concentrations of NaCl (0.15, 0.2, 0.3, and 0.45 M), it was found that 0.3 M NaCl selectively removes F1 histone from the insoluble DNase-treated chromatin (Figure 2). Although all of the histone components remain in the insoluble DNase-treated chromatin a small amount of F1 histone was found in the supernatant after the reaction (not shown here), and it was also found in other experiments that no histone was degraded by DNase I or during incubation of chromatin alone in 5 mM phosphate (pH 6.5)–1 mM $MgCl_2$ for 1 hr.

F1 histone is selectively removed by 0.3 M NaCl only from the insoluble DNase-treated chromatin but not from untreated chromatin. Moreover the chromatin used in this study was washed with 0.3 M NaCl during the isolation, and no F1 histone is dissociated from DNA until concentration of NaCl reaches 0.5–0.6 M (Ohlenbusch *et al.*, 1967). Treatment of the insoluble DNase-treated chromatin with 0.15–0.2 M NaCl does not extract F1 histone but NaCl at a concentration of 0.45 M extracts considerable amounts of all types of histones and DNA from the DNase-treated chromatin.

Figure 2 shows that the 0.3 M NaCl extract contains mainly F1 histone but there is material at the top of the acid–urea gel suggesting that there is other protein in addition to F1 histone. Sodium dodecyl sulfate gel electrophoresis of the 0.3 M NaCl extract shows that in addition to F1 histone there are other histone fractions and nonhistone proteins. However, F1 histone seems to be the main protein component (Figure 3).

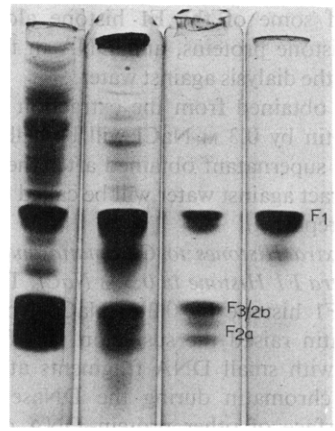

FIGURE 3: Sodium dodecyl sulfate gel electrophoresis of the proteins in the 0.3 M NaCl extract. From left to right: whole rat liver chromatin, the 0.3 M NaCl extract, the proteins in the precipitate, and the supernatant after the dialysis of the 0.3 M NaCl extract against water. The histone bands in the whole chromatin sample were identified by coelectrophoresis of known histone fractions in a separate experiment.

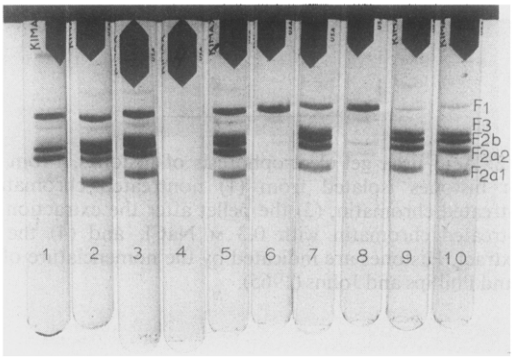

FIGURE 4: Binding of extra calf thymus histones to rat liver chromatin. From left to right: (1) calf thymus histone containing F_1, F2b, and F2a2 fractions; (2) histones in rat liver chromatin; (3) histones after binding of the calf thymus histones to rat liver chromatin; 0.15 M NaCl supernatant (4) and pellet (5); 0.3 M NaCl supernatant (6) and pellet (7); 0.45 M NaCl supernatant (8) and pellet (9); native chromatin treated with 0.45 M NaCl, pellet (10). Rat liver chromatin (1 mg of DNA) in 1 ml of 5 mM sodium phosphate (pH 6.5) was mixed with 2 mg of calf thymus histones in 1 ml of 5 mM phosphate and stirred vigorously on a Vortex mixer. After 10 min standing in ice the resulting precipitate was washed with 5 mM phosphate three times. The final washed precipitate was extracted with NaCl of different concentrations and centrifuged at 20,000*g* for 15 min. Histones were extracted from the pellets with 0.4 N H_2SO_4 as described in Materials and Methods.

