Immunochemical Procedure for Partial Purification of Newly Synthesized Proteins in Polyoma Virus-Infected Mouse Cells

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A broad-spectrum antiserum has been obtained against nuclear proteins from normal mouse fibroblasts. Solid phase immunoabsorbents prepared from the serum specifically bind the corresponding antigens. The removal in this way of a significant fraction of host proteins leads to partial purification of viral and virus-induced polypeptides from polyoma virus-infected cell extracts. Four main peptides can be selected, with respective molecular weights of 90,000, 70,000, 46,000, and 41,000.

Assays of newly appearing enzymatic activities in crude extracts from virus-infected cells are usually inconclusive, for the background of pre-existing cellular enzymatic activities is high. Analytical resolution of newly synthesized polypeptides from cellular proteins, by gel electrophoresis, is also difficult to accomplish because cellular protein synthesis continues at a high level in infected cells (17). Conventional protein purification methods are impracticable with the small amounts of material available from monolayer cell cultures grown *in vitro*.

We have devised a method for removing a large fraction of cellular proteins from infected cell extracts, leaving untouched and in undenatured form proteins which are made only in infected cells. The general principle is to produce a serum containing a broad spectrum of antibodies against the proteins of a given fraction (in the present case, nuclear proteins soluble at low ionic strength) from uninfected mouse cells. The serum is polymerized to form a solid phase immunoabsorbant which can be used either in columns or batchwise to remove the cellular proteins from infected cell extracts. Unadsorbed proteins, which can be either virus-induced cellular proteins or viral-coded proteins, are analyzed by polyacrylamide gel electrophoresis and autoradiography.

MATERIALS AND METHODS

Cell lines and virus strains. 3T6 mouse fibroblasts were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Cell lines were periodically treated with kanamycin and anti-PPLO agent (Gibco), and checked every 6 to 8 weeks for mycoplasma contaminations. Large-plaque polyoma virus, originally obtained from M. Vogt, was routinely grown in baby mice according to Winocour (20).

Infection of cells and radioactive labeling. Subconfluent 3T6 cultures were infected with polyoma virus at a multiplicity of 10 to 50 PFU/cell. Mock infection was carried out by using Dulbecco-Eagle medium instead of virus suspension (16). For radioactive labeling of proteins, Dulbecco-Eagle medium was diluted twofold with Earle solution and supplemented with 1 μ Ci of a mixture of 15 14 C-labeled amino acids per ml (CEA France, catalog no. CB 51; specific radioactivity 100 mCi/mmol). Cells were in most cases labeled from 0 to 20 h postinfection and then harvested.

Preparation of nuclear extracts. Cells were washed three times with Tris-saline buffer solution (16). Cells (5×10^6) were shaken on a Vortex Jr. mixer at 4 C for 1 min in 10 ml of 0.1% Tween-80 in distilled water. Disruption of cytoplasmic membranes was monitored by phase contrast microscopy. A 10-ml amount of twofold concentrated Tris-saline buffer was added, and the nuclei were separated by centrifugation at $900 \times g$ for 10 min at 4 C. The nuclear pellet was suspended and washed three times without the use of pipettes in 0.25 M sucrose, 0.005 M $MgCl_2$, and 0.01 M Tris, pH 7.4, and then stored for 18 h at 4 C in the same buffer. Sonic treatment with a microtip in a Branson apparatus at a power setting of 100 W for 15 s at 4 C destroyed the nuclei, as judged by microscope inspection. After centrifugation at $20,000 \times g$ for 1 h, the supernatant fluid, containing proteins soluble at low ionic strength, was dialyzed for 24 h against 0.15 M NaCl (pH 7).

Protein concentrations were measured according to the method described by Lowry et al. (11), and the radioactivity of labeled extracts was measured by liquid scintillation counting after precipitation with trichloroacetic acid.

