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Reconstitution of Chromatin: Mode of Reassociation of Chromosomal Proteins†

Chi-Bom Chae

ABSTRACT: The mode of reassociation of Ehrlich ascites histones and non-histone proteins during chromatin reconstitution was studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the procedure of Bekhor et al. (I. Bekhor, G. M. Kung, and J. Bonner, (1969), *J. Mol. Biol. 39,* 351) most of histones and non-histone proteins reassociate with DNA in the last dialysis step of the dissociated chromatin, that is, the dialysis of the chromatin in 0.4 *M* NaCl–5 *M* urea against a dilute buffer. The reassociation of histones and non-histone proteins with DNA is more gradual in the procedure of L. Kleiman and R.-C. C. Huang [(1972), *J. Mol. Biol. 64,* 1]. However, in both procedures the bulk of the Ehrlich ascites non-histone proteins reassociate with DNA after the binding of histones to DNA. There are small amounts of non-histone proteins which reassociate with DNA before and at the same time as histones reassociate with DNA.

Chromatin consists of DNA, histones, non-histone proteins, and a small amount of RNA (Bonner et al., 1968). Isolated chromatin mediates the synthesis of tissue-specific RNAs in the presence of RNA polymerase (Axel et al., 1973; Gilmour and Paul, 1973; Steggles et al., 1974), and only a small portion of chromosomal DNA (2–10%) is transcribed into RNA in vivo (Grouse et al., 1972) and in vitro (Tan and Miyagi, 1970). Therefore, it appears that chromosomal proteins regulate the expression and repression of genes. Recently several reports have appeared on the reconstitution of fully dissociated chromatin, and the reconstituted chromatin appears to behave as native chromatin as far as the synthesis of tissue-specific RNAs is concerned (Bekhor et al., 1969; Huang and Huang, 1969; Spelsberg et al., 1971; Gilmour and Paul, 1970). The tissue specificity of chromatin seems to be controlled by non-histone proteins

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rather than histones when the two components from different tissues were exchanged during reconstitution of chromatin (Spelsberg et al., 1971; Gilmour and Paul, 1970), and the correct distribution of chromosomal proteins along DNA is controlled by protein-bound chromosomal RNA (Bekhor et al., 1969; Huang and Huang, 1969). However, the nature of the chromosomal RNA now is in question, and it has been reported that the chromosomal RNA is a mixture of degraded rRNA and tRNA (Von Heyden and Zachau, 1971; Scharpe and Parijs, 1974). The correctness of the reported chromatin reconstitution also is obscure since the transcriptional fidelity of the reconstituted chromatin has been determined by the RNA/DNA hybridization methods which only detect the RNA transcripts from highly repeating sequences of DNA (Melli and Bishop, 1969), and also under the reported conditions of chromatin reconstitution extensive degradation of histones and non-histone proteins might have occurred (Chae and Carter, 1974). Furthermore both bacterial and mammalian RNA polymerases transcribe the genes for rRNA and 5S RNA in Xenopus liver chromatin in an aberrant manner (Reeder, 1973; Honjo and Reeder, 1974). Therefore it is doubtful if the reconstituted chromatin retained integrity of native chromatin as far as the chromosomal proteins and transcriptional fidelity of chromatin are concerned. Nonetheless the reported results are suggestive that chromosomal proteins may return to correct sites along the DNA under the reported conditions of chromatin reconstitution. Therefore, studies on the mode of reassociation of chromosomal proteins with DNA during chromatin reconstitution in conjunction with the studies on transcription of specific genes from the reconstituted chromatin may provide insight into the mechanism of organization of chromatin as well as the function of chromosomal proteins. Kleiman and Huang (1972) have reported the sequence of reassociation of histones with DNA during chromatin reconstitution. In this report the sequence of reassociation of both histones and non-histone proteins with DNA was studied under the two chromatin reconstitution procedures reported by Bekhor et al. (1969) and Kleiman and Huang (1972) by sodium dodecyl sulfate (SDS)[1] polyacrylamide gel electrophoresis of chromosomal proteins. Chromatin isolated from Ehrlich ascites carcinoma was chosen for the study described here because of the very low proteolytic degradation of the Ehrlich ascites chromosomal proteins during reconstitution of chromatin.

Materials and Methods

Preparation of Chromatin from Ehrlich Ascites Carcinoma Cells. Transplantation and collection of Ehrlich ascites carcinoma cells are described in a previous report (Chae et al., 1968). Pure nuclei were prepared as described by Hymer and Kuff (1964) with some modifications as follows: Ehrlich ascites cells were homogenized in 1% Triton X-100 containing 0.25 *M* sucrose, 3 m*M* $MgCl_2$, and 10 m*M* phosphate (pH 5.8) by a tight-fitting Dounce homogenizer and centrifuged at 700*g* for 5 min. This process was repeated twice more. The final nuclei were free of cytoplasmic material when examined under a light microscope. The pure nuclei were washed twice each in the following solutions (Chae, 1974): 0.075 *M* NaCl–0.024 *M* EDTA (pH 7), 0.3 *M* NaCl (pH 6), and 5 m*M* phosphate (pH 6.5). The final chromatin was suspended in cold water and sheared in a VirTis homogenizer at 20–30 V for 90 sec, and the concentration of chromatin was adjusted to 2 mg of DNA/ml. The chromatin, when frozen at −20°, was stable for several months as far as the electrophoretic patterns of proteins were concerned. The chromatin contained 1 mg of histone and 1.2 mg of non-histone proteins per mg of DNA.