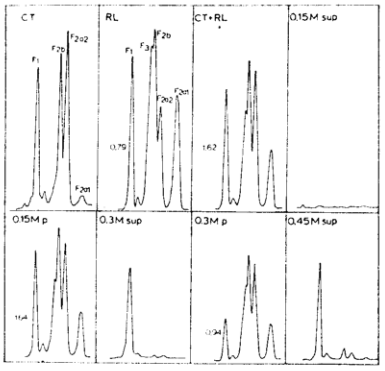

FIGURE 5: Densitometric tracings of the gels shown in Figure 4: CT, calf thymus histones; RL, histones from rat liver chromatin; CT + RL, histones after the binding of calf thymus histones to rat liver chromatin; 0.15 M sup, 0.15 M NaCl supernatant; 0.15 M p, pellet chromatin obtained after the extraction with 0.15 M NaCl; other gels are indicated likewise. The numbers on the left side of F1 histone peak indicate the ratios of F1 histone/F2a1 histone.

The 0.3 M NaCl fraction also contains a significant amount of DNA and the protein/DNA ratio ranged between 4 and 5. When the 0.3 M NaCl fraction was dialyzed against water, a precipitate was formed and the supernatant usually contained protein and DNA in the ratio of 10. The sodium dodecyl sulfate gel electrophoresis showed that the supernatant contained mainly F1 histone, and the precipitate contained some F1 histone, other histone fractions and nonhistone proteins (Figure 3). Since the supernatant contained mainly F1 histone and DNA there was the possibility that the F1 histone and DNA were originally associated together in chromatin and extracted by 0.3 M NaCl after the nuclease digestion of chromatin. Therefore, it was of interest to study (a) the possibility that the F1 histone and DNA in the supernatant are present as a complex form, (b) the possible mechanism of the quantitative extraction of F1 histone by 0.3 M NaCl from the insoluble DNase-treated chromatin, and (c) the mechanism of the precipitation of some of the F1 histone along with other histones, nonhistone proteins, and DNA in the 0.3 M NaCl fraction during the dialysis against water.

The fraction obtained from the extraction of the DNase-treated chromatin by 0.3 M NaCl will be called 0.3 M NaCl extract and the supernatant obtained after the dialysis of the 0.3 M NaCl extract against water will be called the supernatant fraction in this report.

Binding of Extra Histones to Chromatin and Selective Dissociation of Extra F1 Histone in 0.3 M NaCl. The quantitative extraction of F1 histone by 0.3 M NaCl from the DNase-treated chromatin raised the suspicion that free F1 histone or F1 histone with small DNA fragments attached may be released from chromatin during the DNase digestion and bind to the surface of other protein–DNA complexes, and these may be selectively dissolved in 0.3 M NaCl. To test this possibility a large excess of a calf thymus histone preparation, containing very small amounts of F2a1 and F3 histone, was mixed with rat liver chromatin. After washing the insoluble complex resulting from the binding of extra histones to the chromatin, it was extracted with 0.15, 0.3, and 0.45 M NaCl solutions, and the dissociated histones and the histones which remained bound to chromatin were analyzed by acid–urea gel electrophoresis. Figure 4 shows the electrophoretic patterns of the histones and Figure 5 shows the densitometric tracings of the gels. The numbers shown on the left side of F1 histone peaks in Figure 5 are the ratios of F1 histone/F2a1 histone. It is clear from Figure 5 that the heights of F1, F2b, and F2a2 histone peaks increased considerably in the chromatin mixed with extra histones and the ratio of F1/F2a1 increased from 0.79 in rat liver chromatin to a value of 1.62 after the binding of the extra calf thymus histones (CT + RL in Figure 5). The histone/DNA ratio of the chromatin was increased from 1.1 to 1.9. When this chromatin with extra histones was treated with 0.15 M NaCl, no histone was dissociated but 0.3 M NaCl selectively removed F1 histone (0.3 M sup in Figure 5). The F1/F2a1 ratio decreased in the insoluble pellet from 1.62 to a value of 0.94 (0.3 M p in Figure 5). The other extra histones, *viz.*, F2b and F2a2, were not dissociated even in 0.45 M NaCl, but at this concentration a considerable amount of the chromatin F1 histone was dissociated as seen from the weak intensity of F1 histone band in gel No. 9 and 10 in Figure 4. In the other binding experiments with mixtures of F3, F2a2, and F2a1, it was found that no significant amount of chromatin F1 histone was dissociated by 0.3 M NaCl, suggesting that chromatin F1 histone is not displaced by the binding of extra histones.

