The Nature of Protein Association with Chromatin†

D. Doenecke‡ and B. J. McCarthy*

ABSTRACT: The reality of the nonrandom distribution of histones along the chromatin strand was investigated in several ways. It does not appear to derive from histone exchange during shearing and is evident in chromatin fixed with formaldehyde prior to shearing. Endogenous or *Escherichia coli* polymerase are preferentially associated with regions of chromatin with a low protein/DNA ratio. Although RNA polymerase and histones are fixed to chromatin after formaldehyde treatment with high efficiency, only a minor fraction of non-histone protein is fixed under similar conditions. Even after washing in high salt to minimize adventitious association, most remaining non-histone protein fails to be fixed. The utility of this approach for defining chromosomal proteins is discussed.

When chromatin is sheared and centrifuged on a steep sucrose gradient, a broad distribution results in which the more slowly sedimenting fraction is deficient in histones (Doenecke and McCarthy, 1975). This result implies that histones are nonrandomly distributed along the chromatin strand leaving some sections of the DNA essentially uncovered (Varshavsky et al., 1973). It is possible, however, that the result is dependent upon some preparational artifact involving mobility of the chromosomal proteins at some stage of chromatin preparation, shearing, or formaldehyde fixation. Accordingly, experiments have been performed directed at these possibilities, in an attempt to establish the biological reality of the nonrandom arrangement of histones along the chromatin.

In addition we have studied the association of proteins with chromatin. The endogenous RNA polymerase activity appears to be nonrandomly associated with different chromatin fractions as does *Escherichia coli* RNA polymerase when incubated with chromatin in vitro. The nature of the association of non-histone proteins with chromatin was investigated by banding chromatin to equilibrium in CsCl or Conray gradients (Doenecke and McCarthy, 1975) after exposure to high salt concentrations to remove weakly bound protein.

Materials and Methods

Measurement of Endogenous RNA Polymerase Activity. Chromatin was prepared from Schneider's tissue culture cells with the following modifications of a procedure described elsewhere (Doenecke and McCarthy, 1975). Cells were homogenized in a buffer containing 0.3 *M* sucrose, 2 m*M* $MgCl_2$, and 0.5 m*M* dithiothreitol in 10 m*M* Tris (pH 8.0). Afer centrifugation for 5 min at 2000 rpm in a Sorvall HB4 rotor the pellets were homogenized in the buffer described above, containing 0.5% NP40. After standing for 4 min in the cold, nuclei were collected at 4000 rpm in the same rotor. The pelleted nuclei were then resuspended in 25% glycerol, 5 m*M* Tris (pH 8.0), 5 m*M* dithiothreitol, 5 m*M* $MgCl_2$, and 0.1 m*M* EDTA followed by centrifugation at 6000 rpm for 10 min. The resulting pellet was then suspended in 12.5% glycerol, 0.1 m*M* EDTA, 0.5 m*M* dithiothreitol, and 1 m*M* Tris (pH 8.0). After centrifugation for 10 min at 8000 rpm, the pellet was washed once in the same buffer, centrifuged again, and resuspended in 2 ml of this buffer. This suspension was then sheared at 3000 psi in a French pressure cell and centrifuged for 15 min at 10,000 rpm in a Sorvall HB4 rotor and the supernatant was then layered on top of two gradients, ranging from 0.17 to 1.7 *M* sucrose in the 12.5% glycerol-Tris buffer described above. Chromatin loaded onto the gradient represented a 70–80% yield in terms of total cellular DNA.

After centrifugation in a Beckman SW41 rotor for 15 hr at 32,000 rpm and 4°, 0.5-ml fractions were collected and the optical density at 260 nm was determined; 200-μl aliquots of each fraction were assayed for RNA polymerase activity in a 400-μl mixture containing 12.5% glycerol, 5 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 25 m*M* Tris (pH 8.0), 2.5 m*M* dithiothreitol, 0.3 *M* ammonium sulfate, 0.08 m*M* each of ATP, GTP, and CTP, 1 μCi of [^{3}H]UTP (specific activity 15 Ci/mmol), and 10 μg of HeLa DNA.

After incubation for 60 min at 37°, the samples were chilled, one drop of bovine serum albumin (5 mg/ml) was added, and the sample was made 10% in trichloroacetic acid. The samples were then filtered through Whatman (GFC) filters, washed with 5% Cl_3CCOOH and ethanol, and dried. Radioactivity was then measured in a Omnifluor (New England Nuclear, Boston, Mass.)–toluene scintillation fluid.

