THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 256, No. 15, Issue of August 10, pp. 8117-8123, 1981
Printed in U.S.A.

Tissue Specificity and Cellular Distribution of Novikoff Hepatoma Antigenic Proteins p39, p49, and p56*

(Received for publication, February 5, 1981, and in revised form, April 27, 1981)

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Three Novikoff hepatoma protein antigens (approximately M_r = 39,000, 49,000, and 56,000) were partially purified from Novikoff hepatoma chromatin. Rabbit antiserum to these three proteins was used to examine various rat tissues for the presence of these antigens. Immunological specificity of the antiserum was assessed using quantitative microcomplement fixation assay or by visualization of the immunoreactive complexes with peroxidase-antiperoxidase procedure after transferring the electrophoretically separated proteins to nitrocellulose sheets. The immunoreactivity was localized with the three proteins p39, p49, and p56 in Novikoff hepatoma. The p56 protein was found to be present in normal rat liver, 24-h regenerating rat liver, fetal rat liver, or kidney, albeit in much smaller amounts as found in Novikoff hepatoma. The p49 and p39 antigens were specific for Novikoff hepatoma. Immunoabsorption experiments confirmed the specificity of this antiserum. Assessment of various subcellular fractions of Novikoff hepatoma revealed that the p39, p49, and p56 protein antigens are present in the cytoplasmic fractions as well as in isolated chromatin.

The search for antigens specific for neoplasia has received considerable attention due to potential diagnostic and therapeutic applications. Several classes of cancer-associated antigens have been described such as carcinoembryonic antigen (1), preneoplastic antigen (2), and various nuclear antigens such as Epstein-Barr virus-associated antigen (3), "extractable nuclear antigen" (4), and the nuclear and nucleolar antigens reported by Busch and coworkers (5-8).

Previous studies from this laboratory using complement fixation assays have shown Novikoff ascites hepatoma to contain antigens for which the immunological specificity changed during tissue differentiation or carcinogenesis (9-15). Fractionation of isolated Novikoff hepatoma chromatin resulted in the separation of protein components partially responsible for specific immunological activity of antisera to dehistonized Novikoff hepatoma chromatin (16). This communication describes studies in which we have used antisera to three Novikoff hepatoma proteins (p39, p49, and p56) to characterize their tissue specificity and subcellular distribution. Furthermore, we have utilized a modification of the procedure of Towbin *et al.* (17) recently developed in our laboratory (18) for the transfer of proteins from polyacrylamide gels to nitrocellulose sheets and visualization of the immunoreactive components by the peroxidase-antiperoxidase reaction of Sternberger (19).

* This work was supported by National Institutes of Health Grant CA-26412. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

EXPERIMENTAL PROCEDURES

Materials—Novikoff ascites hepatoma was maintained by weekly transplantations in male Sprague-Dawley rats (140-180 g). The hepatoma cells were collected by low speed centrifugation and washed twice with standard saline citrate as described (16). Mature male rat (140-180 g) and fetal rat tissue (16-18 days pregnant) were collected immediately after sacrifice. For regenerating rat liver, mature male rats were partially hepatectomized (20) under light ether anesthesia. Regenerating livers were then excised and used 24 h after surgery (21).

Electrophoretic supplies and the molecular weight standards were purchased from Bio-Rad.

Fractionation of Tissues—All the preparative procedures were done at 0-2 °C, unless indicated otherwise. Animals were killed by cervical dislocation, and tissues were collected in phosphate-buffered saline, washed with several changes of PBS,[1] weighed, and suspended in 4-5 volumes of S-TKM buffer (0.25 M sucrose, 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.5). After mincing with scissors, the tissues were homogenized using a tight fitting Teflon-glass homogenizer. The total amount of cells (broken and whole) in the homogenate was determined by hemocytometer. The homogenate was then centrifuged at 1,000 × *g* for 10 min, the low speed supernatant was collected, and the pellet was rehomogenized as above. After another centrifugation at 1,000 × *g* for 10 min, the supernatant was collected and pooled with the first low speed supernatant. The pellet was processed as described below for the preparation of nuclei and chromatin. The pooled low speed supernatants were centrifuged at 10,000 × *g* for 30 min to yield the mitochondrial pellet and supernatant. The supernatant was then centrifuged at 100,000 × *g* for 1 h to yield the microsomal pellet and high speed supernatant (cytosol). Aliquots of all fractions were solubilized in 0.5 N NaOH for protein estimation or dissolved in the gel electrophoresis sample buffer to a concentration of 1.8×10^7 cells/ml.

Isolation of Nuclei and Chromatin—Nuclei from Novikoff hepatoma were isolated from the washed cells by hypotonic shock in 10 mM Tris-HCl, (pH 7.5), 0.1 mM phenylmethylsulfonylfluoride homogenization and centrifugation in hypertonic sucrose as described previously (22). Nuclei from other tissues were obtained using the method of Blobel and Potter (23). All nuclear preparations were washed once (resuspension and centrifugation at 1000 × *g* for 10 min) with 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 0.2% Triton X-100, 0.1 mM PMSF before the preparation of chromatin. Chromatin was isolated by the method of Bonner *et al.* (24) as modified in our laboratory (25). Isolated chromatin was washed with 0.3 M NaCl and rehydrated in 100 times diluted standard saline citrate (0.14 M NaCl, 0.014 M, sodium citrate).