The mechanism of the binding of extra histones to chromatin is not clearly known. Phillips (1968) proposed that chromatin contains a considerable number of negative charges due to the carboxyl groups of acidic amino acids in histones and most of the extra histones bind to the carboxyl groups. Supporting this view he found that extra F1 histone is removed by 1 mM HCl and histones F2a and F2b by 3.3 mM HCl. The pH of 1 and 3.3 mM HCl is close to the pK value of the carboxyl group. The chromatin histones are not removed from chromatin until the concentration of HCl reaches 0.01 M. Extra histones may also bind to the surface of acidic nonhistone proteins in chromatin. Direct evidence on the binding of extra histones to the surface of histone–DNA complexes was obtained with reconstitutes of histones and double- and single-stranded DNA. For example, a soluble complex of heat-denatured calf thymus DNA and calf thymus F1 histone having a F1/DNA ratio of 0.85 was prepared by salt gradient dialysis in the presence of urea as described before (Chae *et al.*, 1972) and mixed with an excess of a histone mixture

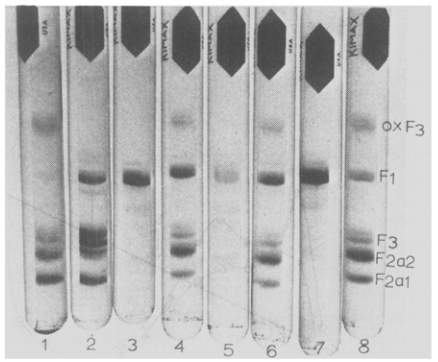

FIGURE 6: Binding of extra histones to a complex of F1 histone and single-stranded DNA. From left to right: (1) a mixture of calf thymus histones lacking F1 histone, (2) total rat liver histones, (3) F1 histone in the F1–DNA complex, (4) histones after adding the mixtures of histones to the F1–DNA complex and washing, (5) supernatant after extraction of the F1–DNA complex and bound histones with 0.3 M NaCl, (6) 0.3 M NaCl pellet, (7) supernatant after extraction with 0.45 M NaCl, and (8) 0.45 M NaCl pellet. A mixture of histones lacking F1 histone in 1 ml of 5 mM phosphate (pH 6.5) (1160 μg) was mixed with 1 ml of F1 histone–DNA complex containing 504 μg of F1 histone and 627 μg of heat-denatured calf thymus DNA, and the resulting precipitate was washed three times with 5 mM phosphate. The washed complex was extracted with 2 ml of 0.3 M NaCl and 0.45 M NaCl and histones were extracted with 0.4 N H_2SO_4 from the pellet. The F1 histone–DNA complex was prepared by salt-gradient dialysis in the presence of urea as described before (Chae *et al.*, 1972).

TABLE I: Removal of Extra Calf Thymus [^{32}P]F1 Histone Bound to Rat Liver Chromatin by 0.3 M NaCl.[a]

No. of Extractions	^{32}P Bound to Chromatin (cpm)	[^{32}P]F1 Bound (mg/mg of Chromatin DNA)
0	62,120	0.99
1	10,100	0.16
2	6,230	0.10
3	5,650	0.09
4	5,600	0.09

[a] Calf thymus F1 histone was phosphorylated in the presence of [^{32}P]-γ-ATP and 3′,5′-cyclic AMP-dependent protein kinase as described before (Chae *et al.*, 1972). The specific radioactivity of the histone was 1250 cpm/μg. About 200 μg of labeled F1 histone in 1 ml of 5 mM phosphate (pH 6.5) was mixed with rat liver chromatin containing 50 μg of DNA in 1 ml of 5 mM phosphate (pH 6.5) and stirred vigorously for 1 min. After standing in ice for 10 min the precipitate was washed with 5 mM phosphate three times each time centrifuging at 10,000g for 10 min. Duplicate pellet samples (total 10 samples), which were extracted 0, 1, 2, 3, and 4 times with 0.3 M NaCl, were dissolved in 1 ml of Hyamine hydroxide and a portion was counted in a scintillation spectrometer. Each value is the average of duplicate samples. The ^{32}P bound to chromatin was still in F1 histone.

