Reassociation of Rat Hepatoma Chromatin Protein Components with DNA

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ABSTRACT. Rat ascites hepatoma chromatin was dissociated in 2 M NaCl–5 M urea–50 mM sodium acetate (pH 6.0) and reconstituted by gradient dialysis against decreasing concentrations of NaCl in the presence of urea. The mode of reassociation of chromatin protein components with DNA was examined mainly by gel electrophoresis. Histone 1 bound to DNA during dialysis against 5 M urea containing decreasing concentrations of NaCl from 0.4 to 0.2 M, and other histone fractions bound to DNA in dialysis at NaCl concentrations from 0.3 to 0.1 M. Binding of histones to DNA was almost completed in 0.1 M NaCl–5 M urea, but about one-half of the dissociated chromatin non-histone proteins (CNH proteins) was still not reassociated with DNA at this step. Some electrophoretically separated CNH proteins reassociated with DNA at almost the same time that histones bound to DNA, and other proteins reassociated with DNA after completion of binding of histones to DNA.

In higher organisms, a large portion of DNA is masked and only a small fraction is transcribed into RNA. This restriction is tissue-specific and well preserved in isolated chromatin (1, 12, 18, 22, 23, 26, 28). Since isolated chromatin consists of DNA, histones, CNH proteins and RNA, it is pertinent to inquire into the role of each chromatin component in determining the specificity of such transcription. Improved methods for reconstitution of chromatin have accelerated advances along this line, and the role of RNA (4, 13, 31) or CNH proteins (2, 10, 11, 27, 29, 30) in regulating template function has been revealed by reconstitution experiments. The electron microscopical appearance of reconstituted chromatin has been reported to be indistinguishable from that of native chromatin (24, 25). In view of the usefulness of the reconstitution procedure, it is of value to investigate the mode of reassociation of dissociated chromatin protein components with DNA during reconstitution and to correlate such data with the changing template functions at various steps of the reconstitution procedure.

Kleiman and Huang (15) studied the sequence of reassociation of histones with DNA during reconstitution and found that the first histone to bind to DNA is H1. Chae (6) studied the mode of reassociation of CNH proteins as well as histones with DNA during reconstitution by SDS-polyacrylamide gel electrophoresis and has pointed out that there are CNH proteins which reassociate with DNA before, simul-

Abbreviations: CNH proteins, chromatin non-histone proteins; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.
taneously and after binding of histones to DNA.

In the present study on reassociation of chromatin protein components with DNA, changing electrophoretic patterns of histones and CNH proteins during reconstitution were analyzed qualitatively and quantitatively by absorbance scannings of the gels. We found that about one-half of the amount of dissociated CNH proteins reassociated with DNA before all histones completed binding to DNA, and the other half after that.

MATERIALS AND METHODS

Growth of cells. Rat ascites hepatoma line (AH 130) used in the following investigation was maintained by serial transplantation in Donryu rats. Cells for experimental use were harvested on the seventh day and stored at −70°C until used.

Preparation of chromatin. Chromatin was prepared as described by Marushige and Bonner (18) with some modifications as follows: Cells were swollen with an equal volume of distilled water for 5 min and then homogenized in 0.075 M NaCl–0.024 M EDTA (pH 8.0). After centrifugation through 1.7 M sucrose, the pellet was dialyzed against 0.01 M Tris–HCl (pH 8.0) and then the chromatin was solubilized by stirring for 1.5 h in 2 M NaCl–10 mM Tris–HCl (pH 8.0). The viscous solution was centrifuged at 10,000 g for 30 min. The supernatant was used as dissociated chromatin.

Reconstitution of chromatin. Urea and sodium acetate were added to the salt–dissociated chromatin to final concentrations of 5 M and 50 mM, and the pH was adjusted to 6.0 by addition of acetic acid. The chromatin dissociated in 2 M NaCl–5 M urea–50 mM sodium acetate (pH 6.0) was dialyzed against the same medium overnight and then dialyzed against 5 M urea–50 mM sodium acetate (pH 6.0) containing NaCl in successively decreasing concentrations of 1.0, 0.6, 0.4, 0.3 or 0.2, and 0.1 or 0 M (4, 13, 27, 32). Each dialysis was for 3 h except for dissociated chromatin in 0.4 M which was dialyzed for 16 h. Finally, the solution was dialyzed against 50 mM sodium acetate (pH 6.0) to remove the urea. At each step of the gradient dialysis, aliquot samples were centrifuged at 156,500 g for 24 h except for the sample in 50 mM sodium acetate (pH 6.0) which was centrifuged for 1.5 h. In investigation of supernatant protein components, the supernatants were dialyzed against 0.01 N acetic acid to remove NaCl and urea, and 0.11 vol of 1 N H₂SO₄ and 4 vol of ethanol were added. After 36 h at −20°C the precipitates were recovered by centrifugation at 10,000 g for 30 min. CNH proteins, as well as histones were quantitatively recovered by this method.