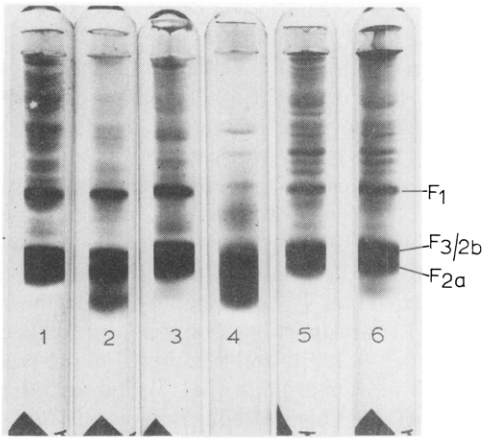

FIGURE 1: Electrophoretic patterns of the proteins of reconstituted chromatin. Rat liver chromatin, native (1) and reconstituted (2); rabbit bone marrow chromatin, native (3) and reconstituted (4); Ehrlich ascites chromatin, native (5) and reconstituted (6). Chromatin dissociated in 3 *M* NaCl–5 *M* urea–10 m*M* phosphate (pH 7) was sequentially dialyzed, 3 hr each, against 5 *M* urea–10 m*M* phosphate (pH 7) containing NaCl of 1, 0.8, 0.6, and 0.4 *M* and then against 10 m*M* phosphate (pH 7). The final reconstituted chromatin was recovered by centrifugation at 30,000*g* for 10 min.

Chromatin from rat liver and rabbit bone marrow was prepared from the nuclei purified by centrifugation through 2.3 *M* sucrose and subsequent washing with 1% Triton containing 0.25 *M* sucrose, 3 m*M* $MgCl_2$, and 10 m*M* phosphate (pH 5.8) as described before (Chae, 1974).

Reconstitution of Ehrlich Ascites Chromatin. Generally two different procedures of chromatin reconstitution have been used, that is, chromatin dissociated in 2–3 *M* NaCl–5 *M* urea was sequentially dialyzed against decreasing concentrations of NaCl up to 0.4 *M* (Bekhor et al., 1969; Gilmour and Paul, 1969, 1970) or 0.1 and 0 *M* (Kleiman and Huang, 1972; Stein et al., 1972; Stein and Farber, 1972) in the presence of 5 *M* urea, and the chromatin sample was finally dialyzed against a dilute buffer (Kleiman and Huang, 1972) or the chromatin in 5 *M* urea was recovered by centrifugation before dialysis of the chromatin against a dilute buffer (Stein et al., 1972; Stein and Farber, 1972). The complete process takes roughly 24 hr, and when the proteins reassociating with DNA at each step of dialysis are to be studied the DNA with reassociated proteins has to be recovered by centrifugation which takes at least 4–6 hr at 200,000–300,000*g*. Therefore, the overall process requires about 30–35 hr. During this time period chromatin samples are exposed to different NaCl and urea concentrations which differently affect the degree of proteolysis of chromosomal proteins (Carter and Chae, in preparation). Therefore, in this study chromatin dissociated in 3 *M* NaCl–5 *M* urea was directly dialyzed into given NaCl and urea concentrations, and the DNA with associated proteins were recovered by a high-speed centrifugation. It was, however, found that the mode of reassociation of chromosomal proteins with DNA is about the same during sequential and direct dialysis of the dissociated chromatin into the same salt and urea concentrations. The chromatin reconstituted by sequential dialysis is somewhat degraded (Figure 1).

[1] Abbreviation used is: SDS, sodium dodecyl sulfate.

Ehrlich ascites chromatin was dissociated at a concentration of 500 μg of DNA/ml in 3 *M* NaCl–5 *M* urea–10 m*M* phosphate (pH 7) for 2 hr. Under this condition 100% of histones and 85% of nonhistone proteins dissociate from DNA. Roughly the same amount of non-histone proteins dissociate in 3 *M* NaCl–5 *M* urea at pH 8. However, significantly more non-histone proteins are degraded at pH 8 than at pH 7. Therefore, all the reconstitution experiments were carried out at pH 7 rather than at pH 8 at which pH most of the reported chromatin reconstitution experiments have been carried out. The dissociated chromatin was divided into 8 to 12 1-ml portions and dialyzed against 1, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, and 0 *M* NaCl in the presence of 5 *M* urea–10 m*M* phosphate (pH 7) and against 10 m*M* phosphate (pH 7). This procedure represents that of Kleiman and Huang (1972). The dissociated chromatin was also dialyzed against 0.3 *M* NaCl–3.75 *M* urea–10 m*M* phosphate, 0.2 *M* NaCl–2.5 *M* urea–10 m*M* phosphate, and 0.1 *M* NaCl–1.25 *M* urea–10 m*M* phosphate which represent 25, 50, and 75% completion of the dialysis of the chromatin in 0.4 *M* NaCl–5 *M* urea against a dilute buffer in the procedure of Bekhor et al. (1969), respectively. After 4 hr of dialysis the chromatin samples were centrifuged for 6 hr at 200,000*g* or 4 hr at 300,000*g*. Ehrlich ascites DNA and commercial calf thymus DNA are quantitatively (>95%) pelleted under the centrifugation conditions. After centrifugation the DNA–protein pellets were rinsed with the respective dialysis solution and dissolved in 1 ml of 1% SDS–10 m*M* phosphate (pH 7)–0.1% β-mercaptoethanol with vigorous stirring for 16 hr at room temperature. The pellet and supernatant fractions were dialyzed against the 1% SDS mixture containing 10% glycerol for at least 6 hr at room temperature. SDS at a concentration of 0.1–1% effectively blocks the proteolysis of chromosomal proteins (Carter and Chae, in preparation), and thus it is expected that no proteolysis of chromosomal proteins occurs during the exposure of chromosomal proteins to SDS at room temperature. The dissociation, reconstitution, and centrifugation of chromatin samples were carried out at 0–4°.