Binding of Labeled RNA Polymerase to Chromatin. DNA-dependent RNA polymerase from *E. coli* was purified by the method of Burgess (1969) without phosphocellulose chromatography. The purified enzyme was then labeled with tritium using the method of reductive alkylation (Rice and Means, 1971) as described elsewhere (Janowski et al., 1972): After the labeling procedure, the enzyme was dialyzed against 0.01 *M* triethanolamine (pH 7.8) and 50% glycerol. Chromatin was labeled by incubating *Drosophila* cells for 20 hr in 100 ml of Schneider's medium, containing 2 μCi of [2-^{14}C]thymidine (specific activity 10 Ci/mol). Chromatin was then prepared as described previously (Doenecke and McCarthy, 1975), washed finally in 0.01 *M* triethanolamine (pH 7.8) and sheared at 3000 psi. Incubation with the enzyme was performed by the procedure described by Cedar and Felsenfeld (1973) in the presence of

† From the Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143. *Received October 31, 1974.* Supported by a grant from the National Science Foundation (GB 35704).

‡ Recipient of a research fellowship from the Deutsche Forschungsgemeinschaft. Present address: Physiologisch-chemisches Institut Lahnberge, D 355 Marburg/Lahn, Federal Republic of Germany.

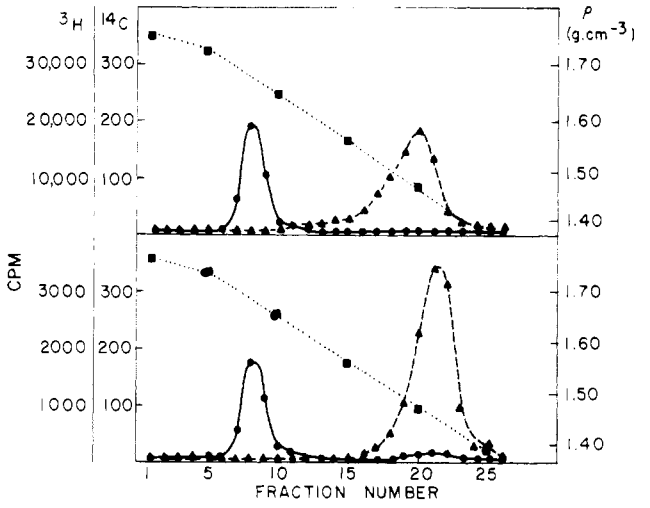

FIGURE 1: Cesium chloride gradient of mixtures of ^3H-labeled *Drosophila* DNA and [^{14}C]thymidine-labeled chromatin, mixed before being sheared at 3000 psi in a French pressure cell, followed by formaldehyde fixation. Upper frame: mixture of chromatin (containing 120 μg of DNA) and 120 μg of purified DNA in 1 ml of 0.01 *M* triethanolamine (pH 7.8) sheared after mixing and subsequently fixed with formaldehyde. Lower frame: mixture of chromatin (containing 120 μg of DNA) and 12 μg of purified DNA, mixed before shearing, the shearing procedure being followed by formaldehyde fixation. Gradient run in SW56 Beckman rotor at 41,000 rpm/10° for 72 hr. (●—●) [^3H]Thymidine radioactivity in purified DNA; (▲- - -▲) [^{14}C]thymidine radioactivity in chromatin; (■. . .■) density.

10 m*M* Tris (pH 7.9), 1 m*M* $MnCl_2$, and 0.08 m*M* each of ATP, GTP, and UTP; 24 μg of chromatin was incubated with 6.4 pmol of ^3H-labeled RNA polymerase, the enzyme concentration being in excess over the template. After 20 min of incubation, the reaction was terminated by chilling and addition of an equal volume of ice-cold 2% formaldehyde in triethanolamine and cesium chloride or Conray gradients run as described previously (Doenecke and McCarthy, 1975).

Cesium Chloride Gradient of [^{14}C]Tryptophan-Labeled Chromatin. Drosophila cells (Schneider, line 2) were labeled in 200 ml of tryptophan-free Schneider's medium, by adding 6 μCi of [3-^{14}C]tryptophan (18 Ci/mol) for 24 hr. Chromatin was prepared from these cells as described (Doenecke and McCarthy, 1975) and fixed with 1% formaldehyde after several washes in 0.01 *M* triethanolamine buffer (pH 7.8) and shearing in a French pressure cell at 3000 psi. After 24 hr at 4° and 20 hr of dialysis against 0.01 *M* triethanolamine buffer, 10-ml cesium chloride gradients were run as described (Doenecke and McCarthy, 1975); 30 fractions were collected, 1 ml of water was added, and optical density at 260 nm as well as Cl_3CCOOH-precipitable radioactivity were determined.