Polyacrylamide gel electrophoreses of proteins by acid-urea gel electrophoresis. The protein samples were electrophoresed on 0.5 × 7 cm, 15% polyacrylamide gels containing 2.5 M urea according to the method of Panyim and Chalkley (21). Aliquots containing histones at 30 μg or less were applied to each gel. Electrophoresis was carried out for 3 h at 150 V. Gels were stained for 1 h in 1% Amido Black 10B–7% acetic acid and destained in 7% acetic acid. Gels were scanned at 600 nm in a Densitrol DMU-2 (Toyo Kagaku Sangyo) with a 2 mm slit, and the relative amount of protein in each histone fraction was determined by integrating the area under each peak of the absorbance scans. Within the range of protein concentration used in this electrophoresis, the amount of bound Amido Black stain varied linearly with the amount of histone applied to the gel as determined by the method of Lowry et al. (17).

Polyacrylamide gel electrophoreses of proteins by SDS-gel electrophoresis. The protein samples were dialyzed against 2% SDS–5% 2-mercaptoethanol–10% glycerol–0.065 M Tris-HCl (pH 6.8) at room temperature overnight and incubated at 50°C for 2 h. Aliquots containing 60 μg or less of CNH proteins were electrophoresed on 0.5 × 10 cm, 10% polyacrylamide gels containing 0.1% SDS at 2 mA per gel using a running buffer of 0.1% SDS–0.192 M glycine–0.025 M Tris (pH 8.3), according to the method of Laemmli (16). After
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Electrophoresis: The proteins were fixed in the gels with 50% trichloroacetic acid (TCA) and washed in 7% acetic acid for 4 h. The washed gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue-50% TCA and destained in 7% acetic acid (14). The gels were scanned, and the relative amount of protein in each band was determined as described above. Within the range of protein concentration used, the amount of bound dye varied almost linearly with the amount of CNH protein applied.

The following standards were used for estimating the molecular weights of CNH proteins: trypsin inhibitor, 21,500 D; bovine serum albumin, 68,000 D; RNA polymerase α subunit, 39,000 D; RNA polymerase β subunit, 155,000 D; and RNA polymerase β' subunit, 165,000 D.

Isolation of histones: Histones were extracted from each nucleoprotein twice for 20 h and 1 h with 0.4 N H₂SO₄. After centrifugation at 25,000 g for 20 min, histones were precipitated from the resulting supernatants with four volumes of ethanol for 36 h at −20°C. The precipitate was recovered by centrifugation.

Chemical determinations: DNA was estimated by the method of Burton (5), using calf thymus DNA as a standard. RNA was estimated by the orcinol method (19), using yeast RNA as a standard. Histones and CNH proteins were assayed by the method of Lowry et al. (17), using rat liver histones and bovine albumin, respectively, as standards. NaCl molarities were determined according to the method of Winkler (33).

RESULTS

Chromatin isolated from rat ascites hepatoma cells was composed in mass ratios of DNA 1.00, histones 1.08, CNH proteins 0.85 and RNA 0.12. The contents of CNH proteins and RNA were higher than those in rat liver chromatin reported previously (32).

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![Figure 1](image-url)

**Fig. 1.** Precipitation of dissociated proteins by centrifugation in the absence of DNA. Chromatin was dissociated in 2 M NaCl-5 M urea-50 mM sodium acetate (pH 6.0) and kept at 4°C overnight. The sample was centrifuged at 156,500 g for 24 h to precipitate DNA. The supernatant was filtered through Toyo filter paper no. 2 and then gradient-dialyzed. The dialysis procedures and centrifugation conditions were the same as those used in the complete system of reconstitution described in Materials and Methods. The amount of protein precipitated is plotted against the amount of DNA present in native chromatin.
Centrifugation of dissociated proteins in the absence of DNA. Chromatin was dissociated in 2 M NaCl-5 M urea-50 mM sodium acetate (pH 6.0) and centrifuged to pellet DNA. The supernatant alone was gradient-dialyzed. The dialysis procedures and centrifugation conditions were the same as those used in the complete system of