SDS Polyacrylamide Gel Electrophoresis. The samples in the 1% SDS mixture (60–70 μl) were applied to 2.5% polyacrylamide gel (0.6 × 0.5 cm) (Wachneldt and Mandel, 1970) on top of 7.5% polyacrylamide gel (0.6 × 10 cm) in 0.1% SDS, 2.5 *M* urea, 5 m*M* EDTA, and 0.1 *M* sodium phosphate (pH 7) (Bhorjee and Pederson, 1972), and electrophoresis was carried out under the conditions which minimize the interference of the separation of proteins by DNA (Smith and Chae, 1973). The gels were stained with Coomassie Blue as described (Fairbanks et al., 1971).

Dissociation of Chromosomal Proteins in NaCl and Urea. When chromatin was dissociated in mixtures of NaCl and urea, chromatin containing 500 μg was centrifuged at 30,000*g* for 20 min. The effective volume of the chromatin pellet was 0.2 ml. To this chromatin pellet was added 1 ml of NaCl–urea–phosphate to give the desired final concentrations of NaCl, urea, and 10 m*M* phosphate as indicated in Figures 6 and 7. The chromatin was stirred at 4° for 2 hr and centrifuged at 200,000*g* or at 300,000*g*.

Preparation of DNA. Chromatin was dissociated in 1% SDS–0.15 *M* NaCl–0.015 *M* sodium citrate by stirring at 37° for 2–3 hr and to this solution was added NaCl to a final concentration of 1 *M*. Protein-free DNA was prepared essentially according to the method of Marmur (1961), and the DNA was further deproteinized by a phenol–cresol mixture (Kirby, 1965). It should be emphasized here that no Pronase was added during the deproteinization since Pronase was found to be present in the final DNA preparations and degrades chromosomal proteins in the presence of urea and NaCl (Chae and Carter, in preparation).

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

Results

Stability of Ehrlich Ascites Chromosomal Proteins during Reconstitution of Chromatin. Bekhor et al. (1969) and Huang and Huang (1969) have reported that salt and urea are necessary for correct reconstitution of chromatin. However, chromatins isolated from certain tissues like liver, thymus, brain, kidney, and testis undergo extensive proteolysis in the presence of salt and urea (Chae and Carter, 1974; Carter and Chae, in preparation). However, it was found that chromatin isolated from the rapidly dividing Ehrlich ascites carcinoma shows very low proteolysis during chromatin reconstitution compared to liver and bone marrow chromatin as shown in Figure 1. Chromatin was dissociated in 3 *M* NaCl–5 *M* urea–10 m*M* phosphate (pH 7) and dialyzed sequentially against decreasing concentrations of NaCl up to 0.4 *M* in the presence of 5 *M* urea–10 m*M* phosphate and then against 10 m*M* phosphate according to the procedure of Bekhor et al. (1969). The final chromatin was recovered by centrifugation and the pelleted, reconstituted chromatin was compared with native chromatin for electrophoretic patterns of proteins. As shown in Figure 1, rat liver non-histone proteins are considerably degraded, and the proteins larger than 80,000 are depleted, and a major degradation product of 10,000 accumulates. The degradation of chromosomal proteins is more extensive in rabbit bone marrow chromatin. However, the chromosomal proteins of Ehrlich ascites chromatin is relatively intact under the same conditions. Acid–urea gel electrophoresis (Panyim and Chalkley, 1969) of the acid-extracted histones shows that no degradation of histones has occurred during the reconstitution of Ehrlich ascites chromatin (not shown here). Rat liver histones also are relatively intact under the conditions as reported earlier (Chae and Carter, 1974). However, bone marrow histones, especially lysine-rich F1 histone, were extensively degraded.