Treatment of Chromatin with High Salt. Two batches of cells were labeled separately with 10 μCi of [^{14}C]leucine (specific activity 312 Ci/mol) and 200 μCi of [4,5-^3H]leucine (specific activity 55 Ci/mmol) for 20 hr in leucine-free Schneider's medium and chromatin was prepared separately as described above. In a similar experiment with [^3H]- and [^{14}C]tryptophan label of chromatin, the cells were incubated for 20 hr with 10 μCi of [^4C]tryptophan (18 Ci/mol), or 300 μCi of [^3H]tryptophan (1.65 Ci/mmol). After the first wash in 0.01 *M* Tris (pH 8.0), the chromatin was washed in 0.01 *M* triethanolamine (pH 7.8). The ^3H-labeled chromatin pellet was then homogenized in a buffer containing 0.131 *M* NaCl, 0.265 *M* KCl, 0.022 *M* $MgCl_2$, 0.004 *M* $CaCl_2$, 0.01 *M* triethanolamine, 0.001 *M* $Na_2S_2O_5$, and 0.1 m*M* $CdSO_4$ as proposed by Comings and Tack (1973), derived from Langendorf et al. (1961).

This treatment was repeated once and both the ^{14}C-labeled and the salt-treated [^3H]chromatin preparations were then finally washed again twice in 0.01 *M* triethanolamine (pH 7.8). Equal amounts were then mixed and this chromatin mixture was subsequently sheared at 3000 psi in a French pressure cell, followed by a 15-min spin at 10,000 rpm in a Sorvall HB4 rotor. Fixation with 0.75% formaldehyde was carried out as described above with part of the chromatin sample. Another aliquot was slowly stirred with 1% sodium dodecyl sulfate at 37° for 1 hr and then dialyzed overnight at room temperature against 0.001 *M* sodium phosphate buffer (pH 7.1), 0.1% β-mercaptoethanol, and 0.1% sodium dodecyl sulfate. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was then performed as described by Weber and Osborn (1969). The gel was stained in Coomassie Blue, destained in 10% acetic acid, scanned at 550 nm in a Beckman Acta CIII spectrophotometer, cut in 1-mm slices, and digested in a 5% solution of Protosol (New England Nuclear, Boston, Mass.) in Omnifluor–toluene scintillation fluid. After 10 hr at 60° and 24 hr in the dark at room temperature, the samples were counted in a Beckman LS counter.

The formaldehyde-treated aliquot of the chromatin was stored for 24 hr in ice, then dialyzed for 24 hr against 0.01 *M* triethanolamine (pH 7.8), and analyzed in a cesium chloride gradient, run for 60 hr at 10° in a Beckman fixed angle rotor, Type 40, at 33,000 rpm. The same preparation was analyzed also in a Conray gradient in the same rotor and under the same conditions, but for 90 hr.

Results

Mobility of Histones during Shearing. In order to obtain subfractions of chromatin, it is necessary to shear to fragments containing a few hundred base pairs of DNA (McCarthy et al., 1973). This is normally accomplished by mechanical shear in the French pressure cell. Since the force required is considerable, normally 3000 psi, the possibility arises that the shearing procedure itself induces changes in the location of the histones. In order to approach this question two kinds of experiments were performed.

In the first, [^3H]DNA was mixed with [^{14}C]chromatin prior to shearing at a ratio of 1:1 or 1:10. After shearing the mixture was fixed with formaldehyde and banded in CsCl. In both cases the vast majority of the [^3H]DNA banded at ρ = 1.69 showing that histones were not released from the chromatin during shearing to be available for binding to the DNA (Figure 1). When the ratio of DNA to chromatin was 1:10 a small fraction of the [^3H]DNA was found in the peak of chromatin at ρ = 1.42 consistent with the release and reassociation of perhaps 1% of the chromosomal protein. Thus, a major reassortment of histones by release into the medium and subsequent binding appears to be excluded. However, this experiment does not eliminate the possibility that shearing facilitates sliding of the histones along the DNA.

In order to investigate this possibility a slightly different approach was used. Chromatin was prepared by the usual procedure and fixed with formaldehyde before shearing. When an aliquot was fractionated on a sucrose gradient, the normal distribution was obtained (Figure 2). Thus, the wide variation in sedimentation behavior of the fragmented chromatin is preserved. When unfractionated chromatin and

