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PURIFICATION FROM CULTURED HEPATOMA CELLS OF TWO NONHISTONE CHROMATIN PROTEINS WITH PREFERENTIAL AFFINITY FOR SINGLE-STRANDED DNA: APPARENT ANALOGY WITH CALF THYMUS HMG PROTEINS*

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SUMMARY

Sequential chromatography on double-stranded DNA and single-stranded DNA columns selects two proteins with marked preference for single-stranded DNA from the complex set of proteins that is released by NaCl from chromatin of cultured hepatoma cells. By a number of criteria, these two proteins appear to be analogous to the calf thymus chromatin proteins HMG-1 and HMG-2.

INTRODUCTION

Proteins that bind more tightly to single-stranded DNA than to double-stranded DNA have been isolated from a variety of prokaryotic and eukaryotic sources. The most thoroughly studied protein of this sort is coded for by gene 32 of bacteriophage T4. The gene 32 protein has been shown by genetic analysis to be required for replication of the phage genome (1). By virtue of its greater affinity for single-stranded DNA than for double-stranded DNA, the gene 32 protein destabilizes the double helix (2), and it is therefore believed that the protein functions to promote strand separation at replication forks (3). Analogs of the gene 32 protein presumably perform the same function in eukaryotic cells, and proteins with preferential affinity for single-stranded DNA have in fact been isolated from a number of eukaryotic sources using sequential chromatography, first on immobilized double-stranded DNA and then on immobilized single-stranded DNA (see, for

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example, reference 4 and references cited there). Whether any of the eukaryotic proteins isolated in this manner functions in DNA replication or any other nuclear process is uncertain, in part because the sequential chromatography scheme has been applied to homogenates of whole cells or organs. In the most comprehensive study of such proteins from a eukaryote, Herrick and Alberts concluded that the proteins they isolated from calf thymus were in fact located in the cytoplasmic fraction of the homogenized thymus glands (4).

In this report, we describe a search for proteins with preferential affinity for single-stranded DNA among the nonhistone chromatin proteins of HTC cells, an established line of cultured hepatoma cells (5). Working, as we have, with a preparation of chromatin proteins rather than with extracts of whole cells or organs should increase the likelihood of isolating proteins involved in nuclear events. Furthermore, chromatin from the rapidly proliferating cultured cells might be a good source of proteins that are part of the DNA replication machinery. As a precautionary measure, we have used NaCl, a mild agent, to dissociate the nonhistone chromatin proteins in order to minimize the possibility of protein denaturation (6).

METHODS

HTC cells were grown in suspension culture, as described previously (6), in Tricine-buffered Swim's 77 medium supplemented with 5% calf serum and 5% fetal calf serum. Under our growth conditions, the cells had a generation time of approximately 20 hr. Calf-thymus DNA (Sigma, Type 2) was purified by repeated extraction in chloroform/isoamyl alcohol (24:1). The purified DNA was sonicated with three 10 sec bursts at a setting of 115 watts on a Branson Sonifier equipped with a microtip. The DNA was dialyzed against 10 mM potassium phosphate (pH 8.5). A portion of the DNA was denatured at 100° for 30 min and then cooled rapidly in an ice bath. Double-stranded and single-stranded DNA samples were covalently linked to cyanogen bromide-activated Sepharose 4B (Sigma) as described by Arndt-Jovin et al. (7). To 5 g portions of activated Sepharose, 20 mg of double-stranded DNA and 50 mg of single-stranded DNA were attached. The double-stranded DNA Sepharose was packed in a glass column and equilibrated to 5% glycerol/10 mM NaCl/1 mM $ZnSO_4$/50 mM sodium acetate (pH 4.5). 10 units of S_1 nuclease (Calbiochem) were circulated through the column for 10 hr at room temperature. Under the conditions used, the S_1 nuclease had no significant activity against double-stranded DNA. Double-stranded and single-stranded DNA columns were equilibrated to 50 mM NaCl/1 mM Tris-Cl (pH 7.5).