Sensitivity of SDS Polyacrylamide Gel Electrophoresis. With the staining method used in this study one can visibly detect 0.1–1 μg quantity of proteins per protein band in SDS gel depending on the type of proteins (Talbot and Yphantis, 1971). Since optimum protein patterns are obtained with chromatin containing about 50 μg of DNA, and also proteins originally associated with 50 μg of DNA have been compared in this study, each protein band shown in Figure 1 and in the subsequent figures represents at least 10^5–10^6 molecules of protein per amount of DNA in the nucleus, that is, 5×10^9 base pairs in the case of mammalian systems (McCarthy, 1965). Therefore, in this study on the mode of reassociation of proteins with DNA during reconstitution of chromatin only a gross picture of the behavior of chromosomal proteins can be obtained, and no attempt was made to identify each non-histone protein band by any nomenclature.

In the SDS gel electrophoresis system used in this study there is no overlap between the bands of histones and non-histone proteins except two non-histone protein bands comigrating with F1 histone and a non-histone protein migrating

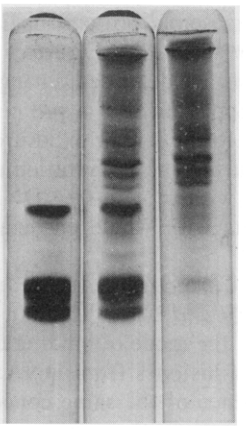

FIGURE 2: Electrophoretic patterns of histones and dehistonized chromatin proteins. From left to right: histones, whole chromatin, and dehistonized chromatin. Ehrlich ascites chromatin was extracted with cold 0.4 *N* H_2SO_4 at 4° for 30 min and centrifuged at 20,000*g* for 15 min. The acid extraction was repeated twice.

with F3 histone as shown in Figure 2. Therefore, no attempt was made to separate histones and nonhistone proteins before electrophoresis.

Mode of Reassociation of Chromosomal Proteins during Reconstitution. In the report by Bekhor et al. (1969) chromatin was dissociated in 2 *M* NaCl–5 *M* urea–10 m*M* Tris (pH 8) and dialyzed against 5 *M* urea–10 m*M* Tris (pH 8) containing 1, 0.8, 0.6, and 0.4 *M* NaCl and finally against 10 m*M* Tris (pH 8). Gilmour and Paul (1969, 1970) and Spelsberg et al. (1971) have used similar methods for reconstitution of chromatin at pH 8.3 and pH 6, respectively. It was reported that at pH 6 most of the non-histone proteins remain associated with DNA in 2 *M* NaCl–5 *M* urea (Spelsberg et al., 1971), but we were unable to confirm this (Chae and Carter, 1974). In this study it was decided to investigate the mode of reassociation of chromosomal proteins during reconstitution of chromatin by direct dialysis of the chromatin dissociated in 3 *M* NaCl–5 *M* urea against given concentrations of salt and urea for the reasons described under Materials and Methods. It was assumed that during the 4-hr dialysis the interactions of protein and DNA have reached an equilibrium.

Figure 3 shows the mode of binding of histones and nonhistone proteins to DNA during reconstitution of chromatin according to the procedure of Bekhor et al. (1969). Figure 3a shows that histones start to reassociate with DNA in 0.4 *M* NaCl–2.5 *M* urea and that the binding of histones to DNA is almost complete in 0.2 *M* NaCl–2.5 *M* urea (gel 9 in Figure 3a and gel 5 in Figure 3b) which corresponds to 50% completion of the dialysis of the chromatin in 0.4 *M* NaCl–5 *M* urea against a dilute buffer in the procedure of Bekhor et al. (1969). The dissociation of non-histone proteins is not complete in 3 *M* NaCl–5 *M* urea in the case of Ehrlich ascites chromatin, and it appears from Figures 3a and b that most of the dissociated non-histone proteins return to DNA after all the histones bind to DNA. It appears also that most of the chromosomal proteins return to DNA during the last dialysis step in the procedure of Bekhor et al. (1969), that is, the dialysis of the chromatin in 0.4 *M* NaCl–5 *M* urea against a dilute buffer rather than the dialysis of the chromatin in 2 *M* NaCl–5 *M* urea against 5 *M* urea containing 1, 0.8, and 0.6 *M* NaCl.

In some reports the chromatin dissociated in 2–3 *M* NaCl–5 *M* urea was dialyzed against 5 *M* urea containing decreasing concentrations of NaCl from 3 to 0.1 *M* (Kleiman and Huang, 1970) or to 0 *M* (Stein et al., 1972; Stein and Farber, 1972). Stein et al. (Stein et al., 1972) recovered the chromatin in 5 *M* urea, but Kleiman and Huang (1972) removed urea from the chromatin. Figure 4 shows the result of an experiment of this type of chromatin reconstitution. In