Fractionation of Chromosomal Proteins—The DNA-associated antigenic complexes were isolated by sequential extraction of chromatin and chromatography of the immunologically active extract. Isolated chromatin was suspended in 5.0 M urea-15 mM sodium

[1] The abbreviations used are: PBS, phosphate-buffered saline (0.15 M NaCl, 10 mM sodium phosphate, pH 7.2); PAP, peroxidase-antiperoxidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonylfluoride.

phosphate buffer-0.1 mM phenylmethylsulfonyl fluoride, pH 7.6. The suspension was gently stirred on ice for 20–30 min or until dissolved. Gentle homogenization (hand-held Teflon pestle-glass homogenizer) can be used to accelerate the dispersion of chromatin. The final concentration of DNA in the suspension should be 0.3–0.4 mg/ml. The suspension was centrifuged at 100,000 × *g* for 16 h, and the supernatants were carefully decanted. This extraction removed 26–30 mg of protein-/100 mg of chromatin DNA. The pellets were carefully resuspended by gentle homogenization and stirring on ice for 30 min in 5.0 M urea-100 mM potassium phosphate-0.1 mM PMSF, pH 7.6, to a DNA concentration of 0.4–0.5 mg/ml. The solution was centrifuged at 100,000 × *g* overnight. The supernatants contained between 40–56 mg of protein/100 mg of chromatin DNA. Most of the immunological activity (after reconstitution to DNA) was associated with this fraction.

The pooled 100 mM phosphate extract was concentrated by ultrafiltration (Amicon Diaflo PM 10 membrane) to 5–6 mg of protein/ml and dialyzed against 2.0 M KCl-5.0 M urea-2 mM Tris-HCl-1 mM potassium phosphate-0.1 mM PMSF, pH 7.0, for 24 h. The dialyzed sample was then fractionated by chromatography on hydroxylapatite. Commercial Bio-Gel (HTP, Bio-Rad Laboratories) hydroxylapatite was freed of fine particles by decantation, suspended in the sample buffer, and left to equilibrate overnight. The equilibrated hydroxylapatite was either packed in columns or used for batch preparation.

For chromatography, the 100 mM phosphate fraction was applied to the column in a ratio of 3–5 mg of protein/ml of packed hydroxylapatite and eluted sequentially with the above 2.0 M KCl-5.0 M urea buffer containing 1.0, 50, and 100 mM potassium phosphate, pH 7.0. The average protein recoveries were 40–45% in 1.0 mM phosphate, 25–30% in 50 mM phosphate, and 5–10% in 100 mM phosphate. Some protein and DNA were retained on hydroxylapatite and could be eluted with 500 mM phosphate. However, this fraction had little immunological activity and was not collected for further processing. All the three fractions were concentrated by ultrafiltration (Amicon Diaflo PM 10 membrane), and appropriate aliquots were reconstituted with purified rat spleen DNA for complement fixation assays.

Further fractionation of the protein components comprising the antigenic complexes was accomplished by gel filtration. Concentrated proteins of the 50 mM phosphate fraction resulting from hydroxylapatite chromatography (30–50 mg) were dialyzed against 5.0 M urea-25 mM Tris-HCl-0.2 mM PMSF-1.0 mM dithiothreitol-0.05% dextran sulfate (1:100 ratio, overnight) and then for 8 h against the gel filtration buffer. Bio-Gel P200 columns (2 × 90 cm) were equilibrated with 25 mM Tris-glycine-1.0 mM dithiothreitol-0.2 mM PMSF-1% sodium dodecyl sulfate, pH 7.0, and the sample was eluted with the same buffer. The fractions were pooled, concentrated, and used for polyacrylamide gel electrophoresis or, after the removal of sodium dodecyl sulfate, for complement fixation assay and amino acid analysis. Active proteins were further purified by rechromatography on Bio-Gel P200.

Ammonium Sulfate Precipitation of Cytosol—Saturated ammonium sulfate was added dropwise to cytosol (1–5 mg/ml) with stirring to a final concentration of 35%. The mixture was stirred slowly for 1 h and centrifuged at 10,000 × *g* for 20 min. The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, to a volume equivalent to that of the original starting volume of cytosol, and the precipitation with ammonium sulfate was repeated. After centrifugation as indicated, the pellets were resuspended in the above buffer and dialyzed overnight against 1000 volumes of 10 mM Tris-HCl buffer, pH 7.5.

Antisera and Immunoassays—Antisera to Novikoff hepatoma chromosomal proteins p39, p49, and p56 were prepared by injecting a mixture of the purified proteins reconstituted with rat spleen DNA (DNA:protein = 1.0:0.4) into New Zealand white rabbits. Blood was obtained by marginal ear venopuncture 7 days after the booster injection. Sera were heat inactivated at 56 °C for 30 min and stored at −20 °C. All the antisera obtained by immunization with Novikoff hepatoma proteins p39, p49, and p56 did not precipitate with the immunogen. Consequently, immunoreactivity was quantitated using the microcomplement fixation assay of Wasserman and Levine (26) as described previously (16). The PAP staining method of Sternberger (19) was used to identify reactive antigens after transfer of chromosomal proteins separated by SDS-PAGE to nitrocellulose strips (Millipore, type HA, 0.45 μm).

For immunoabsorption, isolated chromatins were used as an absorbent in the ratio of 1 mg of chromatin (as DNA) to 1.0 ml of 1:10 diluted antiserum in PBS, pH 7.2. The mixture was gently homogenized by hand with a Teflon-glass homogenizer and incubated on ice for 16 h. Chromatin with the bound antibodies was then removed by centrifugation at 20,000 × *g* for 1 h, and the supernatant was collected and absorbed once more with fresh chromatin as indicated. The absorbed antiserum was used in complement fixation assays or to localize immunoreactive proteins transferred to nitrocellulose sheets.