containing F3, F2b, F2a2, and F2a1 but no F1. The histone/DNA ratio was increased from 0.85 to 1.9 after washing the excess histones from the insoluble complex. As shown in Figure 6, 0.3 M NaCl does not remove F1 histone and any extra histones from the complex. A considerable amount of F1 histone, however, was removed from the complex in 0.45 M NaCl as in the case of chromatin (see Figure 4), and it is possible that F1 histone was dissociated from DNA in 0.45 M NaCl and the extra histones originally associated on the surface of F1 histone–DNA complex now were bound to the DNA in place of F1 histone. F1 histone has a weaker binding affinity for DNA among histone fractions (Ohlenbusch *et al.*, 1967). The amount of F1 histone and other histone fractions required for the titration of DNA phosphates are about equal to the DNA in mass (Olins and Olins, 1971; Shih and Bonner, 1970), and it is very unlikely that most of the histones added to the complex of F1 histone and DNA having a F1/DNA ratio of 0.85 were directly associated with DNA.

In an experiment with [^{32}P]F1 histone, which was phosphorylated in the presence of 3′,5′-cyclic AMP-dependent protein kinase and γ-[^{32}P]ATP (Chae *et al.*, 1972), it was found that the maximum amount of [^{32}P]F1 histone binding to rat liver chromatin was roughly equal to the amount of DNA in chromatin as in the case of the binding of mixtures of histone fractions to chromatin (Figure 4) and about 10% of the bound [^{32}P]F1 histone was resistant to the repeated extraction with 0.3 M NaCl as shown in Table I. The amount of the tightly bound [^{32}P]F1 histone could represent the histone directly bound to free DNA in chromatin. If this is true, then the amount of DNA available for the binding of extra F1 histone could be about 10% of the total chromatin DNA and this is in a similar range as the amount of the DNA available for transcription in rat and mouse liver chromatin (Tan and Miyagi, 1970; Grouse *et al.*, 1972).

The model experiments on the binding of extra histones to proteins of chromatin or of nucleohistone support the possibility that the quantitative extraction of F1 histone from the DNase-treated chromtain by 0.3 M NaCl is due to release of free F1 histone or F1 histone with small fragments of DNA and subsequent binding to other protein–DNA complexes during the nuclease digestion of chromatin. The precipitation of some of the F1 histone along with other histones, non-histone proteins, and DNA in 0.3 M NaCl extract during the dialysis against water (see Figure 3) may also be due to the binding of F1 histone to complexes of DNA and non-histone proteins and other histone fractions in 0.3 M NaCl extract at a low ionic strength.

F1 Histone and DNA in the Supernatant Fraction. As described earlier the supernatant fraction, which was obtained after the dialysis of 0.3 M NaCl extract against water, contained protein and DNA in the ratio of 10, and sodium dodecyl sulfate gel electrophoresis showed that F1 histone was the major protein component in the supernatant fraction (Figure 3). Therefore, there was the possibility that demonstration of the F1 histone and DNA being present as a complex form in the supernatant fraction gives credence to the idea that the DNA might have been the binding sites of F1 histone in chromatin. Since the DNA and F1 histone in the supernatant fraction were originally extracted from the DNase-treated chromatin with 0.3 M NaCl the possibility of the DNA and F1 histone in the supernatant fraction being present as a complex form was investigated in the presence of 0.3 M NaCl. An initial attempt to study chromatographic behavior of the DNA and F1 histone during gel filtration in the presence of 0.3 M NaCl was complicated by the formation of a precipitate when the sample was concentrated to a volume suitable for the chromatography. However, it was possible to study chromatographic behavior of the DNA and F1 histone during ion-exchange column chromatography.

It has been reported that histones bind to a weak cation exchanger, Bio-Rex 70, in the presence of 0.4–0.5 M NaCl and