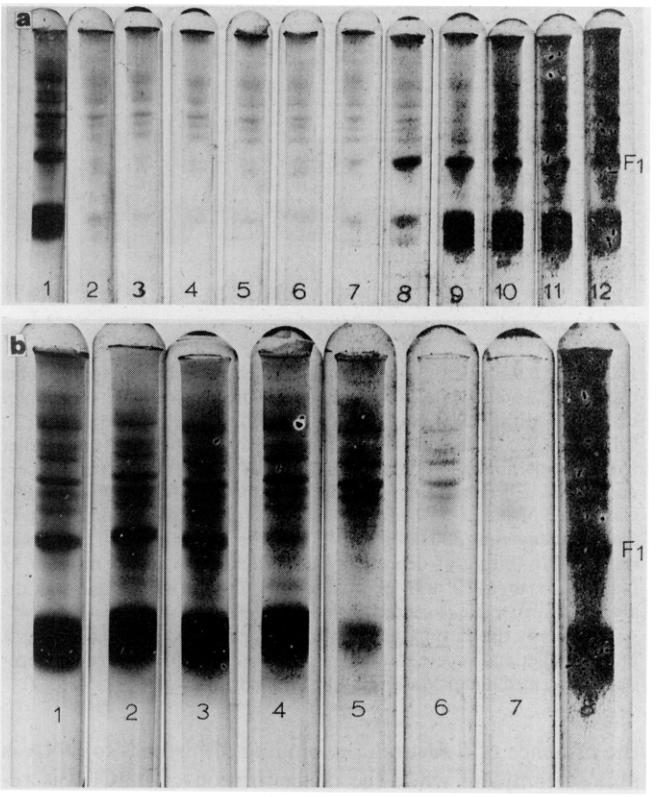

FIGURE 3: (a) Protein patterns of Ehrlich ascites chromatin dissociated in 3 *M* NaCl–5 *M* urea after dialysis into various concentrations of NaCl and urea. DNA–protein pellets: (2) 3 *M* NaCl–5 *M* urea, (3) 1 *M* NaCl–5 *M* urea, (4) 0.8 *M* NaCl–5 *M* urea, (5) 0.6 *M* NaCl–5 *M* urea, (6) 0.5 *M* NaCl–5 *M* urea, (7) 0.4 *M* NaCl–5 *M* urea, (8) 0.3 *M* NaCl–3.75 *M* urea, (9) 0.2 *M* NaCl–2.5 *M* urea, (10) 0.1 *M* NaCl–1.25 *M* urea, (11) 0 *M* NaCl–0 *M* urea; (1) and (12), control chromatin. Experimental details are described under Materials and Methods. (b) Protein patterns of Ehrlich ascites chromatin dissociated in 3 *M* NaCl–5 *M* urea after dialysis into various concentrations of NaCl and urea. Supernatants: (1) 3 *M* NaCl–5 *M* urea, (2) 0.5 *M* NaCl–5 *M* urea, (3) 0.4 *M* NaCl–5 *M* urea, (4) 0.3 *M* NaCl–3.75 *M* urea, (5) 0.2 *M* NaCl–2.5 *M* urea, (6) 0.1 *M* NaCl–1.25 *M* urea, (7) 0 *M* NaCl–0 *M* urea, (8) control chromatin. Experimental conditions were the same as Figure 3a.

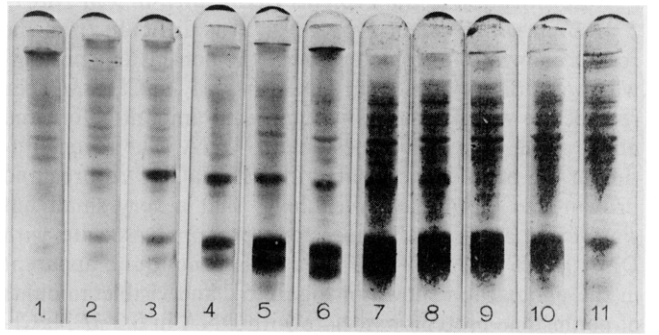

FIGURE 4: Protein patterns of Ehrlich ascites chromatin dissociated in 3 *M* NaCl–5 *M* urea after dialysis into various concentrations of NaCl in the presence of 5 *M* urea. Pellets: (1) 0.5 *M* NaCl–5 *M* urea, (2) 0.4 *M* NaCl–5 *M* urea, (3) 0.3 *M* NaCl–5 *M* urea, (4) 0.2 *M* NaCl–5 *M* urea, (5) 0.1 *M* NaCl–5 *M* urea, (6) 0 *M* NaCl–5 *M* urea. Supernatants: (7) 0.5 *M* NaCl–5 *M* urea, (8) 0.4 *M* NaCl–5 *M* urea, (9) 0.2 *M* NaCl–5 *M* urea, (10) 0.1 *M* NaCl–5 *M* urea, (11) 0 *M* NaCl–5 *M* urea.