![Image of gel electrophoresis](image-url)

Fig. 2. Acid-urea polyacrylamide gel electrophoresis of the protein remaining in supernatant fraction after centrifugation at each step of gradient dialysis. Fig. 2a. Photograph of gels. Fig. 2b. Graphic representation of the amount of each histone fraction. The relative amount of protein in each histone fraction was determined by integrating the area under each peak of the absorbance scans and is expressed as the percentage obtained at a NaCl molarity of 2.0. Each value represents the mean of three determinations. The histone fractions are: H1 (○ ○); H2B (● ●); H2A (■ ■); H3 (△ △); and H4 (▲ ▲).
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Reconstitution described in Materials and Methods. The amount of protein precipitated is shown in Fig. 1. The protein was characterized by acid–urea polyacrylamide and SDS–polyacrylamide gel electrophoreses. The precipitated protein in 0.01 M NaCl–5 M urea contained histone 1 (H1), and the precipitated proteins in sodium acetate buffer contained histone 2B (H2B), 2A (H2A), 3 (H3) and 4 (H4). The CNH proteins were also detectable in all the pellets. The amount of proteins precipitable in the absence of DNA was found to be small, and this centrifugation method for separation of protein from nucleoprotein was demonstrated to be useful and within the limits of small error.

Reassociation of histones. Histones recovered from both supernatant and pellet were electrophoretically examined as described in the methodology section to examine the mode of histone reassociation. Fig. 2 shows the results on supernatant proteins. As shown in Fig. 2b, the decrease of H1 in the supernatant occurred mainly between the NaCl molarities of 0.4 and 0.2, suggesting that the first histone to bind to DNA was H1. At NaCl molarity of 0.2, H1 disappeared completely from the supernatant. The decrease of other histone fractions H2B, H2A, H3 and H4 took place mainly between NaCl molarities of 0.3 and 0.1. At NaCl molarity of 0.1, these histone fractions scarcely remained in the supernatant, suggesting completion of bindings to DNA.

The histones obtained from the pellet were also investigated and the results are presented in Fig. 3. If the recovery of histones was as complete from the pellet as from the supernatant, a decrease of histones in the supernatant should result in a corresponding increase of histones in the pellet. However, some differences from the ex-

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Fig. 4. Absorbance scans of SDS-polyacrylamide gel electrophoresis of protein remaining in the supernatant fraction after centrifugation at each step of gradient dialysis. MW, molecular weight.
pected values were seen (Fig. 3). Relating to this problem, the residues of pellets already treated with 0.4 N H$_2$SO$_4$ to extract histones were electrophoretically examined by the method of Laemmli (16). The study revealed remains of histones in the pellet residues at NaCl molarities of 0.1 and 0 (results not shown). Limited extractability of histones with acid from reconstituted DNA-histone complex has also been reported by other investigators (8). In view of this incomplete acid extraction of histones from some pellet fractions, it is reasonable that greater stress in evaluation should be placed on the results obtained from the supernatant fraction (Fig. 2b) than on those from the pellet fraction (Fig. 3).

Reassociation of CNH proteins. The dissociation of CNH proteins in 2 M NaCl–5 M urea–50 mM sodium acetate (pH 6.0) was not as complete as that of histones. After centrifugation of chromatin dissociated in this solution, about 80% of CNH proteins was dissociated and found in the supernatant and about 20% undissociated in the pellet.

Proteins obtained from supernatant and pellet fractions at each step of gradient dialysis were electrophoresed on SDS-polyacrylamide gel to study the mode of reassociation of CNH proteins during reconstitution. Quantitative recovery and qualitative separation of bands on gels were found to be superior with the supernatant proteins than with the pellet proteins. For these reasons, data obtained from supernatant are used here.

