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Immunoautoradiographic detection of proteins after electrophoretic transfer from gels to diazo-paper: Analysis of adenovirus encoded proteins

(two-dimensional gel electrophoresis/DNA-binding protein/protein imprints)

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ABSTRACT **We describe a method by which complex protein mixtures are fractionated by standard one-dimensional NaDodSO$_4$/polyacrylamide gel electrophoresis or O'Farrell two-dimensional gel electrophoresis and then are efficiently and rapidly transferred electrophoretically to diazobenzyloxymethyl- or diazophenylthioether-paper and analyzed by immunoautoradiography. The method is illustrated with protein extracts of human KB cells infected with adenovirus type 2. Proteins were transferred from gels without decrease in resolution and with an increase in the sensitivity of detection by autoradiography when [^{35}S]-methionine-labeled proteins were used. When unlabeled proteins were transferred, low levels of virus encoded proteins could be detected by sequential treatment of diazobenzyloxymethyl-paper with anti-adenovirus type 2 virion or anti-73,000 DNA binding protein and ^{125}I-labeled *Staphyloccus aureus* protein A. Covalently bound viral proteins retained immunologic reactivity after dissociation of the protein A and antibody. By one-dimensional gel transfer/immunoautoradiography, seven virion proteins were detected as prominent bands and several others as weaker bands. By two-dimensional gel transfer/immunoautoradiography, several additional viral proteins were detected. By use of anti-DNA binding protein serum, the M_r 73,000 protein and M_r 41,000–48,000 subspecies were detected. A protein present at a concentration of approximately 1 part in 100,000 of the total protein can be identified in cell extracts. This method may be applicable to various biological problems requiring resolution and detection of small amounts of specific proteins that can be recognized immunologically or that can be detected by binding to specific radiolabeled DNA or RNA sequences or hormones.**

The detection and analysis of small amounts of specific proteins are important in elucidating the mechanisms of virus replication and cell transformation as well as of many other biological processes. Two-dimensional (2D) gel electrophoresis of cell extracts (1) followed by protein staining or autoradiography makes it possible to detect more than a thousand proteins as well-resolved spots in a single slab gel. However, these procedures do not identify the proteins.

Immunoprecipitation of radiolabeled cell extracts permits the sensitive detection of specific proteins in complex mixtures. However, unrelated proteins may coprecipitate, leading to erroneous conclusions. To avoid this, unlabeled protein mixtures have been separated on a slab gel and then specific proteins were identified by incubation with antiserum followed by a radiolabeled second antibody (2). The high background, extensive washing required, and the fact that only protein present near the surface will react with antibody are drawbacks. Renart *et al.* (3) found that transferring proteins from gels to diazobenzyloxymethyl (DBM)-paper by the Southern blotting procedure greatly improved the potential of this approach. The covalently bound proteins are still capable of reacting with antibodies, and the immune complex may be detected by using a labeled second antibody or iodinated *Staphyloccus aureus* protein A, which binds to the Fc portion of IgG (4). The polyacrylamide gels used by Renart and coworkers were crosslinked by reagents that were then cleaved by periodate or alkali. However, some proteins might be altered by these treatments.

Electrophoresis has been used to facilitate the transfer of proteins to nitrocellulose sheets (5). However, not all proteins will adsorb to nitrocellulose, and the binding is not covalent. Therefore, the use of DBM-paper may be preferable. Bittner *et al.* (6) recently used electrophoresis to assist in the transfer of nucleic acids and proteins to nitrocellulose sheets or DBM-paper.

We report here the development of a system for the detection and analysis of small amounts of specific proteins which combines high resolution and excellent sensitivity. We have separated proteins present in adenovirus type 2 (Ad2)-infected and mock-infected human KB cell extracts by one-dimensional (1D) or 2D polyacrylamide gel electrophoresis and efficiently transferred them electrophoretically to DBM-paper or diazophenylthioether (DPT)-paper. Specific viral proteins were detected by treatment with antiserum to Ad2 virions or to the M_r 73,000 Ad2-encoded DNA binding protein (DBP) followed by treatment with ^{125}I-labeled protein A and autoradiography.

MATERIALS AND METHODS

Preparation of Cell Extracts and Virus. KB cells were grown and infected with Ad2 as described (7). "Late" infected cell extracts were labeled with [^{35}S]methionine (40 μCi/ml; 954 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels; New England Nuclear) from 15 to 21 hr after infection. "Early" infected cell extracts were prepared by using cycloheximide (CHX) and arabinosylcytosine (AraC) as described (8). ^{32}P-Labeled extracts were prepared by labeling cells with inorganic $^{32}PO_4$ (1 mCi/ml; carrier-free; New England Nuclear). Cell pellets (4 × 10^7 cells) were prepared according to the method of O'Farrell (1). Ad2 virions labeled with [^{35}S]methionine were purified as reported (7). Protein concentration was estimated by the method of Lowry *et al.* (9).