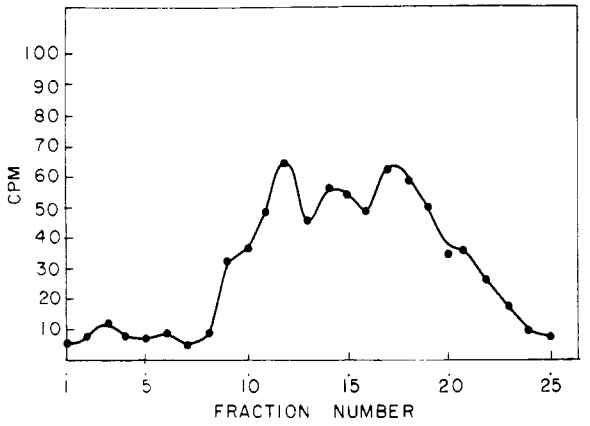

FIGURE 2: Sucrose gradient of chromatin fixed with 1% formaldehyde for 24 hr at 0° and dialyzed for 24 hr against 0.01 *M* triethanolamine buffer before shearing at 3000 psi in a French pressure cell. Sucrose gradients: 0.17–1.7 *M* sucrose in 0.01 *M* triethanolamine (pH 7.8) run for 15 hr at 32,000 rpm in a Beckman SW41 swinging bucket rotor. Fractions of 0.5 ml were collected and Cl_3CCOOH precipitable cpm measured in 50-μl aliquots (●—●).

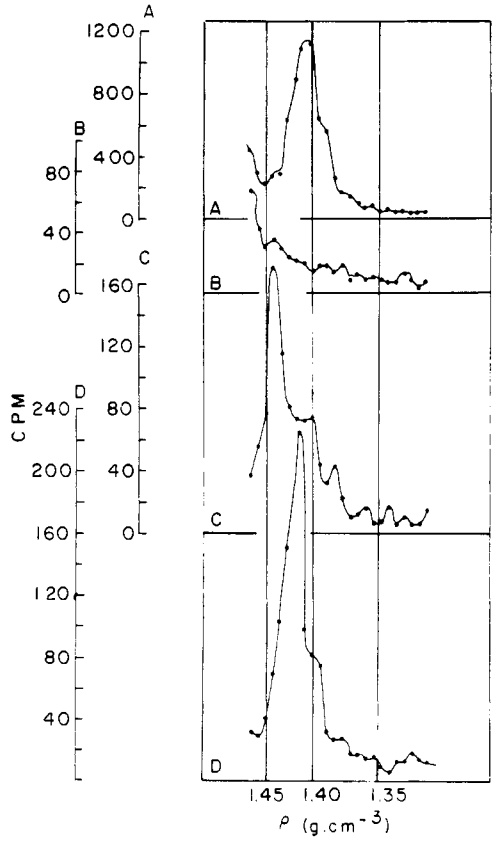

FIGURE 3: Cesium chloride gradient of total chromatin and different chromatin fractions of the sucrose gradient shown in Figure 2. (A) Total unfractionated chromatin, sheared after fixation in 1% formaldehyde; (B) fractions 9–12; (C) fractions 13–16; (D) fractions 17–19. Gradient was run for 60 hr at 10° in a fixed-angle Beckman rotor Type 40 at 33,000 rpm.

fractions representing different parts of the sucrose gradient were centrifuged in CsCl gradients, distributions illustrated in Figure 3 were obtained. Slowly sedimenting chromatin displayed a density of >1.46, while the most rapidly sedimenting material banded at 1.41. This difference in density and protein content is the same as that obtained when the chromatin is fixed after shearing (Doenecke and McCarthy, 1975). Therefore it does not appear that the shearing procedure generates the nonrandom distribution of histones along the DNA.

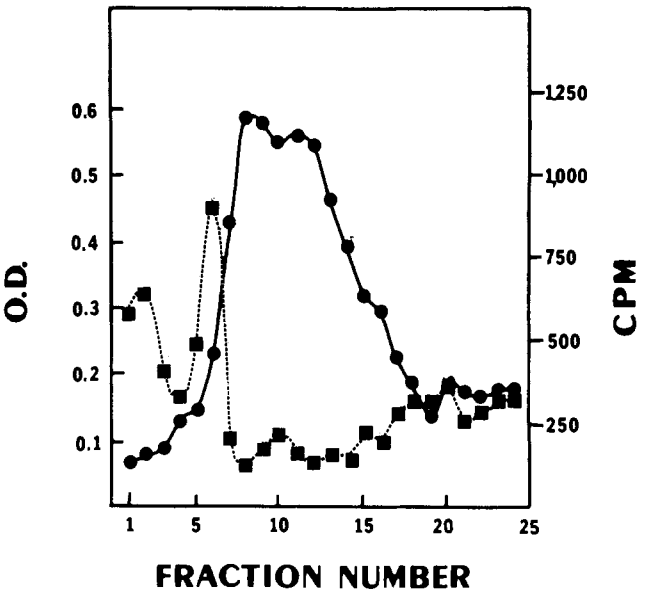

FIGURE 4: Measurement of endogenous RNA polymerase activity of chromatin from *Drosophila* tissue culture cells fractionated on a sucrose gradient containing 12.5% glycerol. Aliquots of the different fractions were incubated with [^{3}H]UTP and unlabeled GTP, ATP, and CTP in the presence of 1 m*M* $MnCl_2$, 5 m*M* $MgCl_2$, 0.3 *M* ammonium sulfate, and 2.5 m*M* dithiothreitol in 25 m*M* Tris buffer (pH 8.0) and 10 μg of HeLa DNA. (●—●) OD_{260}; (■. . .■) Cl_3CCOOH-precipitable radioactivity.

