DISSOCIATION AND RECONSTITUTION OF CHROMATIN WITHOUT APPRECIABLE DEGRADATION OF THE PROTEINS

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When chromatin from Novikoff hepatoma ascites cells was dissociated in 3 M NaCl - 7 M urea either at pH 6 or 8, degradation of chromosomal proteins was observed in two-dimensional gel electrophoretic patterns. This degradation was not prevented by 50 mM $NaHSO_3$ but was prevented by 1 mM PMSF (phenylmethylsulfonyl fluoride). Reconstitution of the chromatin components dissociated in 3 M NaCl - 7 M urea - 0.05 M sodium acetate (pH 6.0) containing 1 mM PMSF resulted in reassociation of DNA, histones and the major nonhistone proteins (B24, B26, B33, BE, BJ, C1, C6, CG, CH, CM, C14, CP, C18, CR, CS and C25). Two-dimensional gel electrophoresis showed that although the proportion of the nonhistone proteins to histones was lower in reconstituted than in native chromatin, the template activity of the reconstituted chromatin was similar to that of native chromatin.

INTRODUCTION

Reconstitution of chromatin components has been employed in studies on the role of the nonhistone proteins (NHP) in the tissue specific restriction of the eukaryotic genome (1-3). RNA-DNA hybridization has shown that the RNA transcribed from reconstituted chromatin with bacterial RNA polymerase contains sequences that compete with the RNA products formed in vivo, although misreading may occur (4,5), and the early studies probably detected only highly reiterated RNA species (5). However, no complete analysis was made of the NHP in reconstituted chromatin (6,7).

Dissociation of chromatin by a number of procedures results in extensive degradation of histones (8,9). The initial goal of the study was to reconstitute the chromatin under conditions that

would prevent this degradation. Inasmuch as the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) (9,10) was effective, further studies were made on the composition and template activity of the reconstituted chromatin.

MATERIALS AND METHODS

Preparation of Chromatin - Chromatin was prepared from citric acid nuclei (11) by three washes with 0.075 M NaCl, 0.025 M sodium EDTA (pH 8.0) and 4-5 washes of the residue with 0.01 M Tris (pH 8.0) and centrifugation at 15,000 x g for 30 minutes.

Dissociation of Chromatin - Chromatin was dissociated in 3 M NaCl - 7 M urea in either sodium acetate (0.05 M, pH 6.0) or Tris-HCl (0.05 M, pH 8.0) in the presence or absence of $NaHSO_3$ or PMSF. The solution was adjusted to an $O.D._{260}$ of 15-20 units, stirred at 4^{o} for 5-6 hours and then centrifuged at 105,000 x g for 36 hours in a Beckman Type 35 rotor to pellet the DNA.

Reconstitution of Chromatin - Chromatin was reconstituted by mixing the dissociated proteins in 3 M NaCl - 7 M urea in a 2:1 ratio with the DNA pellet. After stirring for 2 hours at 4^{o}, the solution was sequentially dialyzed (1,2) for 3 hours each against 2 M, 1.5 M, 1.0 M and 0.6 M NaCl containing 5 M urea - sodium acetate (0.05 M, pH 6.0) - 0.1 mM PMSF; then it was dialyzed 12 hours against 0.4 M and 2 hours against 0.15 M NaCl, 5 M urea - 0.05 M sodium acetate (pH 6.0). The urea was removed by dialysis against 0.15 M NaCl in 0.05 M sodium acetate (pH 6.0) containing 0.1 mM PMSF for 4 hours. The reconstituted chromatin was pelleted at 10,000 x g, washed twice with 0.01 M Tris (pH 8.0) and suspended in 0.05 M Tris (pH 8.0).

To visualize the proteins, two-dimensional gel electrophoresis was carried out as described earlier (12,13).