Immunological techniques. Immunization procedure. A 9-month-old sheep was inoculated by injection in the groin with 2 mg of nuclear proteins from uninfected mouse cells in 2 ml of 0.15 M NaCl and 2 ml of Freund adjuvant. The injections were repeated at 5-week intervals during 1 year. Every week, the animal was bled by puncturing the carotid artery. The number of precipitating antigen-antibody

complexes was determined by double diffusion tests according to Oudin (12), and the serum was then stored at -20°C until used.

Cytological localization of antigens. Antigens were localized by staining with peroxidase-labeled antibodies according to Wicker and Avrameas (19), after fixation either with 2% paraformaldehyde at 4°C for 20 min, or with methanol-ether (60:40) at -20°C for 20 min.

Preparation of immunoadsorbents. The serum was rendered insoluble by the addition of 2.5% glutaraldehyde at pH 5 at room temperature, according to Avrameas and Ternynck (2).

Immunoabsorption of labeled extracts. A 2-ml amount of labeled nuclear extracts dialyzed against 0.15 M NaCl was added to 2 g of insolubilized antiserum and stirred at room temperature for 1 h. The mixture was centrifuged ($3,000 \times g$, 20 min), the supernatant was kept for further tests, and the immunoabsorbant pellet was washed by successive centrifugations in 10 ml of phosphate-buffered saline (PBS) (2). Elution was performed with 10 ml of HCl-glycine buffer (pH 2.8) at 0°C for 15 min. The eluate was immediately neutralized by the addition of 10 ml of 1 M K_2HPO_4 .

Gel electrophoresis. The nuclear proteins were dissolved in 100 μl of 0.0625 M Tris-hydrochloride (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.001% bromophenol blue, and 0.1 M dithiothreitol. The samples were heated for 10 min at 56°C . Equal amounts of protein were applied in individual sample wells of a 15% polyacrylamide slab gel (10-cm long), and were analyzed according to the method of Lämmli (10) and Studier (15). Each well contained 10 to 20 μg of protein in 20 μl of buffer corresponding to approximately 10,000 counts/min. The gels were dried and autoradiographed on Kodirex X-ray film by 2 weeks of exposure. After development, the autoradiogram was scanned with a Joyce-Loebel recording densitometer.

The following proteins were used as markers (18) for the calibration of molecular weights: bovine serum albumin (68,000), ovalbumin (43,000), alcohol dehydrogenase (41,000), pepsin (35,000), and beta-lactoglobulin (18,000). These proteins were prestained with rhemazol blue before electrophoresis (8).

RESULTS AND DISCUSSION

Characterization of nuclear extracts. The yield of proteins and the specific radioactivity at the three stages in the preparation of nuclear extracts are listed in Table 1. Yields of nuclear proteins were not significantly different between mock-infected and virus-infected cells.

Gel electrophoresis resolves 15 to 20 different polypeptides from either normal or polyoma-infected cells (Fig. 1). It should be noted that certain polypeptide bands appear more intense in infected cell extracts than in uninfected cell extracts, and that others appear less intense.

Characterization of the antiserum. Serum

TABLE 1. Recovery of protein during preparation of nuclear extracts

Stages (starting 10^6 cells)	Protein (mg)	Radio-activity (counts/min)	Specific radioactivity (counts per min per mg)
Washed cells ^a	380	1.1×10^8	3×10^6
Whole nuclei	50	1.5×10^8	3×10^6
Low-ionic-strength nuclear extracts	10	2.6×10^7	2.6×10^6

^a Polyoma-infected 3T6 cells (10^6) were labeled, harvested, and fractionated as described in Materials and Methods.