Association of RNA Polymerase with Chromatin. In fragmented chromatin the most slowly sedimented fractions bind *E. coli* RNA polymerase and exhibit all the template activity in vitro (McCarthy et al., 1973; Murphy et al., 1973). It is therefore of interest to localize the endogenous RNA polymerase activity in fractionated chromatin. To this end *Drosophila* chromatin was sheared and fractionated on a sucrose gradient containing glycerol to stabilize the enzyme. When aliquots were assayed for RNA polymerase activity in the presence of excess HeLa DNA as template, much of the activity appeared in the slowly sedimenting region (Figure 4), although fractions near the bottom of the gradient also exhibited high specific enzymatic activity. The activity present in the first three fractions probably represents free enzyme dissociated during the centrifugation. Although a detailed interpretation is difficult, it does appear that the endogenous activity is nonrandomly associated with the chromatin fragments.

The interaction of RNA polymerase with chromatin was studied in another way. *E. coli* polymerase was labeled with ^{3}H by the procedure of Rice and Means (1971) as described earlier (Janowski et al., 1972). The labeled enzyme was incubated with sheared chromatin and an aliquot fixed with formaldehyde and banded to equilibrium in CsCl (Figure 5). Some of the enzyme bands with the main peak of chromatin, while another portion bands at a density where little chromatin is apparent and the apparent protein/DNA ratio is low (i.e., approximately 0.79). We conclude that *E. coli* polymerase binds to chromatin with such an affinity that it may be fixed with formaldehyde and that it binds preferentially to fragments of low protein/DNA ratio.

The same conclusion may be drawn from the distribution in a Conray gradient (Doenecke and McCarthy, 1975) when a parallel aliquot of the polymerase-chromatin com-

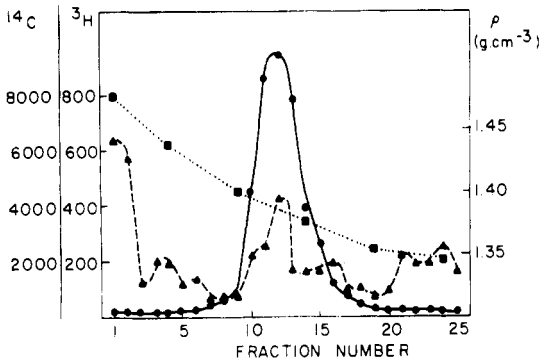

FIGURE 5: Cesium chloride gradient of [^{14}C]thymidine-labeled chromatin, fixed with formaldehyde after 15 min of incubation at 37° with ^{3}H-labeled RNA polymerase in the presence of Mn^{2+}, GTP, ATP, and UTP. Reaction was terminated by chilling in ice and addition of 1 volume of ice-cold 2% formaldehyde solution. (●—●) [^{14}C]thymidine; (▲- - -▲) [^{3}H]RNA polymerase; (■. . .■) density. Gradient was run for 60 hr at 10° in a fixed-angle Beckman rotor Type 40.

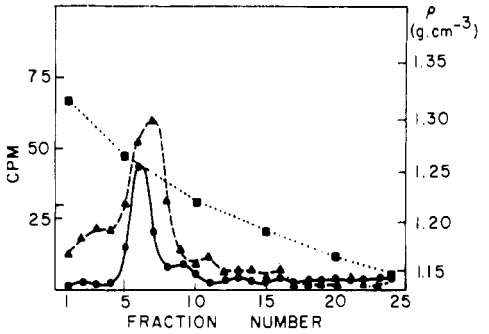

FIGURE 6: Conray density gradient of [^{14}C]thymidine-labeled chromatin after reaction with [^{3}H]RNA polymerase as described in the legend to Figure 5. Gradient was run for 90 hr at 10°, 33,000 rpm in a fixed-angle Beckman rotor Type 40. (●—●) ^{14}C radioactivity; (▲- - -▲) ^{3}H radioactivity; (■. . .■) density.

plex was centrifuged to equilibrium (Figure 6). Again RNA polymerase exhibits a stable association with chromatin and appears to be bound to chromatin of lower than average density; i.e., lower protein/DNA ratio.

Nature of the Proteins Fixed to Chromatin with HCHO. In order to determine the nature of proteins fixed to chromatin with formaldehyde and the efficiency of this reaction, several experiments were performed with chromatin labeled with different amino acids. Tryptophan was used to specifically label non-histone proteins, since histones lack this residue. Lysine was used to preferentially label histones and leucine as a random label for all proteins.