Since antisera to Novikoff antigens p39, p49, and p56 were nonprecipitating, only immunoblocking experiments were employed for studies using cytosol proteins precipitated with 35% ammonium sulfate. Ammonium sulfate pellets mixed in a ratio of 2 mg of protein to 1 ml of 1:10 diluted antiserum were incubated overnight and centrifuged. The absorbed antiserum (supernatant) was treated again with additional 2 mg of ammonium sulfate-precipitated cytosol protein. The treated (absorbed) antiserum could not be used in complement fixation assays and was only employed in the localization of immunoreactive antigens on nitrocellulose sheets containing proteins separated by SDS-PAGE.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Immunolocalization with PAP—Hydrated chromatins or nuclei were suspended in 2 mM Tris-HCl buffer, pH 7.5 (about 1 mg/ml as DNA) and extensively sheared by sonication (18). Sonicated samples were made 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.0625 M Tris-HCl, pH 6.8, resonicated, boiled for 5 min, and then electrophoresed under conditions described by Laemmli (27) using a 3% stacking gel and a 7.5% running gel. All other cellular fractions were dissolved directly in the electrophoresis buffer, without sonication. Protein bands were visualized by Coomassie blue staining (28).

The electrophoretically separated proteins were transferred to nitrocellulose sheets as described by Towbin *et al.* (17). After transfer, the immobilized proteins could be stained with Amido black (29), or the antigens could be visualized using the PAP method (18, 19). The molecular weights of the immunoreactive bands were calculated from the mobility of high molecular weight standards (Bio-Rad) electrophoretically transferred to nitrocellulose and stained with Amido black (29).

Determination of Protein and DNA—Protein was determined in subcellular fractions solubilized with 0.5 N NaOH by the method of Lowry *et al.* (30). All other protein determinations employed the Coomassie dye binding method of Bradford (31). Bovine serum albumin was used as standard. DNA was estimated in chromatin samples by ultraviolet absorbance at 260 nm.

RESULTS

Fractionation of the Nuclear Antigenic Proteins p39, p49, and p56—Using antiserum to dehistonized Novikoff hepatoma chromatin, the fractionation of chromosomal proteins was followed by monitoring the complement fixing activity of the respective fractions. We have found in our previous experiments (10, 15, 16) that the antigenic complexes remain principally associated with the DNA during the extraction of chromatin with 5.0 M urea-50 mM sodium phosphate buffer. This procedure was further simplified by first extracting the chromatin with 5.0 M urea-15 mM sodium phosphate and then with 5.0 M urea-100 mM potassium phosphate. The 5.0 M urea-100 mM potassium phosphate extract contained most of the antigenic proteins (after reconstitution with rat spleen DNA). The advantage of this modification was in reducing the amount of DNA associated with the active 100 mM fraction. This considerably simplified the subsequent chromatography on hydroxylapatite. The concentrated 100 mM potassium phosphate fraction dialyzed against 2.0 M KCl-5.0 M urea-2 mM Tris-HCl-1 mM potassium phosphate-0.1 mM PMSF was chromatographed on a hydroxylapatite column equilibrated with the same buffer. Complement fixation of proteins eluted with 1, 50, and 100 mM potassium phosphate in the above buffer (after their reconstitution with rat spleen DNA) showed that the principal immunological activity was eluted with the 50 mM potassium phosphate. Polyacrylamide gel electrophoresis of this fraction revealed the three major protein peaks (approximate M_r = 39,000, 49,000 and 56,000) previously identified with the complement fixing activity in Novikoff hepatoma (16). To confirm their immunological specificity, the region containing these three proteins (p39, p49, and p56) was isolated by preparative gel electrophoresis and reconstituted

with native rat spleen DNA. The complexes were immunologically reactive with the antiserum to Novikoff hepatoma-dehistonized chromatin. When normal rat liver chromatin was subjected to the fractionation procedure used for the isolation of Novikoff hepatoma proteins p39, p49, and p56, no complement fixation in the presence of Novikoff hepatoma antiserum could be observed with either the 100 mM potassium phosphate chromatin extract or any of its hydroxylapatite fractions, including the 50 mM potassium phosphate eluate. Interestingly, polyacrylamide gel electrophoresis of the 50 mM potassium phosphate fraction revealed the presence of three bands migrating with mobilities very similar to those of the Novikoff hepatoma proteins p39, p49, and p56. However, the rat liver 50 mM potassium phosphate fraction did not fix complement in the presence of antiserum to dehistonized Novikoff hepatoma chromatin.

Gel filtration of the Novikoff hepatoma 50 mM hydroxylapatite fraction on Bio-Gel P200 resulted in three broad peaks which were collected in five pools. Repeated rechromatography of large quantities (10–20 mg) of the enriched Bio-Gel P200 fractions produced small quantities of the individual components p39, p49, and p56. A mixture of these three proteins was used (after reconstitution with rat spleen DNA) to elicit antisera employed in studies described in this report.

Because of limited amounts of the purified proteins p39, p49, and p56, we were able to perform only initial estimates of their amino acid composition. Preliminary data revealed a close similarity of their individual amino acid composition and relatively high contents of glutamic acid, serine, and aspartic acid. Although all three components contained cystine or cysteine and tryptophan, additional analyses are needed for more accurate determinations of these two amino acids due to expected losses during acid hydrolysis. Additionally, amino sugars have been detected in the ninhydrin patterns of acid hydrolysates.