FIG. 1. Autoradiogram and densitometer recording of a polyacrylamide gel of nuclear extracts of polyoma-infected (i and —) and mock-infected (m and - - -) cells. Subconfluent 3T6 cells infected with 10^6 PFU/cell polyoma virus were labeled with a ^{14}C -amino acid mixture ($1 \mu\text{Ci/ml}$) from 0 to 20 h post-infection. Mock-infected cultures were treated identically. Nuclear extracts were prepared as described. Proteins were heated at 56°C for 10 min in the presence of SDS (2%) and β -mercaptoethanol (1%). Approximately 10,000 counts/min were applied in each well and electrophoresed on a 10-cm 15% acrylamide slab gel for 4 h at 40 mA. Standard proteins, prestained with rhemazol dye, were electrophoresed on the same gel. The dry gel was exposed to X-ray film for 2 weeks. Position of marker peptides are indicated by their molecular weights ($\times 10^{-3}$): bovine serum albumin (68), alcohol dehydrogenase (41), pepsin (35), β -lactoglobulin (18).

obtained from an animal immunized by repeated injections produces at least six different bands when tested against the corresponding antigenic mixture in the Oudin double diffusion assay (12) (Fig. 2). This yields a minimal estimate of the number of antigen-antibody complexes: some antigens may not have been concentrated enough for a detectable band to appear; in addition, nonprecipitating complexes would not be detected in this way. Gel electrophoresis in SDS reveals 15 to 20 polypeptides (Fig. 1) in the antigenic mixture; under nondenaturing conditions, it is likely that some of them constitute multisubunit structures or aggregates. Some components, however, are

known to be poor antigens (e.g., histones), and the number of antibody species, although in a comparable range with the number of protein species, does not cover at the present stage their complete spectrum.

The localization of antigens has been checked by staining with peroxidase-coupled antibodies (19) (Fig. 3). The reaction was strongly positive with immune serum: staining was seen at the intranuclear as well as perinuclear areas, with a strong reinforcement of the nuclear membrane (Fig. 3a). Background staining was very faint in cells treated with blank serum (Fig. 3b).

Immunoabsorption of labeled extracts from mock- and virus-infected cells. To adsorb antigens from nuclear extracts, the serum was insolubilized according to Avrameas and Ternynck (2).

A ^{14}C -amino-acid-labeled cell extract was incubated at room temperature in PBS in the presence of the insolubilized immunoabsorbant (see Materials and Methods). The same results were obtained whether sepharose-linked antibodies (3) or glutaraldehyde-treated sera were used. At different times, the mixture was centrifuged and the radioactivity of the supernatant was measured. As shown in Fig. 4, 65 to 80% of the radioactivity was adsorbed in 2 h on the insolubilized complex. As a control, adsorbants made from sera of nonimmunized animals retained only 10 to 15% of the radioactivity. Increasing the amount of immunoabsorbant added led to 90% efficiency of adsorption. In this case, however, the level of adsorption by nonimmunized sera was increased, and the recovery of nonantigenic protein species was lower.

The disappearance of antigens from the supernatant can also be followed by assaying back the supernatants in a double diffusion test against the original serum. All bands rapidly disappear from adsorbed extracts (Fig. 5).

Electrophoretic analysis of unadsorbed proteins. After exhaustive adsorption of extracts of polyoma-infected cells on insolubilized antiserum, unadsorbed proteins were concentrated and analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 6, most of the radioactive polypeptides in the original extracts (Fig. 2) are absent, the remaining radioactivity being distributed among four major bands (A to D) and a number of minor bands. By estimation from the known molecular weight of the marker polypeptides (Fig. 7), the approximate molecular weight of these polypeptides are: A, 90,000; B, 70,000; C, 46,000; and D, 41,000.

An induced cellular protein (X) of the same

FIG. 2. Characterization of the immune serum: immunodiffusion assay before immunization (0) and after 1, 2, 3, and 10 injections. From bottom to top were layered in a capillary tube: a, 0.25% agar containing antiserum; b, 0.25% agar; and c, the antigenic nuclear extract (1 mg of proteins/ml). After 8 days at 20 C in a sterile atmosphere, antigen-antibody precipitating complexes form visible bands, the position of which is a function of the relative concentration of their two components. Large aggregates do not diffuse in the agar layer and produce a precipitate at the upper interface.

