Purification of nuclear antigens in Novikoff hepatoma

(chromatin/DNA binding/nonhistone proteins)

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ABSTRACT Nuclear antigen in Novikoff hepatoma chromatin was partially purified and characterized. As indicated by complement fixation assay, this antigen was present in chromatin of embryonic livers and several transplantable tumors. It was not detected in normal tissue chromatins of the same animals. For its immunological specificity this protein antigen (molecular weight 45,000–80,000) had to be complexed with DNA. Preliminary experiments indicate that specific nuclear protein antigens are also present in human tissues and spontaneous malignancies.

Nuclear transplants as well as other experiments indicate that malignant transformation may reflect a profound change in cellular differentiation. The new (cancerous) phenotype of the newly differentiated cells is maintained through the cellular progenies during the proliferation of the tumor. In view of the widely accepted notion that certain chromosomal nonhistone proteins may be responsible for the tissue-specific regulation of genetic expression, the identification of such regulatory proteins associated with malignant growth is a necessary step toward the basic understanding of carcinogenic mechanism at the level of gene transcription. Assuming that the regulatory proteins may be specific for the particular types of cells and tissues, we have used an immunological approach in the search for such macromolecules. We report here a partial purification of a growth-associated nuclear antigen in Novikoff hepatoma.

MATERIALS AND METHODS

Isolation of Nuclei and Chromatin. Rat liver nuclei were isolated by a modification of the hypotonic sucrose procedure of Blobel and Potter (1). Novikoff hepatoma nuclei were isolated by hypotonic shock, high pressure shearing, and centrifugation in hypotonic sucrose (2). The isolation of chromatin was described previously (3).

Fractionation of Chromosomal Proteins. For immunization, isolated chromatins were dehydrized by dissociation with 5 M urea and 2.5 M NaCl in 50 mM sodium succinate buffer (pH 5.0) (4). After ultracentrifugation, the pellets containing chromosomal nonhistone proteins and DNA were homogenized in 0.1 mM MgCl₂/2 mM Tris-HCl buffer (pH 7.5). The suspension was stirred overnight at 4° and centrifuged at 1000 × g for 5 min to sediment a small amount of particulate material. The supernatant was used for immunization.

For fractionation by hydroxylapatite chromatography, the bulk of chromosomal nonhistone proteins was first removed by extraction with 5.0 M urea/0.1 M phenylmethylsulfonyl fluoride in 50 mM sodium phosphate buffer (pH 7.6). The mixture was gently homogenized (0.3–0.5 mg of DNA per ml) and stirred for 2 hr in an ice bath. Chromatin, partially deproteinized by this procedure (removal of nearly 50% of total chromatin protein content), was sedimented by centrifugation at 15,000 × g for 60 min. The pellet containing DNA, histones, and a small amount of nonhistone proteins was rehydrated by homogenization in 1.5 mM NaCl/0.15 mM sodium citrate/0.1 mM phenylmethylsulfonyl fluoride at a concentration of 2 mg of DNA per ml. The mixture was sonified with a Bronson Sonifier cell disruptor in 15-sec intervals for a total of 90–120 sec at 80–90 W output. The sonication was monitored by mixing a small aliquot of the sonicated sample with an equal volume of 5 M NaCl in 0.1 M Tris-HCl (pH 8.0) to which reagent-grade urea was added to saturation. The shearing was discontinued when viscosity of the solubilized sample became sufficiently low for application to the hydroxyapatite column.

Hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) was freed of fine particles by sedimentation, suspended in 2.0 M KCl/5.0 M urea/0.1 M phenylmethylsulfonyl fluoride/2 mM Tris-HCl/1 mM potassium phosphate buffer (pH 7.0) and left to equilibrate overnight.

The sonicated, partially deproteinized chromatin, suspended in 1.5 mM NaCl/0.15 mM sodium citrate, was solubilized in the KCl buffer described above by adding concentrated buffer mixture to the suspension.

For chromatography, the hydroxyapatite was gravity packed in a column (2.7 × 10–12 or 5.1 × 12–15 cm) and washed with the elution buffer containing 1 mM potassium phosphate. The chromatin sample was applied to the column and eluted sequentially with KCl buffer containing 1 mM, 50 mM, 100 mM, 200 mM, and 500 mM potassium phosphate (pH 7.0). The eluted fractions were concentrated by filtration through Amicon Diaflo PM-10 membrane. Samples in small volumes were concentrated by reverse dialysis against dry Sephadex G-200.

Electrophoresis. For analytical polyacrylamide gel electrophoresis, the system of Shapiro et al. (5) was modified to include urea in the gel and sample buffer (2). The gels were stained with Coomassie brilliant blue and scanned with a Gilford gel scanner at 600 nm.

The method of Laemmli (6) was followed for preparative separation of high-molecular-weight nonhistone proteins eluted with buffer containing 50 mM potassium phosphate. A slab gel apparatus (Schoeffler Scientific Inst., model SE 500) was used with 3-mm thick 13 × 10-cm slabs of 8.75% polyacrylamide and 3% stacking gel. Concentrated proteins of the 50 mM potassium phosphate fraction (total of 8 mg) were applied to two such gels and electrophoresed simultaneously at 70 mA per gel until the bromphenol blue tracking dye reached approximately 5 mm from the end of the gel. Three 5-mm longitudinal strips cut out from both sides and the middle portion of the gel were stained and the remaining portions of the gel were cut to separate major groups of protein bands. Crushed gels were eluted with 0.2% sodium dodecyl sulfate and 1% 2-mercaptoethanol in 50 mM Tris-HCl (pH 8.0), dialyzed extensively against 0.6 M NaCl/5.0

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M urea/0.1% 2-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride/50 mM Tris-HCl (pH 8.0), and finally concentrated to approximately 1 mg of protein per ml. A modification of the method of Weber and Kuter (7) was used for the removal of sodium dodecyl sulfate by chromatography on Dowex AG1X2 resin. Over 99% of the protein-bound detergent was removed by this procedure.