Cell and Tissue Specificity of Novikoff Hepatoma Antigens p39, p49, and p56—Quantitative microcomplement fixation tests of antiserum raised against Novikoff chromosomal proteins p39, p49, and p56 demonstrated its specificity for Novikoff chromatin compared to chromatins prepared from a variety of tissues, including fetal and 24-h regenerating rat liver (Fig. 1). Interestingly, this antiserum no longer required DNA in the complement fixation assays. The specificity of this antiserum was further confirmed with immunoabsorption experiments. Antisera were absorbed with two successive treatments of Novikoff or nonspecific chromatin as described under "Experimental Procedures." Absorbed sera were then tested using complement fixation as shown in Fig. 2. Novikoff hepatoma chromatin was the only absorbent capable of removing the complement-fixing ability of the antiserum while liver, 24-h regenerating liver, or kidney chromatins were ineffective.

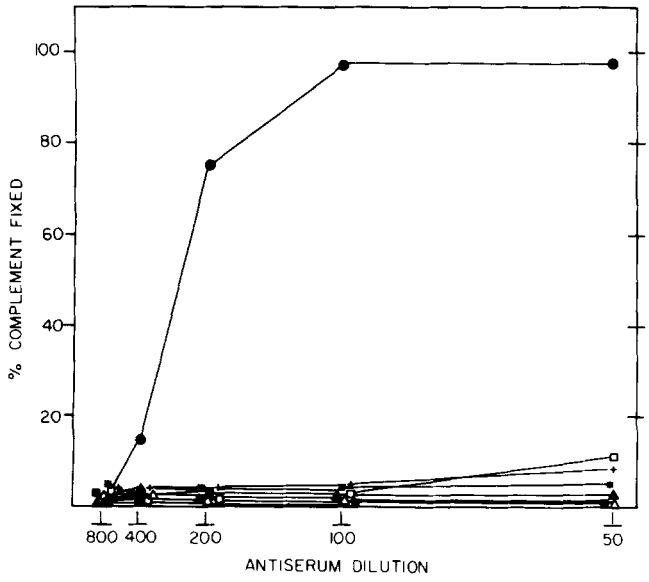

FIG. 1. **Complement fixation of antiserum to Novikoff hepatoma antigens p39, p49, and p56 in the presence of 10 μg/ml (as DNA) of Novikoff hepatoma (●), mature rat liver (▲), fetal rat liver (*), 24-h regenerating rat liver (+), immature (22 days old) rat liver (□), fetal rat brain (■), and fetal rat kidney (△) chromatins.**

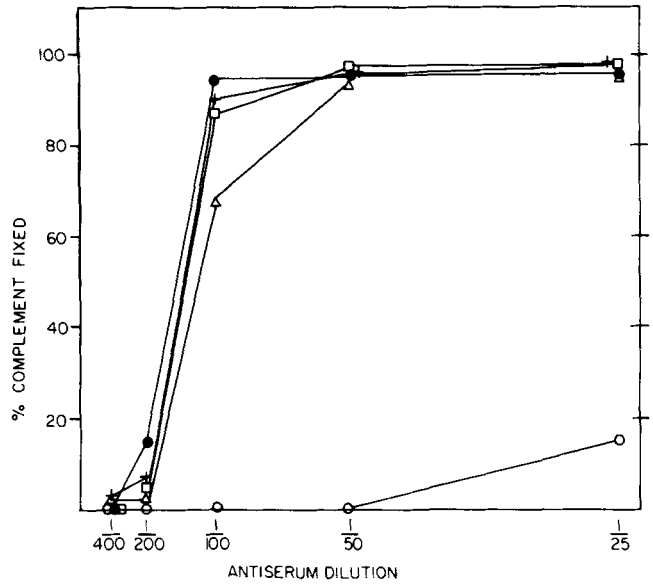

FIG. 2. **Immunoabsorption of antiserum to Novikoff hepatoma antigens p39, p49, and p56 with various chromatins.** Antiserum was absorbed twice as described under "Experimental Procedures," with Novikoff hepatoma (○), 24-h regenerating rat liver (□), mature rat liver (△), or mature rat kidney (+) chromatins. The absorbed sera were then tested for complement fixing ability in the presence of 10 μg/ml (as DNA) of Novikoff hepatoma chromatin. Unabsorbed antiserum (●) was tested as above but absorbed with buffer only.

We have previously demonstrated the feasibility of identification of chromosomal nonhistone protein antigens on SDS-PAGE electrophoretic transfers to nitrocellulose sheets by the second antibody PAP method (18). In a two-step procedure, immunoreactive antigens are expediently identified and initially characterized by their respective denatured molecular weights. In Fig. 3 chromatin proteins were resolved by SDS-PAGE and either stained directly in the gels with Coomassie (Fig. 3*A*), transferred to nitrocellulose sheets and stained with Amido black (Fig. 3*B*), or transferred to nitrocellulose and reacted with the PAP after incubation with antiserum to Novikoff antigens p39, p49, and p56 (Fig. 3*C*). Heterogeneity of the total chromosomal proteins in each chromatin is illustrated in Fig. 3*A*, and the Amido black-stained nitrocellulose sheet of Fig. 3*B* shows that a representative transfer of proteins from the polyacrylamide gel to the nitrocellulose sheet was achieved. Fig. 3*C* indicates that Novikoff hepatoma proteins p39, p49, and p56 are easily identified on nitrocellulose strips containing Novikoff hepatoma chromatin after incubation with antiserum and the PAP reaction but that chromatins from other tissues appear to contain small amounts of antigen p56, most notably those of mature or 24-h regenerating rat liver. The molecular weights of the Novikoff hepatoma antigens were calculated from the mobilities of protein standards

electrophoresed on identical gels, transferred to nitrocellulose sheets, and stained with Amido black. This analysis indicated the immunoreactive bands to be about 56,000, 49,000, and 39,000 daltons. These molecular weights correspond to the mobility of the partially purified immunoreactive antigens of our report (16).