Template Activity - Escherichia coli RNA polymerase pur-

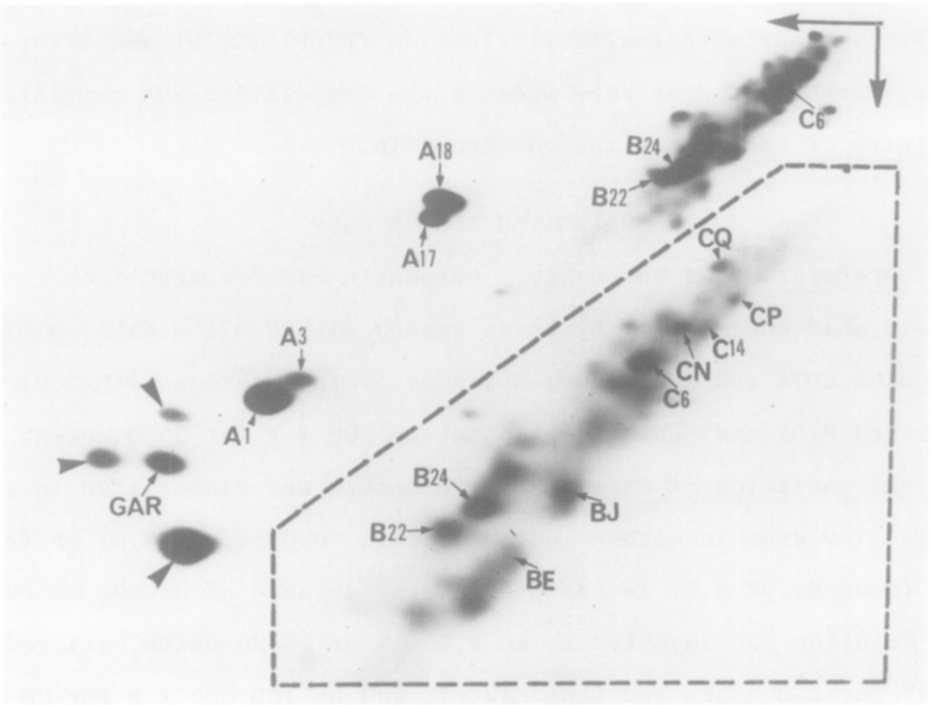

Fig. 1 Two-dimensional gel electrophoresis of chromatin proteins dissociated in 3 M NaCl, 7 M urea, 0.05 M Tris-Cl (pH 8.0) containing sodium bisulfite (50 mM). Approximately 800 µg protein was subjected to first dimensional electrophoresis on 9.5 cm gel rods (10% acrylamide) under acid-urea conditions for 6 hours and longitudinal slices from these rods were then run in the second dimensional SDS slab gels (12% acrylamide) (12). The inset shows the resolution of the higher molecular weight nonhistone proteins in 6% first dimension and 8% second dimensional conditions (13). Pointers in the figures indicate degraded proteins.

chased from Sigma Chemical Co. (St. Louis, Mo.) was used to assay the template activity of the chromatin preparations. The assay mixture contained in 0.2 ml, 50 µmoles Tris-Cl (pH 8.0) 25 µmoles KCl, 2 µmoles $MgCl_2$, 1 µmole $MnCl_2$, 0.2 µmoles of ATP, CTP, GTP and UTP; 0.5 µCi ^{3}H-UTP (Schwarz/Mann, 19 Ci/mmole), 1 µmole β-mercaptoethanol, chromatin containing 1-5 µg DNA, and 5 units of RNA polymerase. ^{3}H-UTP incorporation was measured by the DEAE filter disc method of Blatti et al (14). Assays under these

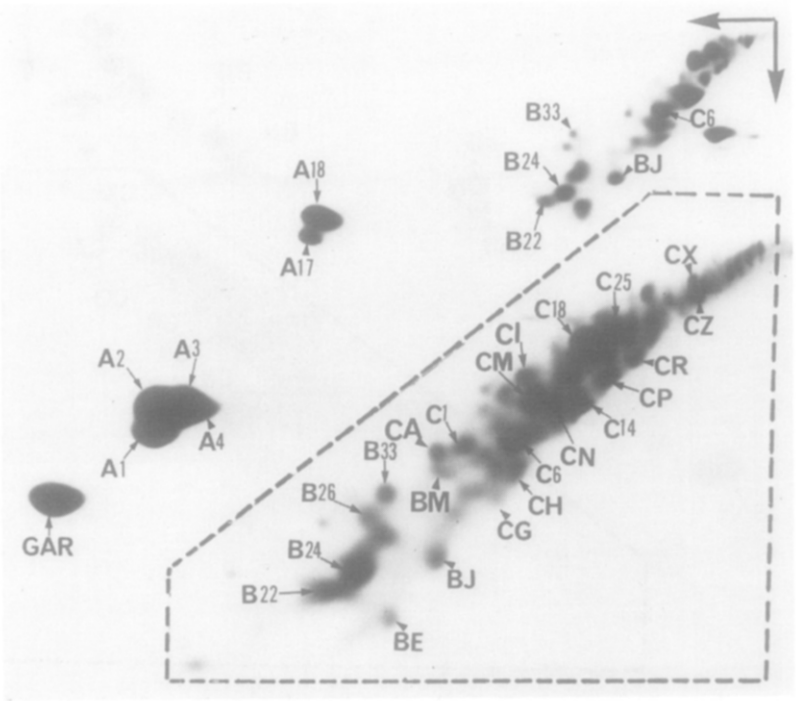

Fig. 2 Two-dimensional gel electrophoresis of chromatin proteins dissociated in 3 M NaCl, 7 M urea, 0.05 M sodium acetate (pH 6.0) containing 1 mM PMSF. Conditions of electrophoresis were as described in Figure 1.

conditions were template limiting. The incubations were carried out at 37° for 20 minutes. Standard assays were used for DNA and protein (15,16) determinations. Deproteinized DNA (17) was kindly provided by Dr. T. O. Sitz.