When cells are labeled with [^{14}C]tryptophan, the radioactively labeled proteins are only partially associated with the main chromatin band in cesium chloride gradients (Figure 7). Table I indicates that the specific activity of chromatin in the peak region is reduced to less than 30% of the value before formaldehyde fixation. This effect can be quantitated either by calculating the ratio of [^{3}H]tryptophan to [^{14}C]thymidine radioactivity before and after the cesium chloride centrifugation or by directly measuring the ^{14}C radioactivity of tryptophan containing protein per unit DNA.

When, on the other hand, the ratio of [^{3}H]lysine to [^{14}C]thymidine is calculated before fixation with formaldehyde and after cesium chloride centrifugation, a slight increase is evident. This might be due to the fact that the peak region of chromatin in these cesium chloride gradients

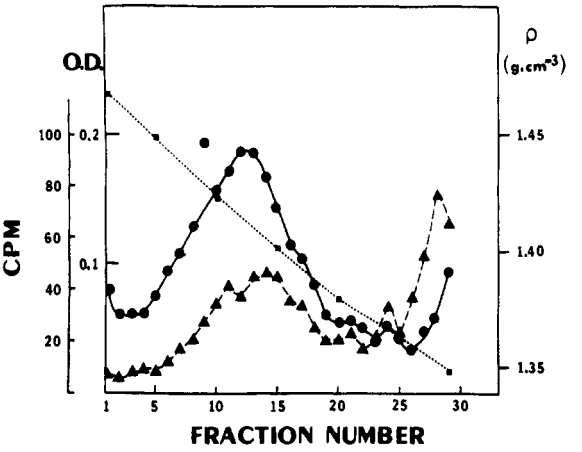

FIGURE 7: Cesium chloride gradient of [^{14}C]tryptophan-labeled chromatin, sheared at 3000 psi in 0.01 *M* triethanolamine buffer (pH 7.8) and fixed with 1% formaldehyde; 10-ml gradient, run for 60 hr at 33,000 rpm in a fixed-angle Beckman 40 rotor at 15°. (●—●) OD_{260}; (▲- - -▲) ^{14}C radioactivity; (■. . .■) density.

Table I: Comparison of Retention of Lysine and Tryptophan Labeled Proteins in Chromatin after Formaldehyde Fixation.

		Tryptophan	
	Lysine[a]	Expt 1[a]	Expt 2[b]
Purified chromatin	4.82	1.64	15.9
Chromatin in CsCl gradient at ρ = 1.41 g cm^{-3}	5.10	0.46	4.4
Ratio of specific radioactivity before and after CsCl centrifugation	1.06	0.32	0.28

[a] Chromatin was labeled with [^{14}C]thymidine and either [^{3}H]-lysine or [^{3}H]tryptophan. The figures tabulated represent ^{3}H/^{14}C ratios. [b] Chromatin labeled with [^{14}C]tryptophan. The figures tabulated are cpm of ^{14}C/μg of DNA.

is impoverished in chromatin segments of lower protein/DNA ratio (Doenecke and McCarthy, 1975).

A similar experiment was performed to determine whether protein which fails to be fixed to the chromatin can be removed by washing with a salt solution. Two batches of chromatin were prepared, one labeled with [^{3}H]tryptophan and the other with [^{14}C]tryptophan. The ^{3}H-labeled preparation was treated with so-called Langendorf salts (Langendorf et al., 1961), a solution which has been shown to remove a large fraction of the non-histone protein (Comings and Tack, 1973). This resulted in a decrease of specific radioactivity from 420 to 220 cpm/μg and a loss of 48% of the [^{3}H]tryptophan-labeled protein. The two chromatin preparations were then mixed, fixed in HCHO, and banded in CsCl (Figure 8). The distribution of ^{3}H and ^{14}C was then determined and the fraction of the tryptophan radioactivity banding in the chromatin region, i.e., ρ from 1.38 to 1.43, was calculated. In the case of the ^{3}H salt-treated preparation no more than 26% of the original protein label was fixed to and banded with the chromatin. Likewise, only 35% of the ^{14}C radioactivity banded in the chromatin region. We therefore conclude that most of the tryptophan labeled non-histone proteins are not so firmly associated as to band with the chromatin after fixation in HCHO; nor is the high salt wash sufficient to remove all the loosely bound proteins.