Immunoabsorption experiments were used to confirm the specificity demonstrated by the immunolocalization of Novikoff chromosomal antigens p39, p49, and p56 on nitrocellulose sheets. Twice absorbed antiserum was incubated with nitrocellulose sheets containing SDS-PAGE-separated chromosomal proteins from various tissues. The results of the PAP reaction are shown in Fig. 4. Absorption of the antiserum with Novikoff chromatin efficiently removed all detectable immunoreactivity, while absorption with normal rat liver or 24-h regenerating rat liver chromatin removed the antibodies recognizing antigen p56 only. Chromatins from normal kidney or fetal rat liver were completely ineffective in removing even the p56 protein staining. These results indicate that Novikoff hepatoma antigen p56 may be present in normal or regenerating rat liver chromatins, albeit in reduced quantities to those found in Novikoff hepatoma chromatin. Antigens p49 and p39, however, appear entirely cell specific for Novikoff hepatoma.

Subcellular Distribution of Novikoff Hepatoma Antigens p39, p49, and p56—Proteins present in fractions from normal rat liver or Novikoff hepatoma were separated by SDS-PAGE and transferred to nitrocellulose. Antiserum that had been absorbed with liver chromatin was used to localize the immunoreactive species in each fraction. As can be seen in Fig. 5, the antigens were present in every subcellular fraction from Novikoff hepatoma that was examined. The p56 antigen is not visualized in this figure since antiserum immunoabsorbed with normal liver chromatin was used to stain the nitrocellulose sheets. Although the transfer technique does not yet allow quantitative evaluation, if one examines the intensity of staining seen when the subcellular fractions are compared on a constant cell number (Fig. 5*B*) or constant protein (Fig. 5*D*) basis, antigens p39 and p49 appear reduced in the 10,000 × *g* pellets.

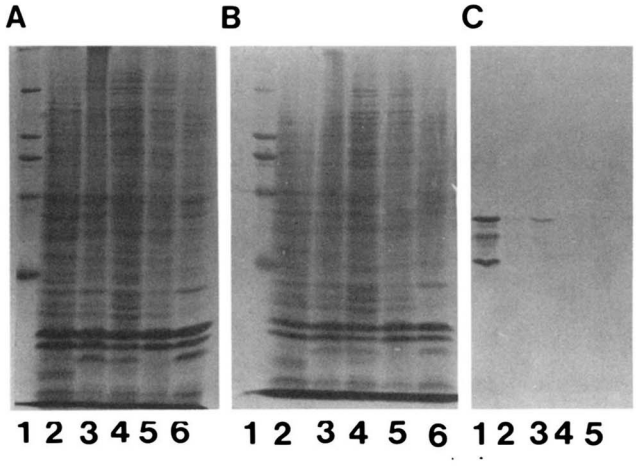

FIG. 3. **Identification of Novikoff hepatoma antigens in chromosomal proteins transferred to nitrocellulose sheets.** *A*, SDS-PAGE of various chromatins. Chromatins were prepared in SDS gel sample buffer, sonicated, and electrophoresed as described under "Experimental Procedures." Each lane contained 25 μg (as DNA) of the respective chromatin. Lane *1*, high molecular weight standards (Bio-Rad) (myosin, M_r = 200,000; β-galactosidase, M_r = 116,500; phosphorylase β, M_r = 94,000; bovine serum albumin, M_r = 68,000; ovalbumin, M_r = 43,000); lane *2*, Novikoff hepatoma; lane *3*, mature rat liver; lane *4*, 24-h regenerating rat liver; lane *5*, fetal rat liver; lane *6*, mature rat kidney chromatins. The origin is at the top of the gels. *B*, SDS-PAGE of various chromatins electrophoretically transferred to nitrocellulose and stained with Amido black. Chromatins were electrophoresed as in *A* and then transferred to nitrocellulose and stained with Amido black as described under "Experimental Procedures." Lanes are as described in *A*. *C*, localization of immunoreactive antigens on nitrocellulose containing SDS-PAGE-separated chromatins. Chromatins were electrophoresed as in *A*, transferred to nitrocellulose, and then the immunoreactive antigens were localized by incubation with antiserum to Novikoff hepatoma proteins p39, p49, and p56 (1:500 dilution) followed by the PAP procedure as described under "Experimental Procedures." Lane *1*, Novikoff hepatoma; lane *2*, mature rat liver; lane *3*, 24-h regenerating rat liver; lane *4*, fetal rat liver; lane *5*, mature rat kidney chromatins.