HTC cell nuclei and chromatin were isolated as described elsewhere (6). Liver nuclei from Buffalo rats were isolated using a slight modification (8) of the method of Blobel and Potter (9). Unsheared HTC cell chromatin was adjusted to 0.75 M NaCl/10% sucrose/10 mM Tris-Cl and subjected to centrifugation at 150,000 g for 3 hr at 4° in a Spinco SW-27 rotor. (In this and all subsequent steps, the pH of Tris-Cl solutions was 7.5 at room temperature.) A large portion of the DNA was pelleted in the centrifugation. The supernatant was removed and applied to a Sepharose CL-2B column (2.5 x 83 cm) that had been equilibrated to 0.75 M NaCl/10 mM Tris-Cl. The elution profile contained two peaks; the first contained nucleoprotein and the second contained dissociated protein that was essentially free of DNA, as judged by absorbances at 220 and 260 nm. The protein in the second peak was diluted to 0.5 M NaCl by adding one-third volume of 10 mM Tris-Cl. The sample was then passed over a Bio-rex 70 column that had been equilibrated to 0.5 M NaCl/10 mM Tris-Cl. The unbound material (consisting entirely of nonhistone proteins) was dialyzed against 50 mM NaCl/1 mM Tris-Cl. Approximately 50% of the protein precipitated during the dialysis. The dialysate was centrifuged at 10,000 g for 10 min, and 82 A_{220} units of the protein in the supernatant were applied to the double-stranded DNA column. The material that failed to bind to the column was applied directly to the single-stranded DNA column. Protein was eluted from the single-stranded DNA column in two steps with 0.25 M NaCl/1 mM Tris-Cl and with 0.5 M NaCl/1 mM Tris-Cl.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was conducted in 15% slab gels (6), using the electrophoresis system described by Laemmli (10). Proteins used in estimating polypeptide molecular weights were bovine serum albumin, ovalbumin, chymotrypsinogen A, and myoglobin. Amino acid analyses were conducted on a Beckman 120C amino acid analyzer after hydrolysis for 24 hr in 6 N HCl at 110°.

RESULTS AND DISCUSSION

We routinely used 0.75 M NaCl/10 mM Tris-Cl (pH 7.5) for chromatin dissociation on the basis of our finding that 0.75 M NaCl is as effective as higher NaCl concentrations in releasing nonhistone protein mass from HTC cell chromatin (6). 0.75 M NaCl released a complex set of proteins (Track 1, Figure 1). The dissociated proteins were treated as described in Methods to obtain a sample for chromatography on double-stranded DNA Sepharose. Approximately 70% of the protein in the sample was retained by the double-stranded DNA column. Essentially all of the protein that failed to bind was retained in the subsequent chromatography on single-stranded DNA Sepharose. A rather heterogeneous set of polypeptides was eluted from the single-stranded DNA column by 0.25 M NaCl/1 mM Tris-Cl (Track 2, Figure 1), but the material eluted by subsequent application of 0.5 M NaCl/1 mM Tris-Cl consisted almost exclusively of two polypeptides