The same experiment was repeated using [^{3}H]- and [^{14}C]leucine so that both histones and non-histones would

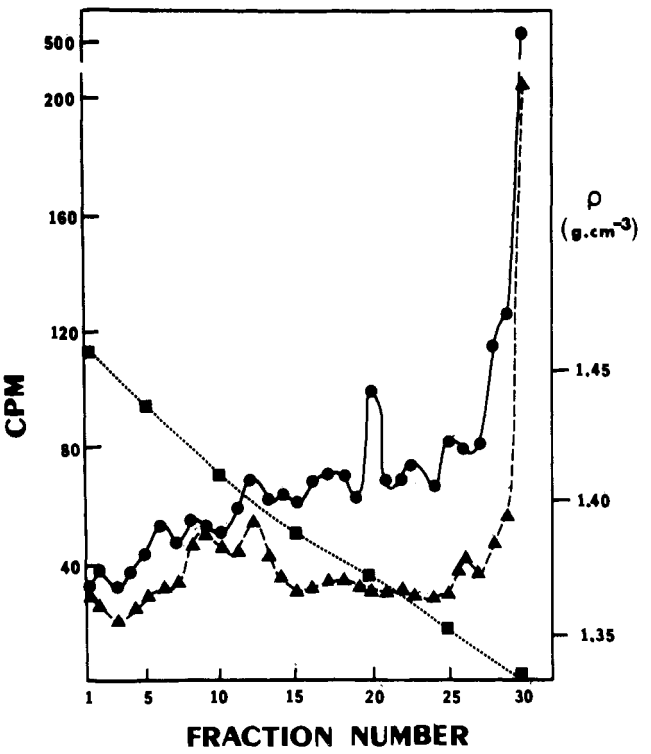

FIGURE 8: Cesium chloride gradient of a mixture of untreated [^{14}C]tryptophan-labeled chromatin (▲- - -▲) and [^{3}H]tryptophan-labeled chromatin (●—●), washed in high salt as described under Materials and Methods. After fixation with formaldehyde (1%), the chromatin mixture was centrifuged for 60 hr at 32,000 rpm and 10° in a fixed-angle Beckman Type 40 rotor. (■. . .■) Density.

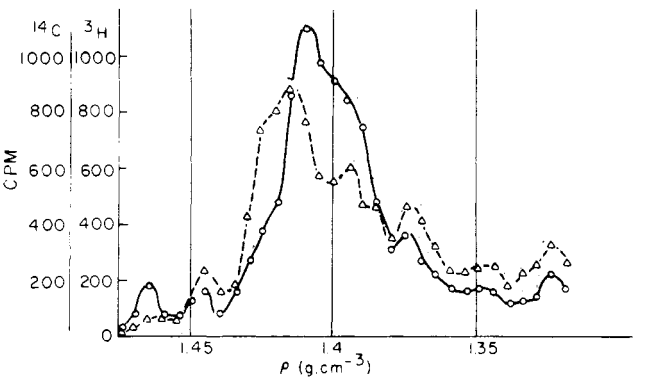

FIGURE 9: Cesium chloride gradient of a mixture of untreated [^{14}C]leucine-labeled chromatin (O- - -O) and [^{3}H]leucine-labeled chromatin, washed in high salt as described under Materials and Methods (Δ- - -Δ). Gradient was run for 60 hr at 10° in a fixed-angle Beckman Type 40 rotor at 30,000 rpm.

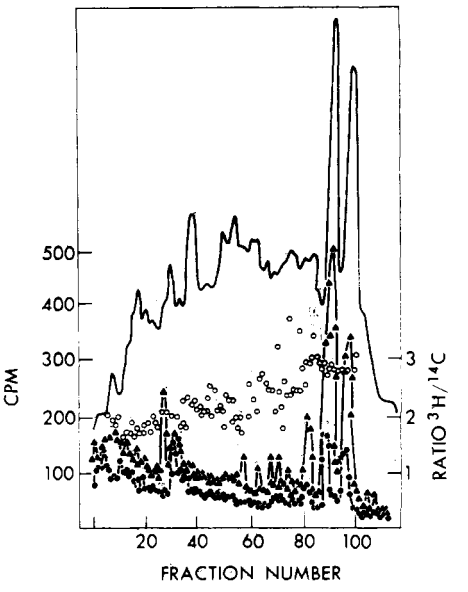

FIGURE 10: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of total nuclear proteins, extracted with 1% sodium dodecyl sulfate at 37° for 1 hr from a mixture of untreated [^{14}C]leucine-labeled chromatin and [^{3}H]leucine-labeled, high salt washed chromatin. OD_{550} (—) was scanned after staining the gel with Coomassie Blue and destaining in 10% acetic acid. Radioactivity was measured in 1-mm gel slices after incubation at 60° for 10 hr in a 5% mixture of Protosol (New England Nuclear, Boston, Mass), in Omnifluor (New England Nuclear)/toluene scintillation fluid. (●—●) ^{14}C radioactivity; (▲—▲) ^{3}H radioactivity; (O- - -O) ratio ^{3}H/^{14}C.

be labeled. In addition a sample was taken for sodium dodecyl sulfate polyacrylamide gel electrophoresis after salt treatment of the ^{3}H-labeled chromatin and mixing with the ^{14}C control. The distribution in CsCl after fixation and banding shows that on the average the salt-washed chromatin bands at a higher density, i.e., peak at 1.42 rather than 1.41 (Figure 9). Thus, in terms of total protein the difference between salt-washed and untreated chromatin is minor. In the case of the ^{3}H-labeled salt-treated chromatin about 60% of the protein banded with the chromatin peak compared to 70% for control untreated [^{14}C]chromatin.