Electrophoretic patterns of the supernatant proteins are shown in Fig. 4. The bands were assigned arbitrary numbers for convenience in order of decreasing molecular weight. As shown in Fig. 5, a decrease of about 20–25% was found in the amount of supernatant CNH proteins at a NaCl molarity of 0.4. This might suggest that a small amount of dissociated CNH proteins could reassociate before histones bind to DNA, since histone binding does not yet occur at a molarity of 0.4 except for H1. When the
The molarity of NaCl was lowered to 0.1 at which binding of all histones to DNA was almost complete, a decrease of about 40–45% was found in the amount of supernatant CNH proteins. The remaining 55–60% of supernatant CNH proteins decreased and disappeared during dialyses against 0 M NaCl–5 M urea and 50 mM sodium acetate buffer. Assuming that a decreased amount of CNH proteins in the supernatant indicates the approximate amount of CNH proteins which reassociaated with DNA,
these results suggest that about one-half of dissociated CNH proteins reassociated with DNA before the completion of binding of histones to DNA and the remaining one-half after that.

As shown in Fig. 6a, a slight reassociation of CNH proteins in bands 3, 8, 10 and 11 seems to be already present at NaCl molarity of 0.4, and then the reassociation was completed at NaCl molarity of 0. It is noted that 60% or more of these CNH proteins reassociated at a NaCl molarity of 0.1, at which all histone fractions completed bindings to DNA. On the other hand, as shown in Fig. 6c, reassociation of CNH proteins in bands 21, 23, 25, 26, 28 and 29 scarcely occurred until the molarity of NaCl decreased below 0.1, and the reassociation was completed at the final step of dialysis against acetate buffer. The mode of reassociation of CNH proteins in bands 18 and 20 (Fig. 6b) was intermediate between the lower bands (Fig. 6a) and the higher bands (Fig. 6c).

**DISCUSSION**

In study of chromatin reconstitution, the method of centrifugation (6, 32) or column chromatography (15) has been used for separation of protein from nucleoprotein. Column chromatography may be superior qualitatively to the centrifugation method. Quantitatively, however, a relatively low recovery has been reported (15) at low NaCl concentration. For both a qualitative and quantitative analysis, a good recovery is necessary. Under the centrifugation conditions used in this study, the DNA associated with proteins precipitated completely. A problem to be clarified was the extent of proteins that did not reassociate with DNA. The usefulness of the centrifugation procedure was shown in the experiment with the sample without DNA, as mentioned in the beginning of the Results.

It has been shown previously that isolated chromatin contains protease activity capable of degrading histones in chromatin (3, 7, 9). Bartley and Chalkley (3) have found that the enzyme is essentially inactive below pH 7.0, and a rapidly replicating mouse ascites tumor line is devoid of enzyme activity in isolated chromatin. Fig. 2a indicates that there is no significant degradation of histones during reconstitution under the conditions described in this paper.

Our results concerning reassociation of histones with DNA show that the first histone to bind to DNA is H1, and this occurs mainly between NaCl molarities of 0.4 and 0.2 in the presence of 5 M urea–50 mM sodium acetate (pH 6.0), and the other histone fractions start to reassociate from a NaCl molarity of 0.3 and complete reassociation at NaCl molarity of 0.1 in the presence of 5 M urea–50 mM sodium acetate (pH 6.0). These results are consistent with the results of Kleiman and Huang (15), who showed that the first histone to bind to DNA was H1 in the presence of 5–7 M urea.

In addition to the band characteristics of normal rat liver H1, a slower migrating band was examined by acid-urea polyacrylamide gel electrophoresis of H1 extracted from rat ascites hepatoma chromatin (unpublished result). Similar band microheterogeneity has been reported in H1 extracted from exponentially growing hepatoma tissue culture cells (20). It is of interest to note that both components of H1 were shown to be similarly reassociated with DNA at the same steps of gradient dialyses during reconstitution (Fig. 2a).
It has been pointed out by Chae (6) that there are CNH proteins which reassociate with DNA before, at the same time and after binding of histones to DNA during chromatin reconstitution and that the bulk of the CNH proteins reassociate with DNA after the binding of histones to DNA. It was shown that about one-half of dissociated CNH proteins reassociate with DNA before all histones complete binding to DNA and about one-half after that. However, the amount of CNH proteins which reassociate with DNA before binding of H1 to DNA remains to be determined.

As for the electrophoretically separated CNH proteins, it was found that CNH proteins in some bands scarcely reassociated until the molarity of NaCl reached 0.1, at which all histones completed binding to DNA, and that CNH proteins in some other bands mainly reassociated before completion of binding of all histones to DNA. Possible areas to be explored in future studies include differences that might exist in direct binding of proteins to DNA and their effect on the DNA-histone interaction.

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