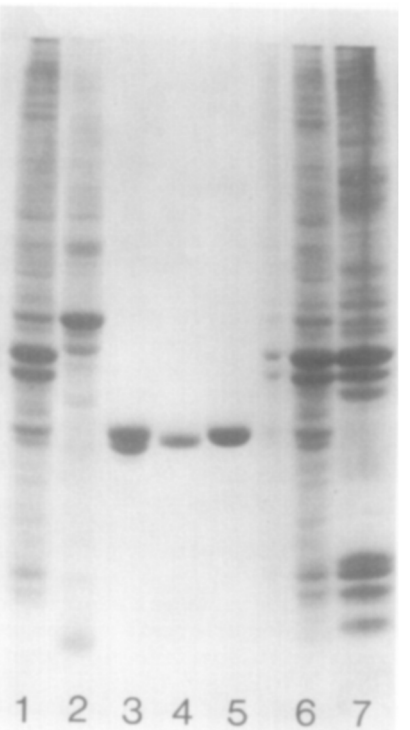

Figure 1. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of chromatin proteins. The samples were: Track 1, unfractionated HTC cell chromatin proteins dissociated by 0.75 M NaCl; Track 2, material eluted from single-stranded DNA column with 0.25 M NaCl/1 mM Tris-Cl; Track 3, material eluted from single-stranded DNA column with 0.5 M NaCl/1 mM Tris-Cl; Track 4, NH-2 from DEAE cellulose (see Figure 2); Track 5, NH-1 from DEAE-cellulose (see Figure 2); Track 6, same sample as in Track 1; Track 7, unfractionated proteins dissociated from rat liver chromatin by 0.75 M NaCl.

(Track 3, Figure 1). We have designated the major polypeptide of lower mobility NH-1 and the other major polypeptide NH-2. By gradient elution, we estimate that NH-1 and NH-2 require approximately 0.35 M NaCl for their elution from the single-stranded DNA column (11). Double-stranded DNA columns used in this isolation scheme were treated with S_1 nuclease as described in Methods. If a double-stranded DNA column was not exposed to S_1 nuclease, NH-1 and NH-2 were retained by the column, presumably by binding to single-stranded regions of the nominally double-stranded DNA.

NH-1 and NH-2 were separated from each other by chromatography on DEAE-cellulose (Figure 2, and Tracks 4 and 5 in Figure 1). The two proteins,

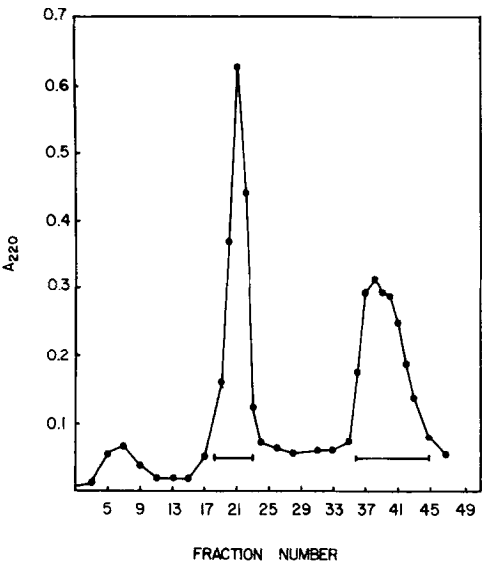

Figure 2. Separation of NH-1 and NH-2 by DEAE-cellulose column chromatography. The material eluted from single-stranded DNA with 0.5 M NaCl/1 mM Tris-Cl was dialyzed against 1 mM Tris-Cl (pH 7.5) and applied to a DE-32 column (0.5 X 20 cm) that had been equilibrated to 1 mM Tris-Cl. At fraction number 12, an 80 ml linear gradient in NaCl (0 to 0.15 M) was applied. Fraction volume, 2.0 ml. Bars indicate fractions that were combined for gel electrophoresis (Figure 1) and amino acid analysis (Table I): the peak centered at tube 20 contained NH-2.

which evidently possess marked preference for single-stranded DNA, are among the most prominent nonhistones that are extracted by 0.75 M NaCl from HTC cell chromatin (Track 1, Figure 1). We obtained 2 μg of NH-1 per mg of HTC cell chromatin DNA. Using an estimated molecular weight of 28,000 obtained by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, the NH-1 content of HTC cell chromatin can be calculated to be 4 X 10^5 molecules per haploid genome (assuming 100% yield in the isolation scheme). An essentially identical recovery was obtained in a single preparation in which chromatin was dissociated in 2 M NaCl rather than in 0.75 M NaCl. In that preparation, no additional polypeptides were observed in the material eluted from the single-stranded DNA column with 0.5 M NaCl/1 mM Tris-Cl.