**Immunosassay Procedures.** Because the immunological reactivity of the nuclear nonhistone proteins described here depends on their association with homologous DNA (4, 8-10), all protein samples for immunosassays were reconstituted to DNA. Purified rat spleen DNA was mixed with the protein functions at a ratio of 0.4 mg of protein per 1 mg of DNA in 2 ml of 2.5 M NaCl/5 M urea/50 mM Tris-HCl buffer (pH 8.0), and NaCl was slowly removed by gradient dialysis against buffered urea. Finally, urea was removed by dialysis against 1.5 mM NaCl/0.15 mM sodium citrate (11).

The complement fixation technique of Wasserman and Levine (12) was used to test the immunospecificity of antibodies. The antisera were decomplemented by heating at 56°C for 30 min. Lyophilized guinea pig serum complement, washed sheep erythrocytes, and rabbit anti-sheep erythrocyte serum were purchased from Capel Laboratories (Downington, PA).

**Analytical Procedures.** The protein, DNA, and RNA concentrations were determined by the methods of Lowry et al. (13), Burton (14), and Mejbaum (15), respectively. Sodium dodecyl sulfate was assayed by titration with methylene blue (16).

**RESULTS**

Isolated chromatin contains antigens specific for differentiated cells and organs. The process of carcinogenesis results in a phenotypically new tissue, the tumor. This change is reflected in the chromatin composition of the affected cells and can be detected by complement fixation of chromatin in the presence of appropriate antisera (4). In rat, the antisera against dehistonized Novikoff hepatoma chromatin reacted strongly with chromatin of several transplantable rat tumors (Fig. 1A and refs. 9 and 17). While chromatin from the normal liver and thymus did not fix the complement, chromatin from embryonic liver was reactive. A different situation was observed for spontaneous human malignancies (Fig. 1B). Here only the breast carcinoma chromatin fixed complement in the presence of antisera against dehistonized human breast carcinoma chromatin. Lung carcinoma, normal breast tissue, benign breast tumor, HeLa cell, Novikoff hepatoma, and human placenta chromatin were all nonreactive.

In search for a tumor-specific antigen, Novikoff hepatoma chromatin was partially deproteinized, sheared, dissociated, and fractionated on hydroxylapatite columns (Fig. 2). A nonhistone protein fraction eluting with 50 mM potassium phosphate contained the immunoreactive material (Fig. 3). This fraction represented less than 4% of the total protein content of Novikoff hepatoma chromatin. Because of its considerable electrophoretic heterogeneity, this fraction was subjected to preparative electrophoresis in polyacrylamide gel. After electrophoresis, the protein bands were located and cut according to the scheme in Fig. 4 and the eluted proteins were freed of sodium dodecyl sulfate by ion exchange chromatography. As shown in Fig. 5, proteins represented by three major bands (molecular weight 45,000-60,000) were immunoreactive when reconstituted with rat spleen DNA. Digestion of the active complexes with DNase or protease abolished their immunological activity.

**DISCUSSION**

Production of antibodies against a vast variety of antigens and their subsequent identification and analysis represents perhaps the most powerful tool for probing the diversity and specificity of cellular components. While most of the published immunogenic materials described are of extracellular or cytoplasmic origin, the presence of specific antigens in the cell nucleus became known relatively recently. Since essentially all nuclear macromolecules can be potentially immunogenic, investigations into their tissue and cell specificity are especially important.

Poor solubility and extensive heterogeneity of chromosomal nonhistone proteins discouraged serious studies of their immunological specificity. Early reports on immunological tissue specificity of nuclear proteins and nucleohistone (18, 19) did not stimulate much interest until Chytil and Spelsberg (20) showed that dehistonized chromatin can elicit the formation of tissue-specific antibodies in rabbits. Their results were con-
confirmed by other investigators (8, 21), and it was shown in our laboratory that the exceptional specificity of the nuclear protein antigens depends on their association with DNA of the same species (4, 8–10). Indeed, when the antigenic complexes of chromosomal nonhistone protein from Novikoff hepatoma with rat DNA were digested by DNase or trypsin, the immunoreactivity was lost.

These observations are extended in this contribution in which the antigen present in Novikoff hepatoma and other tumors of the rat is identified as a complex of rat DNA with protein(s) migrating on polyacrylamide gel in the molecular weight range between 45,000 and 60,000. The relatively high molecular weight as well as its dependence on the presence of homologous DNA distinguished this antigen from other antigenic nuclear
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FIG. 5. Complement fixation of fractions obtained by preparative electrophoresis of the hydroxylapatite fraction of Novikoff hepatoma eluted with 50 mM phosphate buffer (see Fig. 4). All samples were reconstituted with rat spleen DNA and assayed in the presence of antisera against dehistonized chromatin of Novikoff hepatoma. ●, Unfractionated 50 mM eluate; ▲, electrophoretic fraction 4; ▼, electrophoretic fraction 7; □, all other electrophoretic fractions.

molecules, e.g., the 26,000-dalton nuclear antigen described by Yeoman et al. (22) or the DNA binding proteins isolated from human sera (23, 24).

The specificity of DNA-binding chromosomal nonhistone proteins changes dramatically during cell differentiation (4, 25), chemical (4) or viral (21) carcinogenesis, and spontaneous neoplasia (26). It can be speculated that if these DNA-dependent nuclear antigens represent a part of mechanisms by which cells express and maintain their phenotype, our experiments may open new ways for detailed biochemical and immunological studies on cell differentiation and carcinogenesis.

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