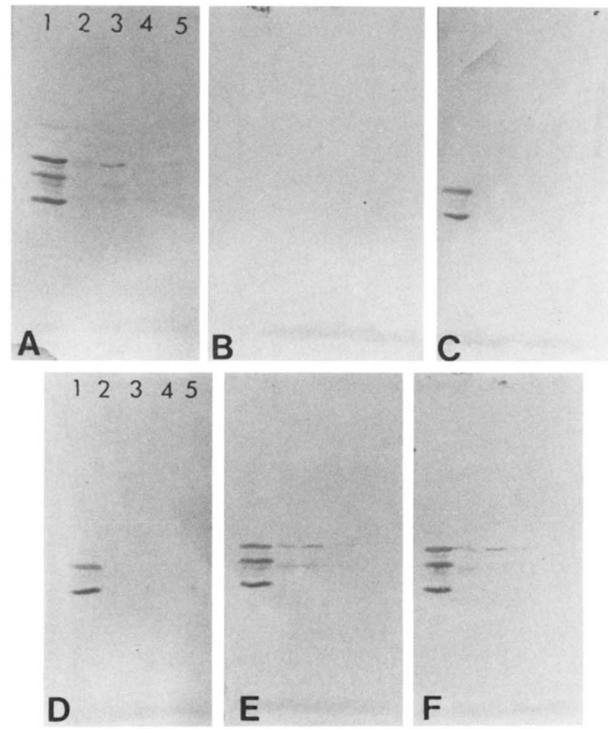

FIG. 4. **Immunoabsorption of antiserum to Novikoff hepatoma proteins p39, p49, and p56 with various chromatins.** Antiserum was absorbed twice as described under "Experimental Procedures." Absorbed antisera were then incubated with nitrocellulose sheets containing SDS-PAGE-separated chromatins (25 μg as DNA of each chromatin), and immunoreactive species were then visualized with the PAP reaction. Each nitrocellulose sheet contains (lanes *left* to *right*), Novikoff hepatoma, rat liver, 24-h regenerating rat liver, fetal rat liver, and rat kidney chromosomal proteins with the origin at the top of the sheet. The absorbed antisera used to stain each sheet were: *A*) unabsorbed; *B*) absorbed with Novikoff hepatoma chromatin; *C*) absorbed with rat liver chromatin; *D*) absorbed with 24-h regenerating rat liver chromatin; *E*) absorbed with rat kidney chromatin; *F*) absorbed with fetal rat liver chromatin.

We have found that Novikoff hepatoma antigens p39, p49, and p56 present in the cytosol fraction can be easily precipitated with low concentrations of ammonium sulfate. Nearly complete precipitation of these antigens was achieved with 35% ammonium sulfate resulting in about 10- to 15-fold enrichment as assessed by complement fixation assays. The tissue specificity of the cytosol Novikoff hepatoma antigens was then examined using 35% ammonium sulfate pellets isolated from various tissues. Results of these experiments were identical with those shown in Fig. 3 for chromatin except that 35% ammonium sulfate pellets from various cytosols were assayed and the antiserum against Novikoff hepatoma antigens p39, p49, and p56 was absorbed with normal liver chromatin before use. The results again indicated that only Novikoff hepatoma contains the cell-specific antigens p39 and p49.

Confirmation of the tissue specificity exhibited by Novikoff

hepatoma antigens p39 and p49 present in cytosol was obtained from immunoblocking experiments exploying cytosol 35% ammonium sulfate pellets from various tissues. Antiserum against Novikoff antigens p39, p49, and p56 was pretreated with the respective ammonium sulfate pellets and then used to localize immunoreactive antigens on nitrocellulose strips containing SDS-PAGE separated chromosomal proteins (Fig. 6*A-F*). Interestingly, every tissue examined was active in removing activity directed against antigen p56, and these results are in contrast to those observed when various chromatins were used as immunoabsorbents (Fig. 4). However, the cell specificity of antigens p39 and p49 was further verified in these experiments.