RESULTS

Dissociation of Chromatin - Dissociation of chromatin in 3 M NaCl - 7 M urea solubilized most of the chromosomal proteins either at pH 6.0 or at pH 8.0 (Fig. 1-3). Two-dimensional gel electrophoresis (12) provided patterns for both histones (GAR, A1-4, A17, A18) and nonhistone chromosomal proteins (Fig. 1-3). Sodium bisulfite (50 mM) at pH 6 or 8.0 did not inhibit proteolysis (2,18) in these preparations (Fig. 1) even when added just

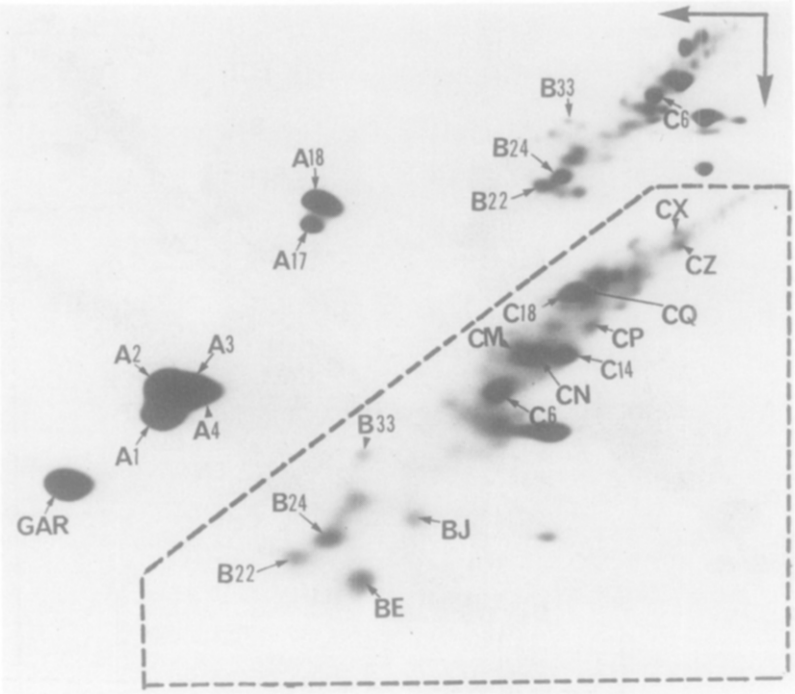

Fig. 3 Two-dimensional gel electrophoresis of chromatin proteins dissociated in 3 M NaCl, 7 M urea, 0.05 M Tris (pH 8.0) containing 1 mM PMSF. Conditions of electrophoresis were as described in Figure 1.

prior to dissociation without readjusting the pH (19). When 1 mM PMSF was added to the dissociating buffer at pH 6.0 (Fig. 2), the degraded protein spots (Fig. 1,pointers) were eliminated. With PMSF, degradation was also reduced at pH 8.0 but to a lesser extent than at pH 6.0 (Fig. 3).

The insets in Figures 1-3 show the protein patterns from the same extracts with the improved resolution provided by using 6% and 8% polyacrylamide in the first and second dimensions, respectively (13). The presence of proteins of high molecular weight such as CX and CZ, as well as the less hazy background in Figure 2, indicates that PMSF also minimized degradation of the NHP.