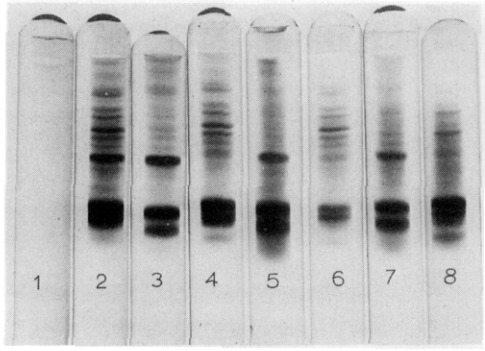

FIGURE 5: Reconstitution of Ehrlich ascites chromosomal proteins dissociated in 3 *M* NaCl–5 *M* urea with protein-free DNA. Samples dialyzed against 0.5 *M* NaCl–5 *M* urea, pellet (1) and supernatant (2); 0.1 *M* NaCl–5 *M* urea, pellet (3) and supernatant (4); 5 *M* urea, pellet (5) and supernatant (6); 0.2 *M* NaCl–2.5 *M* urea, pellet (7) and supernatant (8). Ehrlich ascites chromatin (1 mg of DNA/ml) was dissociated in 3 *M* NaCl–5 *M* urea–10 m*M* phosphate (pH 7) for 2 hr at 4° and centrifuged at 300,000*g* for 4.5 hr. The supernatant (0.5 ml) was mixed with 400 μg of deproteinized Ehrlich DNA in 0.5 ml of 3 *M* NaCl–5 *M* urea–10 m*M* phosphate (pH 7) and the sample was dialyzed against various concentrations of NaCl and urea for 4 hr at 4°. After dialysis the chromatin sample was centrifuged at 300,000*g* for 4 hr, and pellet and supernatant were prepared for SDS gel electrophoresis as described under Materials and Methods.

the presence of 5 *M* urea the binding of histones to DNA is almost complete when the concentration of NaCl was reduced to 0.1 *M*, and the bulk of non-histone proteins and a small amount of histones are still not associated with DNA in 5 *M* urea in the absence of NaCl. Therefore, most of the non-histone proteins return to DNA during the dialysis of the chromatin in 0.1 *M* NaCl–5 *M* urea against a dilute buffer in the procedure of Kleiman and Huang (1972), and it is very likely that in the experiments of Stein et al. the reconstituted chromatin recovered in 5 *M* urea lacks most of the non-histone proteins and a small amount of histones. Kleiman and Huang (1972) have reported that among histone fractions F1 histone first binds to DNA at an ionic strength of 0.34 in the presence of 5–7 *M* urea, and the results shown in Figure 4 seem to support their finding.

Reconstitution of the Proteins Dissociated in 3 M NaCl–5 M Urea with Protein-Free DNA. It is difficult, from the results shown so far to determine if the non-histone proteins which are dissociated from DNA in 3 *M* NaCl–5 *M* urea reassociate with DNA in any significant amount before and at the same time as histones bind to DNA. The reconstitution of the proteins dissociated in 3 *M* NaCl–5 *M* urea and protein-free DNA, however, shows that a very small but significant amount of non-histone proteins associate with DNA in 0.5 *M* NaCl–5 *M* urea (this is not quite apparent in the photograph shown in Figure 5), and visible amounts of nonhistone proteins associate with DNA in 0.2 *M* NaCl–2.5 *M* urea, 0.1 *M* NaCl–5 *M* urea, and 5 *M* urea as shown in Figure 5. These results suggest that there are non-histone proteins which reassociate with DNA before and at the same time as histones bind to DNA during the stepwise dialysis of the dissociated chromatin against decreasing concentrations of NaCl and urea according to the methods of Bekhor et al. (1969) and Kleiman and Huang (1972). It appears that significantly more histones are not bound to DNA in 0.1 *M* NaCl–5 *M* urea, 5 *M* urea, and 0.2 *M* NaCl–2.5 *M* urea than in the previous experiments with whole chromatin (see Figures 3 and 4). This is due to the presence of a smaller amount of DNA per chromosomal proteins in this experiment than the amount of DNA present per total chromosomal proteins in Ehrlich ascites chromatin. It is also shown in Figure 5 that considerable amounts of proteins are degraded during this experiment as apparent by the appearance of a protein smaller than histones. This is due to the longer exposure of the proteins to NaCl and urea during the preparation of the proteins and the reconstitution with protein-free DNA compared to the earlier experiments with whole chromatin (see Figures 3 and 4).

Dissociation of Chromosomal Proteins in Different Concentrations of NaCl and Urea. Kleiman and Huang (1972) have reported that the mode of binding of histones to DNA and dissociation of histones from DNA are somewhat different in the presence of the same concentrations of NaCl and urea. For example, in the presence of 6 *M* urea F1 histone shows the strongest affinity for DNA during reconstitution, but F1 histone is dissociated after histones F2b and F2a2, but before histones F2a1 and F3 during extraction from chromatin by NaCl in the presence of 6 *M* urea (Kleiman and Huang, 1972). Therefore, it was of interest to compare the mode of reassociation and dissociation of non-histone proteins in the presence of the same concentrations of NaCl and urea. In the procedure of Bekhor et al. (1969) for chromatin reconstitution, the binding of histones is almost complete in 0.2 *M* NaCl–2.5 *M* urea (see Figure 3b) but most of the non-histone proteins returns to DNA after histones in 0.1 *M* NaCl–1.25 *M* urea (see Figure 3b). During extraction of chromatin no histone dissociates from DNA in 0.2 *M* NaCl–2.5 *M* urea as shown in Figures 6a and b, but a considerable amount of non-histone proteins dissociates in this solution. Only small amounts of non-histone proteins dissociate in 0.1 *M* NaCl–1.25 *M* urea during extraction of chromatin. Therefore, there seems to be no apparent differences in the binding affinity of histones and non-histone proteins during dissociation and reassociation of chromosomal proteins in 0.1 *M* NaCl–1.25 *M* urea and 0.2 *M* NaCl–2.5 *M* urea.