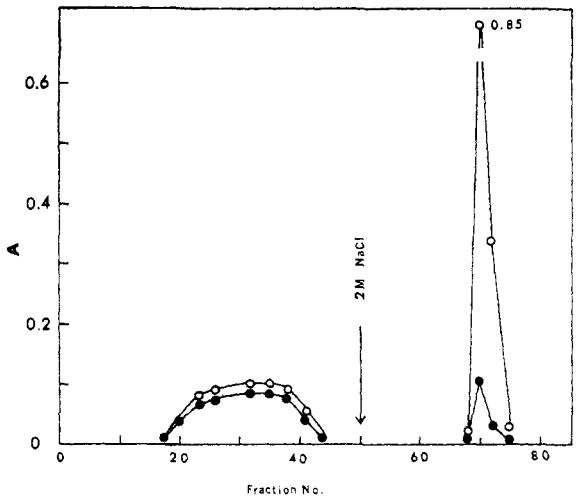

FIGURE 7: Bio-Rex 70 column chromatography. The supernatant fraction was dialyzed against 0.3 M NaCl–10 mM Tris (pH 7) and applied to a 1 × 20 cm Bio-Rex 70 column equilibrated with the same buffer. After all the run-off fractions were collected the column was eluted with 2 M NaCl–10 mM Tris (pH 7). The fractions were read at 230 nm (○) and 260 nm (●). The sample applied to the column had absorbance of 0.295 at 230 nm and 0.121 at 260 nm. The pooled run-off fraction had a protein/DNA ratio of 3.5 and the fraction eluted by 2 M NaCl was negative for diphenylamine reaction (Burton, 1956).

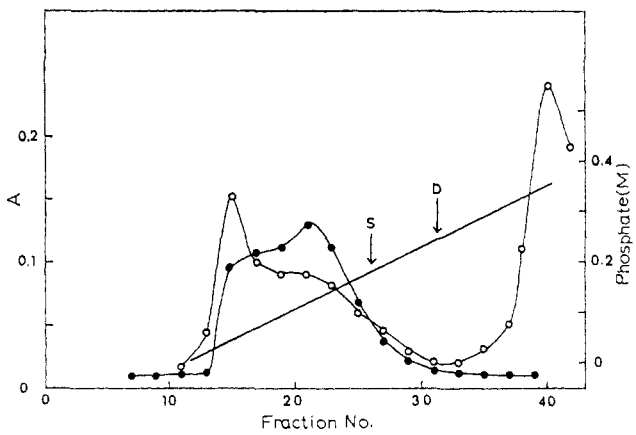

FIGURE 8: Hydroxylapatite column chromatography. The supernatant fraction in water was applied to a 1 × 9 cm hydroxylapatite column which was equilibrated with 1 mM sodium phosphate (pH 6.8) and the column was eluted with a gradient of 0.01–0.4 M sodium phosphate (pH 6.8) (total 100 ml). Absorbance read at 230 nm (○) and 260 nm (●). Phosphate (–) was determined by reading the absorbance at 400 nm of the color developed after mixing with ammonium molybdate (Boltz and Mellon, 1948). The sample (12.5 ml) applied to the column had absorbance of 0.41 at 230 nm and 0.20 at 260 nm. The positions for the elution of single- and double-stranded calf thymus DNA are indicated by S and D, respectively.

are eluted by higher concentrations of NaCl, 1.5–2 M (Balhorn *et al.*, 1971; Van den Broek *et al.*, 1973). When the supernatant fraction was applied to a Bio-Rex 70 column in the presence of 0.3 M NaCl–10 mM Tris (pH 7) quantitatively all the DNA in the supernatant fraction was found in the run-off fraction with a considerable amount of protein, and there also was protein which was tightly bound to the column and eluted by 2 M NaCl–10 mM Tris (pH 7) (Figure 7) but not by 1 M NaCl–10 mM Tris. The protein/DNA ratio of the pooled run-off fraction was 3.5 suggesting that about 35% of the protein in the supernatant fraction does not bind to the column. Sodium dodecyl sulfate polyacrylamide gel electrophoresis shows no protein band in the run-off fraction (pooled and lyophilized) and only F1 histone in the fraction eluted by 2 M NaCl–10 mM Tris (pH 7). This type of experiment has been repeated several times with the same result. It is possible that the protein in the run-off fraction is of a high-molecular weight (>130,000) which does not migrate into the 10% polyacrylamide gel used in this study. If the DNA and F1 histone in the supernatant fraction are present as a complex in 0.3 M NaCl it is expected that the DNA and F1 histone would be either in the run-off fraction or bind to Bio-Rex 70 depending on overall charges of the complex. However, the result of the Bio-Rex 70 chromatography strongly suggests that the F1 histone and the DNA in the supernatant fraction are not present as a complex form but the DNA could be bound to the nonhistone protein in the supernatant fraction. The same conclusion was also obtained from hydroxylapatite chromatography of the supernatant fraction (Figure 8): the supernatant fraction in water was applied to a column of hydroxylapatite and the column was eluted with a gradient of 0.01–0.4 M sodium phosphate (pH 6.8) (Figure 8). All the DNA in the supernatant fraction was eluted by 0.1 M phosphate but F1 histone was eluted by phosphate of 0.35–0.4 M. Nonhistone protein was eluted very closely with DNA and again the nonhistone protein showed no visible protein band in sodium dodecyl sulfate gel. The results from the hydroxylapatite and Bio-Rex 70 column chromatography seem to suggest that most of the F1 histone is freed from DNA during the long-term digestion of chromatin with DNase I.