Reconstitution of Chromatin - Chromatin dissociated at pH

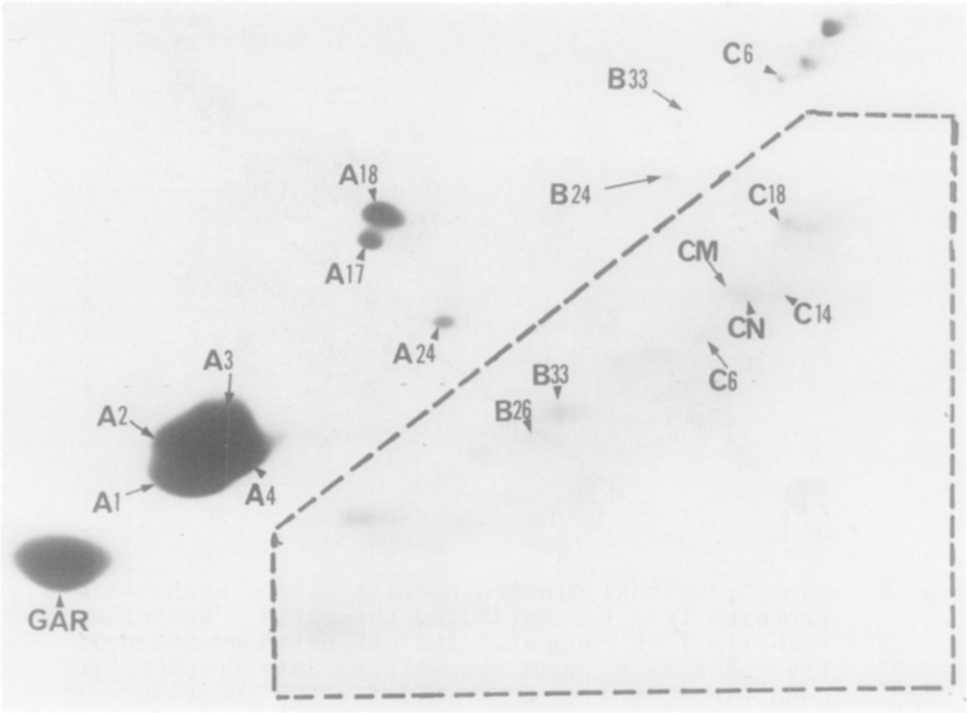

Fig. 4 Two-dimensional electrophoresis of proteins from reconstituted chromatin. Proteins were dissociated from reconstituted chromatin in 3 M NaCl, 7 M urea, 0.05 M sodium acetate (pH 6.0) containing 1 mM PMSF. Electrophoretic conditions were as described in Figure 1.

6.0 in the presence of PMSF was reconstituted by gradient dialysis. To analyze the proteins present, it was redissociated in 3 M NaCl - 7 M urea, sodium acetate (0.05 M, pH 6.0), 1 mM PMSF and centrifuged at 105,000 x g for 36 hours to pellet the DNA. Figure 4 shows the two-dimensional gel pattern of proteins in the supernatant. The amount of NHP reassociated with the DNA was lower than that of the histones (GAR, A1-4 and A17-18). Analysis of the nonhistone proteins from the "dehistonized" Chromatin II fraction (20,21) of reconstituted chromatin showed that the major NHP spots were present including BE, BJ, B24, B33, C1, CG, CH, C6, CM, C14, CP, C18, C25, CR and CS (Fig. 5). By contrast, the

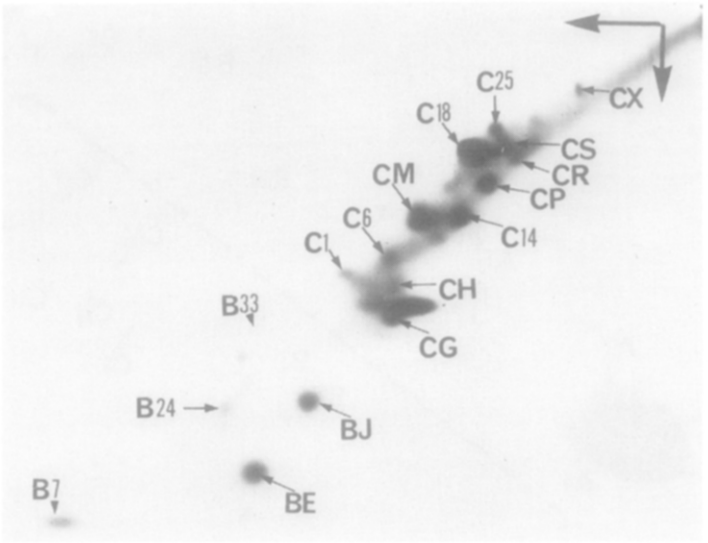

Fig. 5 Two-dimensional electrophoresis of the nonhistone proteins from reconstituted chromatin. Proteins were released from reconstituted chromatin by DNase I treatment after acid extraction. Electrophoretic conditions were as described for the inset in Figure 1.

densities of spots B24, B26, and B33 were much lower and spots BM, CA, CI and CZ were absent.