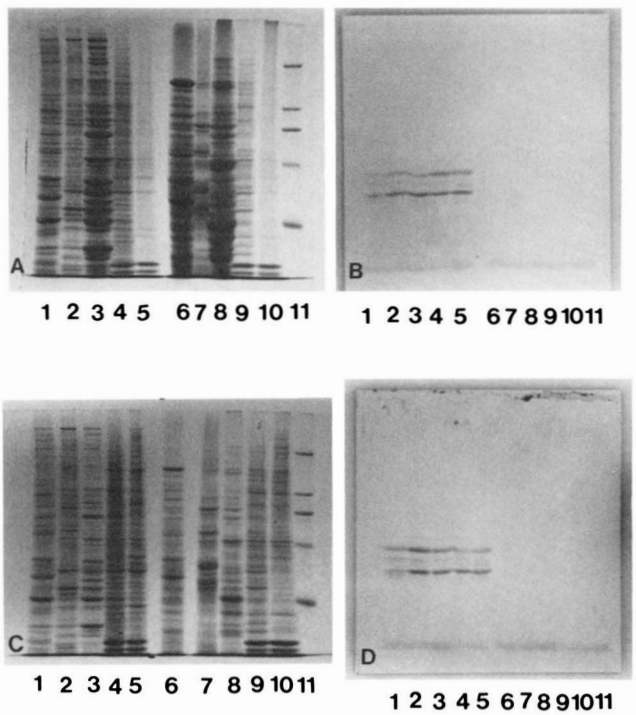

FIG. 5. **Identification of Novikoff hepatoma antigens in various subcellular fractions from Novikoff hepatoma and rat liver.** *A*, SDS-PAGE of various subcellular fractions from Novikoff hepatoma and normal rat liver. Fractions were prepared and solubilized in SDS gel sample buffer as described under "Experimental Procedures." Each lane contained the amount of subcellular fraction equivalent to 5.4×10^5 cells in a volume of 30 μl. Lanes *1-5* are from Novikoff hepatoma (lane *1*, 10,000 × *g* pellet; lane *2*, 100,000 × *g* pellet; lane *3*, 100,000 × *g* supernatant; lane *4*, nuclei; lane *5*, chromatin). Lanes *6-10* are from normal rat liver (lane *6*, 10,000 × *g* pellet; lane *7*, 100,000 × *g* pellet; lane *8*, 100,000 × *g* supernatant; lane *9*, nuclei; lane *10*, chromatin). Lane *11* contains molecular weight standards which are as described in Fig. 3. *B*, localization of immunoreactive antigens on nitrocellulose containing SDS-PAGE-separated proteins from various subcellular fractions of Novikoff hepatoma or normal rat liver. Fractions were prepared and electrophoresed as in *A*, transferred to nitrocellulose, and then the immunoreactive antigens were localized with antiserum to Novikoff hepatoma antigens p39, p49, and p56 (1:500 dilution) and the PAP procedure. Antiserum was absorbed twice with liver chromatin before use. Lanes are as shown in *A*. *C*, SDS-PAGE of various subcellular fractions from Novikoff hepatoma and normal rat liver. Fractions were prepared and electrophoresed as described in *A*; however, each lane contained an equivalent amount of protein from the respective fraction (20 μg). The identity of each lane is as described in *A*. *D*, localization of immunoreactive antigens on nitrocellulose containing SDS-PAGE-separated proteins from various subcellular fractions of Novikoff hepatoma or normal rat liver. Fractions were prepared and electrophoresed as in *C*, transferred to nitrocellulose, and then the immunoreactive antigens were localized with antiserum to Novikoff hepatoma antigens, p39, p49, and p56 (1:500 dilution) and the PAP procedure. Antiserum was absorbed twice with liver chromatin before use. Lanes are as indicated in *A*.

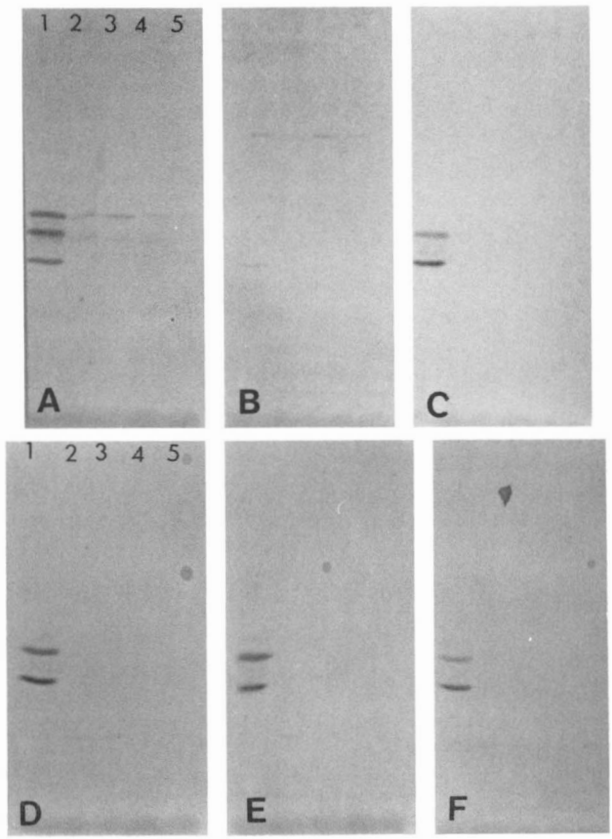

FIG. 6. **Immunoblocking of antiserum to Novikoff hepatoma antigens p39, p49, and p56 with cytosol 35% ammonium sulfate pellets.** Antiserum was treated twice with cytosol ammonium sulfate pellets from various tissues as described under "Experimental Procedures." Treated antisera were then incubated with nitrocellulose sheets containing SDS-PAGE-separated chromosomal proteins (25 μg as DNA of each chromatin), and immunoreactive species were visualized with the PAP reaction. Each nitrocellulose sheet (*A-F*) contains (lanes *left* to *right*) Novikoff hepatoma, mature rat liver, 24-h regenerating rat liver, fetal rat liver, and mature rat kidney chromosomal proteins with the origin at the top of the sheet. The antisera used to stain each sheet were blocked with an ammonium sulfate pellet from (*A*) buffer only, (*B*) Novikoff hepatoma, (*C*) mature rat liver, (*D*) fetal rat liver, (*E*) 24-h regenerating rat liver, and (*F*) mature rat kidney cytosol.

DISCUSSION

The antigenicity of dehistonized chromatins is well documented in the literature (9, 10, 13-16), and antibodies to specific chromosomal proteins can provide powerful reliable probes for studying the diversity of chromosomal architecture. We have described in this report a second generation antiserum, directed against three Novikoff hepatoma antigenic proteins p39, p49, and p56, purified from chromatin as described in the previous communication (16), with an additional step of chromatography on Bio-Gel P200 in the presence of SDS. Using methods developed for identifying immunoreactive components in crude mixtures of proteins separated by SDS-PAGE and transferred to nitrocellulose (17, 18) we have confirmed that the principal immunoreactivity of this antiserum is directed against three protein species of about 56,000, 49,000 and 39,000 daltons, which is in agreement with the estimates of the mobility of the purified immunoreactive antigens reported by Fujitani et al. (16).

Although the antisera employed in the reported experiments have been elicited to proteins obtained by fractionation of isolated chromatin, our results show that the p39, p49, and p56 proteins are also present in Novikoff hepatoma cytoplasm.