FIG. 3. Cytological localization of antigens. 3T6 cells were grown on cover slips. After fixation, they were treated with peroxidase-coupled antibodies from immunized (a) or nonimmunized (b) sheep. After extensive washing, peroxidase-coupled complexes were revealed by a brown deposit after incubation with 3,3'-diaminobenzidine in the presence of hydrogen peroxide.

FIG. 4. Immunoadsorption of ^{14}C -labeled nuclear extracts. Extracts from virus- (●) and mock-infected cells (○) were incubated in the presence of insolubilized sheep serum, and the proportion of radioactivity left in solution was plotted as a function of time. Control: same extracts in the presence of a nonimmunized sheep serum (+).

molecular weight as D, different from the viral capsid components VP_1 and VP_2 , has been recorded in SV40-infected cells extracts by Anderson and Gesteland (1).

As judged from its electrophoretic mobility, band C (46,000) is likely to correspond to the major polyoma capsid polypeptide (VP_2) (9, 13). The B polypeptide (70,000) may correspond to the NVP1 component described by Walter et al. (17), or to the material purified as T-antigen by Del Villano and Defendi in the case of SV40 (4), or both. A polypeptide of molecular weight close to that of component A (90,000) has been described as a minor constituent of the viral particle (13).

Faint bands with electrophoretic mobilities comparable to those of A, B, and D (but not C) can also be seen in adsorbed extracts from uninfected nuclei. This suggests that proteins A, B, and D may be normal cell proteins whose synthesis is stimulated on infection. Alternatively, the minor components present in uninfected cell extracts may represent different polypeptide species which coincidentally migrate with the same mobility as A, B, and D. Experiments are in progress to clarify this point.

Band g, whose molecular weight corresponds to that of one of the known cellular histones (5), is present in roughly equal amounts in both infected and uninfected cell extracts after immunoadsorption. Since histones are known to be poorly antigenic, it is not surprising that they would remain unadsorbed. It has repeatedly been found that two other bands in the histone region (e and f) are of a much lower intensity in extracts from infected cells than those from

uninfected cells. Further studies are required to determine if this corresponds to a real difference, or to a technical artifact.

Availability of partially purified viral proteins opens now the possibility of studying their functions. True virus-coded enzymes may be distinguished from virus-induced cellular gene products using conditional defective or deletion mutants of the virus. Immunochemical

FIG. 5. Immunoadsorption of nuclear extracts followed by immunodiffusion. Same experiment as in Fig. 4: the supernatant after 0.5 and 1 h of incubation being tested for the presence of antigenic proteins as described in the legend of Fig. 2.

FIG. 6. Autoradiogram and densitometer recording of the peptides after immunoadsorption of nuclear extracts. Supernatants obtained after two successive adsorptions for 1 h (see Fig. 4) were analyzed as described in the legend of Fig. 1. The sample order is: mock-infected cells (m and --), PY-infected cells (i and —), purified ^{14}C -labeled PY virus (V). Positions of marker peptides are indicated by their molecular weights ($\times 10^{-3}$): bovine serum albumin (68), alcohol dehydrogenase (41), pepsin (35), β -lactoglobulin (18). The dry gel was exposed to X-ray film for 2 weeks. A, B, C, and D are overexposed.

FIG. 7. Determination of the molecular weights of peptides A, B, C, and D. The marker proteins are

fractionation has two main advantages over conventional purification methods: it can be used efficiently on small amounts of material, and it leaves viral proteins unaltered, under mild, nondenaturing conditions suitable for a good recovery of enzymatic and antigenic activities. It depends essentially upon the availability of a broad-spectrum antiserum. In the present study, only a fraction of the total proteins of the infected cells has been studied (low ionic strength nuclear extract). It should be possible in the future to extend this methodology to any given cell fraction (chromatin, membrane proteins, ribosomal proteins, etc.) in various systems.

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bovine serum albumin (BSA) and its dimeric form (BSA dimer), ovalbumin (OVA), liver alcohol-dehydrogenase (AD), pepsin (P), and β -lactoglobulin (BLG). Relative mobility of peptides A to D is indicated by arrows.

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