Template Activity - When the chromatin was dissociated and reconstituted under conditions used in earlier studies (1-3), the template activity of the chromatin reconstituted in the presence of $NaHSO_3$ was much higher than that of the undissociated chromatin (Table I). The template activity of the chromatin reconstituted with NHP prepared at pH 6.0 in the presence of PMSF was approximately the same as undissociated chromatin (Table I). The protein to DNA ratios of isolated chromatin and reconstituted chromatin were 2.23 and 2.27, respectively.

DNA Pellet - After centrifuging the chromatin dissociated at pH 6.0 for 36 hours at 105,000 x g, the DNA pellet contained a small amount of acid-insoluble proteins. This pellet had 85% of

TABLE I

TEMPLATE ACTIVITIES USING E. COLI RNA POLYMERASE

Template	[^{3}H]-UMP(C.P.M.) incorporated/μg DNA	Percent Template Activity
DNA	42,000	100
DNA pellet	35,000	85
Chromatin	1,066	2.50
Reconstituted Chromatin[1]	850	2.02
Reconstituted Chromatin[2]	5,500	13.10

[1]Dissociation and reconstitution in the presence of PMSF.

[2]Dissociation and reconstitution in the presence of sodium bisulfite (50 mM).

template activity of deproteinized DNA (Table I). The DNA pellet was treated with 0.4 N H_2SO_4 and then with DNase I to release these NHP (21); two-dimensional gel analysis revealed only protein spots BE, BJ, CG' and CH in this fraction (Fig. 6).

DISCUSSION

The two-dimensional polyacrylamide gel electrophoretic method (12,13) permits ready identification of degraded nuclear protein products and provided a basis for these studies on prevention of nuclear protein degradation by PMSF. Reconstitution of chromatin demonstrated that many NHP isolated in the presence of PMSF readily reassociated with DNA, although to a lower extent than the histones. Other NHP either reassociated with DNA to a lesser extent or not at all. The template activity was much lower in chromatin reconstituted with undegraded than with de-

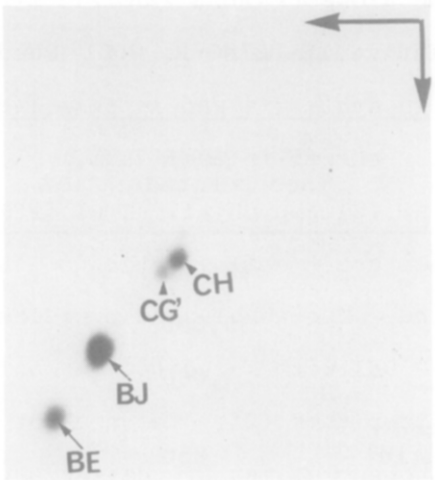

Fig. 6 Two-dimensional gel electrophoresis of proteins associated with the DNA pellet obtained after dissociation of the chromatin. Proteins were solubilized from the DNA pellet by DNase I treatment as described in the text. Electrophoretic conditions were as described for the inset in Figure 1.

graded nuclear protein preparations. Interestingly, the template activity of chromatin reconstituted with undegraded proteins was 2.0% of that of free DNA compared with 2.5% of free DNA with intact, freshly isolated chromatin. It remains to be determined whether RNA produced in these systems bears a meaningful structural and functional relationship to RNA produced *in vivo* or with native chromatin (4,5).

It is not clear which, if any, of the proteins that reassociate with DNA serve as gene control proteins (1-3). The small number of proteins that do not dissociate from DNA (Fig. 6) with 3 M NaCl - 7 M urea repress DNA template activity by 15% (Table I), but their possible activity as gene activators has not been determined. It has been assumed that other NHP are gene activators because of the greater template activity of chromatin reassociated in the presence of NHP and histones than in the

presence of histone alone (1-3). Specific systems for evaluation of specific gene activation are now being sought including globin synthesis, ovalbumin synthesis and in this laboratory, synthesis of rRNA on nucleolar chromatin templates.

Spelsberg et al (2) and Chytil et al (22) indicated that at pH 6.0 only histones and a small portion of the nonhistone proteins were extracted. The nonhistone proteins that remained associated with the DNA were suggested to confer tissue specificity on chromatin templates (2). The present results show that most of the nonhistone proteins are dissociated along with the histones at pH 6.0 (Fig. 2,6). The 4-5 proteins (Fig. 6) that remain associated with DNA may function as tightly bound gene repressors or may be structural proteins.

PMSF did not appreciably effect the reassociation of histones and DNA. Of the NHP, about 25 proteins demonstrated by gel electrophoresis reassociated with DNA but a substantial number of proteins did not. The template activity of the reconstituted chromatin was also quite similar to that of native chromatin; it remains to be determined whether the selected group of proteins that reassociated with DNA determine the specificity of gene readouts.

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