Presently, we cannot ascertain whether the cytoplasmic presence of these antigens is caused by cross-contamination or leakage during chromatin isolation or whether they are present in both cellular compartments, *e.g.* a situation similar to the high mobility group proteins (32). The intensity of immunological staining exhibited by the nuclear and cytoplasmic transfers appears to support the latter possibility. However, the p39 and p49 antigens exhibited cellular specificity regardless of which cellular compartment was examined. In complement fixation assays none of the nonspecific chromatins tested were active in absorbing the complement-fixing ability of the antiserum; however, when immunoreactivity was assessed by the PAP reaction on SDS-PAGE-separated proteins transferred to nitrocellulose, both normal rat liver and 24-h regenerating rat liver chromatin removed antibodies directed against antigen p56. These results suggest that with these antisera this antigen is not a major complement-fixing antigen and that normal rat liver or 24-h regenerating liver chromatins contain reduced amounts of the p56 antigen or antigen(s) of very similar nature.

The initial antiserum by which antigens p39, p49, and p56 were identified was obtained to dehistonized chromatin. We have previously shown that such antisera frequently recognize components of fetal or regenerating liver chromatins (16, 21). A likely explanation for the specificity observed for the antigens p39, p49, and p56 is the heterogeneity of the initial immunogen. Indeed, our recent work (18) has shown that antisera prepared against dehistonized chromatin from Novikoff hepatoma recognize many tissue-specific components as assessed by the immunodetection of antigens on nitrocellulose sheets containing electrophoretically separated and immobilized chromosomal proteins. It is likely that other antigens demonstrated by this procedure correspond to species which are also present in fetal or regenerating liver. Furthermore, it must be emphasized that during the purification of antigens p39, p49, and p56 using an antiserum against dehistonized chromatin as a probe, only complement fixation assays were available to us. Thus, our preparative procedures were aimed at recovering the maximal complement-fixing activity in the individual fractions, and other lesser activities were not pursued further. We believe that methods using the immunodetection of SDS-PAGE-separated antigens immobilized on nitrocellulose will greatly facilitate the identification of immunoreactivity in antisera to chromatin fractions and in the present system will help to reveal antigens common to Novikoff hepatoma and both fetal and regenerating livers.

We have previously reported that antisera obtained against dehistonized chromatin preparations recognized selectively complexes between chromosomal nonhistone proteins and DNA as detected by microcomplement fixation assays (13–15). Since, unless covalently linked, the DNA becomes separated from proteins during electrophoresis in the presence of SDS, it is obvious that the Novikoff hepatoma antigens p39, p49, and p56 do not need to associate with DNA to be immunologically cell specific. The remote possibility that association with nitrocellulose affords the antigenic proteins a conformation essentially identical with that in the DNA-protein complexes is eliminated by our unpublished findings that these three antigens present in cytosols fixed the complement with the same specificity as observed for chromatin preparations from the same tissues. Apparently, we have lost during the purification of Novikoff hepatoma antigens p39, p49, and p56 the antigenic components which interreact with DNA and form immunologically cell-specific complexes. Preliminary experiments comparing the complement-fixing activity of antisera to dehistonized Novikoff hepatoma chromatin with their electrophoretic patterns on nitrocellulose transfer sheets support this conclusion.

The absence of Novikoff hepatoma proteins p39 and p49 from electrophoretic transfers of the total rat liver and other rat tissue chromatins indicates that these antigens may be specific for Novikoff hepatoma. Although our immunoabsorption experiments support this notion, it is conceivable that the p39 and p49 proteins may be present in normal tissues, albeit in a chemically modified form which is not recognized by our antisera. Consequently, although the antisera are specific for Novikoff hepatoma, the cell specificity of the p39 and p49 proteins must be further confirmed by their structural analysis. Indeed, close inspection of electrophoretically separated chromosomal protein fractions from rat liver indicated the presence of proteins similar to the p39 and p49 antigens; however, these rat liver proteins did not react with the Novikoff hepatoma antiserum.

Busch and co-workers have identified a number of antigenic Novikoff hepatoma proteins by differential extraction of chromatin or nucleoli with buffered salt solutions (5–8, 33) or by isolation from Novikoff hepatoma cytosol (34). Of particular interest is the B1 antigen (M_r = 37,000) from Novikoff chromatin, the NoAg 1 antigen from Novikoff nucleoli (M_r = 60,000) (7), and the 45,000-dalton species purified from Novikoff cytosol (34), all of which have molecular weights similar to the Novikoff hepatoma antigens described in this report. Concerning the former nuclear and nucleolar antigens, the behavior of these proteins during isolation, extraction, and immunization suggests that Novikoff hepatoma antigens p39, p49, and p56 are different. Raju *et al.* (34) reported a specific antigen in Novikoff cytosol closely corresponding to the molecular weight of Novikoff antigen p49. While the link between the latter antigen and antigen p49 is so far only in molecular weight, it is noteworthy that this antigen does not exhibit the solubility properties of the Novikoff hepatoma antigen p49. Raju *et al.* (34) needed relatively high concentrations of ammonium sulfate (60%) to precipitate the antigen from Novikoff hepatoma cytosol. This is in contrast to antigen p49 which precipitates from cytosol nearly completely at 35% ammonium sulfate concentration.

We have as yet assigned no cellular function for the Novikoff antigens described here. Their solubility in only high concentrations of urea and other denaturing solvents along with their apparent presence in all the subfractions tested suggests that they may represent structural elements of the cell.

Acknowledgments—We thank Michele Tutt and Cheryl Saggese for skillful technical assistance.

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