In the procedure of Kleiman and Huang (1972) for chromatin reconstitution, the concentration of salt is decreased from 3 to 0.1 *M* in the presence of 5 *M* urea, and most of the non-histone proteins does not reassociate even in 5 *M* urea (Figure 4). However, 5 *M* urea alone does not extract any significant amount of histones and non-histone proteins from Ehrlich ascites chromatin (Figure 7). This is in contrast to our earlier report on the quantitative extraction of non-histone proteins by 5 *M* urea at pH 7 and 8 from rat liver chromatin (Chae and Carter, 1974). It is not certain at this time if this difference in the extractability of non-histone proteins by 5 *M* urea is due to the possible difference in the property of the proteins or due to the difference in the property of chromatin. Most of the non-histone proteins, however, dissociate from Ehrlich ascites chromatin in 0.1 *M* NaCl–5 *M* urea at pH 7 (Figure 7). Therefore, there is a difference in the binding affinity of non-histone proteins to DNA during dissociation and reassociation in 5 *M* urea in the case of Ehrlich ascites chromatin.

The mode of reassociation of histones to DNA and dissociation of histones from DNA is significantly different in the presence of NaCl and urea as reported by Kleiman and Huang (1972). During reconstitution of chromatin no histone reassociates with DNA until the concentration of NaCl reaches 0.3–0.4 *M* in the presence of 5 *M* urea. However, during the extraction of chromatin there are some histones which do not dissociate from DNA in 0.5–0.6 *M* NaCl–5 *M* urea (Figure 6a and b). The undissociated his-

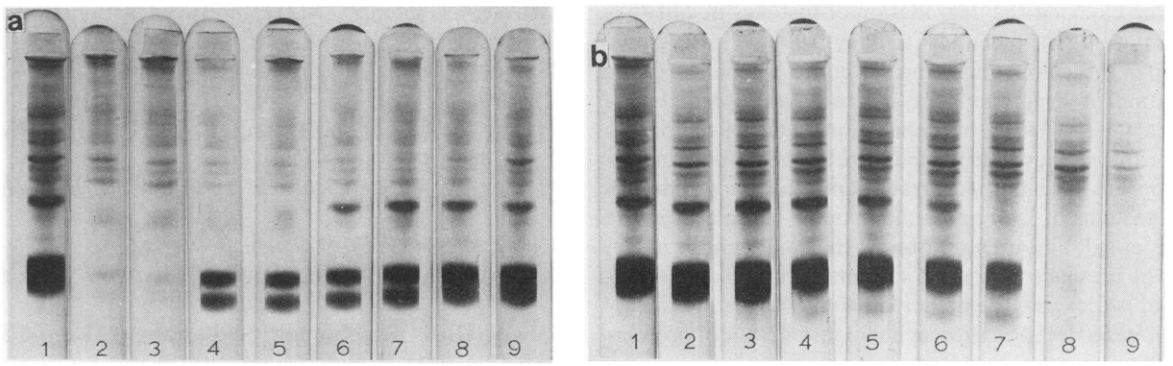

FIGURE 6: (a) Extraction of Ehrlich ascites chromatin with various concentrations of NaCl and urea. The proteins associated with DNA: (2) 3 *M* NaCl–5 *M* urea, (3) 2 *M* NaCl–5 *M* urea, (4) 0.6 *M* NaCl–5 *M* urea, (5) 0.5 *M* NaCl–5 *M* urea, (6) 0.4 *M* NaCl–5 *M* urea, (7) 0.3 *M* NaCl–3.75 *M* urea, (8) 0.2 *M* NaCl–2.5 *M* urea, (9) 0.1 *M* NaCl–1.25 *M* urea, (1) control chromatin. (b) Extraction of Ehrlich ascites chromatin with various concentrations of NaCl and urea. The proteins dissociated from DNA: (2) 3 *M* NaCl–5 *M* urea, (3) 2 *M* NaCl–5 *M* urea, (4) 0.6 *M* NaCl–5 *M* urea, (5) 0.5 *M* NaCl–5 *M* urea, (6) 0.4 *M* NaCl–5 *M* urea, (7) 0.3 *M* NaCl–3.75 *M* urea, (8) 0.2 *M* NaCl–2.5 *M* urea, (9) 0.1 *M* NaCl–1.25 *M* urea, (1) control chromatin. Experimental conditions were the same as Figure 6a.

tones were found to be histones F2a1 and F3 by acid–urea gel electrophoresis (not shown here). The first histones which dissociate from DNA in the presence of NaCl and urea (Figures 6b and 7) were found to be histones F2a2 and F2b by acid–urea gel electrophoresis. Overall the order of dissociation of histones from DNA in the presence of 5 *M* urea appears to be F2b = F2a2, F1, F3 = F2a1. Similar results on the dissociation of histones from DNA in the presence of salt and urea have been reported by Bartley and Chalkley (1972) and Kleiman and Huang (1972).