We did not observe polypeptides with the mobilities of NH-1 or NH-2 in the proteins dissociated from adult rat liver chromatin by 0.75 M NaCl (Track 7, Figure 1). Hence, if NH-1 and NH-2 are present in adult rat liver

TABLE I

Amino Acid Compositions of NH-1, NH-2, HMG-1, and HMG-2[a]

	NH-1[b]	NH-2[b]	HMG-1[c]	HMG-2[c]
Lys	17	16	21.8	21.8
His	2.5	1.5	1.6	1.9
Arg	3.7	3.8	3.3	4.5
Asp	11	8.4	11.0	10.0
Thr	3.4	3.4	2.9	2.3
Ser	6.1	6.6	5.4	7.4
Glu	19	16	18.3	15.7
Pro	5.7	5.6	5.5	8.0
Gly	9.3	14	5.8	6.5
Ala	9.5	7.9	8.9	7.7
Cys	d	d	0.0	0.0
Val	2.4	4.4	2.2	1.8
Met	0	0.5	1.9	1.9
Ile	2.0	3.2	2.1	1.7
Leu	2.9	4.2	2.5	2.3
Tyr	1.4	1.9	2.7	2.6
Phe	3.6	2.8	4.0	3.8

[a]Values are given in mole percent. [b]Average of single analyses on three different preparations. No corrections have been made for hydrolytic losses. [c]Taken from Goodwin et al. (12). [d]Half-cystine values were not determined for NH-1 and NH-2.

chromatin, they occur in substantially lower amounts than in HTC cell chromatin. Amino acid compositions of NH-1 and NH-2 are presented in Table I.

NH-1 and NH-2 appear to be analogous to the calf thymus chromatin proteins HMG-1 and HMG-2, first described by Goodwin et al. (12). Like HMG-1 and HMG-2, NH-1 and NH-2 are dissociated from chromatin at moderate NaCl concentrations; all four proteins are soluble in 2% trichloroacetic acid and insoluble in 10% trichloroacetic acid. The estimated molecular weights of NH-1 and NH-2 (around 28,000) are close to the molecular weights reported for HMG-1 and HMG-2 (13). Finally, the amino acid compositions of NH-1 and NH-2 resemble those of HMG-1 and HMG-2 (Table I). The similarities in composition include a rather high total content of Glx, Asx, Lys, and Arg, which is a characteristic feature of the calf thymus HMG proteins (12).

The functions of HMG-1 and HMG-2 and their analogs from sources other than calf thymus are not yet known. Goodwin et al. (12) have suggested that the proteins play as yet ill-defined structural roles. Results pre-

sented by Levy et al. (14) and Vidali et al. (15) suggest that HMG proteins are preferentially associated with transcribed DNA sequences. [Goodwin and Johns (16) recently reported conflicting results, however.] Javaherian et al. (17) reported that calf thymus HMG-1 and HMG-2 are capable of reducing the linking number of circular PM2 DNA molecules when the DNA molecules are ligated in the presence of either protein. They pointed out that one mechanism by which the proteins might reduce the linking number is by preferentially binding to single-stranded DNA. Our results with apparently analogous proteins from rat hepatoma cells are consistent with calf thymus HMG-1 and HMG-2 possessing preferential affinity for single-stranded DNA. The two studies taken together suggest that preferential affinity for single-stranded DNA may be a general property of high molecular weight HMG proteins. That property could be important to the proteins' carrying out structural roles or participating in active processes, including transcription or replication. The enrichment of NH-1 and NH-2 that we have observed in chromatin of rapidly dividing, cultured HTC cells would be consistent with these proteins' being involved in DNA replication (possibly in a manner analogous to the gene 32 protein) or in other nuclear events characteristic of proliferating cells. It should be noted that Seyedin and Kistler (18) have reported that cessation of cell proliferation in rat testes results in disappearance of a protein analogous to HMG-2.

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