The fact that the DNA in the supernatant fraction is eluted from hydroxylapatite by a phosphate concentration lower than that necessary for the elution of single-stranded calf thymus DNA suggests that the DNA may be oligonucleotides. In a separate experiment the amount of total phosphate in the DNA and the amount of terminal phosphate released from the DNA by *E. coli* alkaline phosphatase (Garen and Levinthal, 1960) were compared and it was found that the DNA in the supernatant fraction has an average length of 30 nucleotides (not shown here). The DNA has a base composition identical with that of whole chromatin DNA (G + C = 42%, A = T and G = C) thus discounting the possibility of the DNA being of any special class.

Discussion

Recently Clark and Felsenfeld (1972) reported the isolation of complexes of DNA and mixtures of F2a1 and F3 histones from calf thymus chromatin, and they found that this DNA is rich in guanine and cytosine and contains 40–50 base pairs. They first removed all other histones and nonhistone proteins from chromatin except F2a1 and F3 histones, and the final protein–DNA complex was treated with nuclease to remove the free portions of DNA.

However, experiments described in this study show that probably all F1 histone is released from chromatin in a free form and associates with other protein–DNA complexes produced during the digestion of rat liver chromatin with DNase I. This is in contrast to the report by Mirsky and his group that among histone fractions F1 histone is most effective for protecting the DNA in the nucleus or chromatin against nuclease attack (Mirsky and Silverman, 1972; Mirsky *et al.*, 1972). It is not certain at this time if the difference in the results obtained in this study and by Mirsky's group is due to the possible differences in the reaction conditions or chromatin preparations. However, it is clear that under the conditions very similar to those of Clark and Felsenfeld (1971) the

digestion of roughly 50% of chromatin DNA resulted in the release of free F1 histone, and the actual amount of DNA covered by histones and other proteins could be much larger than 50% in chromatin. Itzhaki also reported that proteins are released during the digestion of rat thymus chromatin by DNase I (Itzhaki, 1971) but she did not identify the released proteins. Mirsky found that the degree of digestion of chromatin DNA is variable depending on the reaction conditions and chromatin preparations (Mirsky, 1971).

The weak protection of DNA by F1 histone during the nuclease digestion of chromatin in this study may be in agreement with the view that F1 histone molecules are most exposed to aqueous environment among the various histone fractions in the isolated chromatin (Smart and Bonner, 1971), and that F1 histone is most easily displaced from the original binding sites in chromatin in the presence of an excess amount of RNA and DNA (Jensen and Chalkley, 1968; Ilyin *et al.*, 1971) and also urea (Ilyin *et al.*, 1971).

The selective dissociation of the extra F1 histone bound to chromatin by 0.3 M NaCl may be very useful for determining the amount of free DNA in chromatin isolated from various tissues. The amount of the extra F1 histone not removable from chromatin, after its binding, by 0.3 M NaCl may represent the amount bound to free DNA. In an experiment with [^{32}P]F1 histone the amount of extra [^{32}P]F1 histone tightly binding to rat liver chromatin after the washings with 0.3 M NaCl was 10% of the chromatin DNA in mass. In view of the fact that 1:1 mass ratio of F1 histone and DNA is required for the titration of all DNA phosphates (Olins and Olins, 1971; Shih and Bonner, 1970) the value obtained above from the binding of extra F1 histone to chromatin may indicate that 10% of rat liver chromatin DNA may be the "free" zone available for binding of proteins. This is in agreement with the values obtained for the transcribable regions of rat and mouse liver chromatin (Tan and Miyagi, 1970; Grouse *et al.*, 1972).

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