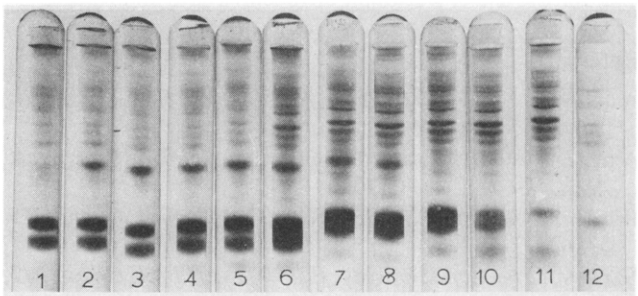

FIGURE 7: Extraction of Ehrlich ascites chromatin with various concentrations of NaCl in the presence of 5 *M* urea. Proteins associated with DNA: (1) 0.5 *M* NaCl–5 *M* urea, (2) 0.4 *M* NaCl–5 *M* urea, (3) 0.3 *M* NaCl–5 *M* urea, (4) 0.2 *M* NaCl–5 *M* urea, (5) 0.1 *M* NaCl–5 *M* urea, (6) 0 *M* NaCl–5 *M* urea. Proteins dissociated from DNA: (7) 0.5 *M* NaCl–5 *M* urea, (8) 0.4 *M* NaCl–5 *M* urea, (9) 0.3 *M* NaCl–5 *M* urea, (10) 0.2 *M* NaCl–5 *M* urea, (11) 0.1 *M* NaCl–5 *M* urea, (12) 0 *M* NaCl–5 *M* urea.

Discussion

It appears that most of the chromosomal proteins, which dissociate in 3 *M* NaCl–5 *M* urea, do not reassociate with DNA during the dialysis of the dissociated chromatin against decreasing concentrations of NaCl until 0.4 *M* in the presence of 5 *M* urea. Since Bekhor et al. (1969) and Gilmour and Paul (1969, 1970) have dialyzed the chromatin in 0.4 *M* NaCl–5 *M* urea against a dilute buffer to obtain reconstituted chromatin, it appears that most of the non-histone proteins and histones might have reassociated with DNA in a matter of a few hours in their experiments. The reassociation of histones and non-histone proteins with DNA during reconstitution of chromatin is more gradual in the procedure of Kleiman and Huang (1972) than in the procedure of Bekhor et al. (1969). Stein et al. (Stein et al., 1972; Stein and Farber, 1972) dialyzed the chromatin dissociated in 3 *M* NaCl–5 *M* urea against decreasing concentrations of NaCl up to 0 *M* in the presence of 5 *M* urea, and the chromatin in 5 *M* urea was recovered by centrifugation. This chromatin most likely lacks most of the non-histone proteins and a small amount of histones.

In the case of Ehrlich ascites chromatin, in addition to the non-histone proteins which do not dissociate in 3 *M* NaCl–5 *M* urea, there are the non-histone proteins which reassociate with DNA before, at the same time as, and after the binding of histones to DNA. It is possible that the non-histone proteins which do not dissociate in 3 *M* NaCl–5 *M* urea and the non-histone proteins which reassociate before histones may prevent the binding of histones to the DNA sites already occupied by the non-histone proteins. The presence of rat liver non-histone proteins which have greater binding affinity for DNA than histones in the presence of urea and NaCl has also been reported (Wakabayashi et al., 1974). It is not certain at the present time whether the non-histone proteins which reassociate with DNA at the same time as and after histones bind to DNA actually bind to DNA or to the histone surface of histone–DNA complex. Extra histones can tightly bind to the histone surface of histone–DNA complex (Chae, 1974), and it is likely that some non-histone proteins do likewise. Also the amount of non-histone proteins which have the property of binding to DNA is reported to be about 40% (Van den Broek et al., 1973), and a significant amount of non-histone proteins is the proteins of the heterogeneous nuclear RNA–protein complex associated with standard chromatin preparation (Pederson, 1974a,b). Therefore, clear picture of the mode of binding of non-histone proteins can only be obtained from the studies on the binding of isolated non-histone proteins to DNA and DNA–histone complex. This is being pursued in this laboratory.

It also is desirable to reexamine the correctness of the reconstitution procedure of chromatin using the chromatin which permits study on transcription of specific genes from isolated chromatin. It has been possible to determine the transcription of globin gene from chromatin isolated from avian reticulocytes (Axel et al., 1973), fetal liver (Gilmour and Paul, 1973), and bone marrow (Steggles et al., 1974). The transcription of simian virus 40 DNA from the chromatin isolated from cells transformed by the virus has also been reported (Astrin, 1973; Shih et al., 1973). The main problem may be, however, the degradation of chromosomal proteins during reconstitution of chromatin by chromatin-bound protease. Sodium bisulfite has been reported to be ef-

fective for blocking the degradation of histones in calf thymus chromatin (Bartley and Chalkley, 1970). However, we found that the compound is not effective for blocking the degradation of liver and bone marrow chromosomal proteins (Carter and Chae, in preparation). During the preparation of this manuscript we found that phenylmethanesulfonyl fluoride inhibits the degradation of histones and nonhistone proteins of liver and bone marrow chromatin in the presence of salt and urea. However, the compound is active only in the presence of organic solvents such as isopropyl alcohol and *p*-dioxane. We do not know to what extent the organic solvent affects the hydrophobic interaction of proteins and DNA.

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