The same difference in protein content is evident when the mixture of salt-treated and control chromatin is banded to equilibrium in a Conray gradient. Here the peak of ^{3}H is at $\rho = 1.25$ while that of ^{14}C is at about $\rho = 1.26$ suggesting that some 38% of the protein was removed by the salt wash.

Finally the nature of the proteins removed by the salt wash was determined by sodium dodecyl sulfate gel electrophoresis. The distribution of total protein and ^{3}H and ^{14}C label is shown in Figure 10. Since histones other than f1 are not removed by the salt wash, the ratio of ^{3}H/^{14}C in the histone region (fractions 84–100) may be taken as a reference. Here the ratio is approximately 3 while in other parts of the gel the ratio is lower than 2. Thus it appears that at least one-third of the non-histone protein is removed by the high salt wash.

Discussion

The most slowly sedimenting fractions of chromatin fragmented by shear exhibit many of the attributes expected for "active" or transcribable chromatin. It has a much higher template activity in vitro after the addition of exogenous polymerase (Murphy et al., 1973; McCarthy et al., 1973), as well as a lower histone/DNA ratio. However, some questions exist as to the significance of the template activity measurements and to the differences in protein content. These doubts arise from the difficulty of defining the native structure of chromatin and eliminating the possibility of reassortment of the protein moiety of chromatin during preparation and manipulations involved in fractionation. In the present paper we take up the question of preparational artifact and examine the nature of the association of chromosomal proteins with DNA.

The occurrence of a large scale movement of chromosomal protein from its original site into solution and back onto chromatin can probably be discounted as a result of the experiments described. Thus DNA added before shearing does not become associated with protein to any appreciable degree. However, a much more likely type of histone move-

ment during experimental manipulation is not so readily eliminated for histones may simply slide along the DNA strand from a configuration in which they are regularly distributed to form clusters leaving regions impoverished in protein. This possibility was investigated by fixing the chromatin after preparation and before shearing and sucrose gradient centrifugation. Since the same sucrose gradient profile was obtained, indicating the same unequal distribution of histones, we conclude that movement along the DNA during shearing is not responsible for the observed pattern of distribution. However, the possibility remains that movement of histones occurs during preparation. Attempts have been made to exclude this possibility by HCHO fixing of nuclei or cells, but it has proved difficult to obtain demonstrably clean preparations of chromatin. Finally we cannot dismiss the possibility that continuous movement of histones along the DNA is a normal process in vivo.

Preferential binding of *E. coli* RNA polymerase to chromatin subfractions has often been used to discriminate template active chromatin (Murphy et al., 1973; Billing and Bonner, 1972; Marushige and Bonner, 1971). In the present study it is apparent that both the endogenous and exogenous polymerases are preferentially associated with slowly sedimenting chromatin. However, this is to be expected if this fraction is in fact deficient in histones, bearing in mind the high affinity of RNA polymerase for DNA. Thus this finding does not necessarily prove that slowly sedimenting chromatin is representative of in vivo template active material.

However, it is clear that RNA polymerase is more firmly associated with chromatin than most of the so-called non-histone chromosomal protein. After fixation with formaldehyde the enzyme remains associated with chromatin in a CsCl gradient. Similarly, it bands with chromatin in a non-ionic equilibrium density gradient of Conray. On the other hand, only a minor fraction of the total tryptophan-labeled protein behaves in this manner. Most fails to be fixed with HCHO or to band with the chromatin.

A major fraction of the non-histone protein can be removed by washing in salt (Goodwin and Johns, 1972). In the experiments reported here, Langendorf salts (Langendorf et al., 1961) were used as this solution is purported to represent the intranuclear ionic environment. As reported by Comings and Tack (1973), a major fraction of the leucine-labeled non-histone protein was removed. However, even after this treatment, protein remains which fails to be fixed to the chromatin with formaldehyde. Since histones are fixed to the chromatin with high efficiency (Brutlag et al., 1969; Ilyin et al., 1970) it seems reasonable to take formaldehyde fixation as a criteria for intimate association with the chromatin. By this token, most of the non-histone proteins are not firmly associated and may represent mere adventitious association.

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