1D and 2D Gel Electrophoresis. For 1D gels, extracts containing 5 × 10^5 cpm or ≈150 μg of protein were applied per lane. For 2D gels, 5 × 10^6 cpm or ≈600 μg of protein was used. [^{35}S]Methionine-labeled (2 × 10^5 cpm) virion was applied to both 1D and 2D gels. ^{14}C-Labeled proteins were used as molecular weight markers (New England Nuclear). 1D gels and the

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; DBM, diazobenzyloxymethyl; DPT, diazophenylthioether; Ad2, adenovirus type 2; DBP, DNA binding protein; CHX, cycloheximide; AraC, arabinosylcytosine; TENG-N, 50 mM Tris·HCl, pH 7.4/5 mM EDTA/150 mM NaCl/0.25% gelatin/0.05% Nonidet P-40.

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second dimension of 2D gels consisted of 10–18% $NaDodSO_4$/polyacrylamide crosslinked with *N,N'*-methylenebisacrylamide. Gel electrophoresis buffer was 25 mM Tris/192 mM glycine/0.1% $NaDodSO_4$. 2D gels were run according to O'Farrell (1) with minor modifications. The pH range was approximately 4–7. After electrophoresis, slab gels were equilibrated with gentle rocking for 1 hr in three changes (400 ml each) of transfer buffer (25 mM sodium phosphate buffer at pH 6.5), and either dried and autoradiographed or immediately used for transfer to DBM-paper. Autoradiography was done with Kodak X-Omat R film.

Electrophoretic Transfer of Proteins from $NaDodSO_4$/Polyacrylamide Gels to Paper. DBM-paper was prepared according to Alwine *et al.* (10) and DPT-paper was prepared according to Brian Seed (personal communication). The latter paper is prepared by diazotization of 2-aminophenylthioether paper. The apparatus and transfer conditions were similar to those described by Bittner *et al.* (6). Briefly, dialysis membranes were glued to two plastic frames. A sheet of Whatman 3MM paper was placed on each side of the gel/diazo-paper. This was assembled under buffer and then placed between the dialysis membranes; air bubbles were excluded. The apparatus was fastened together by plastic bolts and immersed vertically in 5 liters of buffer. The diazo-paper faced the positive electrode. Transfer was performed at 30 V (about 2.5 A) for 2 hr. A magnetic stirring bar was used to circulate the buffer. After transfer, the diazo-paper was gently rocked in 250 ml of 0.1 M Tris·HCl, pH 9.0/10% (vol/vol) ethanolamine/0.25% gelatin (3) for 2 hr at 37°C.

Antiserum and ^{125}I-Labeled Protein A. Goat antiserum was prepared against purified Ad2 virions. Guinea pig antiserum was prepared against purified M_r 73,000 DBP (11). Rabbit anti-bovine serum albumin was from Miles–Yeda. ^{125}I-Labeled protein A was prepared by the chloroglycouril method (12). Labeled protein A was separated from unreacted ^{125}I by exclusion chromatography with a 10-ml Bio-Gel P-10 column.

Immunoautoradiography of Diazo-Paper Containing 1D and 2D Gel Protein Transfers. After washing in Tris/ethanolamine/gelatin solution, diazo-paper protein transfers were rinsed in water and blotted on Whatman 3MM paper. Antiserum was diluted 1:20 in 50 mM Tris·HCl, pH 7.4/5 mM EDTA/150 mM NaCl/0.25% gelatin/0.05% Nonidet P-40 (TENG-N) (3), and 0.5 ml was added per lane of diazo-protein transfer. The transfer was wrapped in SaranWrap and incubated overnight with gentle rocking at 37°C. The transfer was rinsed in 50 ml of TENG-N and rocked in 250 ml of TENG-N for 2 hr at 37°C. It was then blotted and incubated with ^{125}I-labeled protein A (5×10^5 cpm/ml) in TENG-N in a Pyrex dish for 2 hr at 37°C with rocking. The transfer was rinsed in water and washed in 250 ml of 50 mM Tris·HCl, pH 7.4/5 mM EDTA/1 M NaCl/0.25% gelatin/0.4% Sarkosyl with rocking at 37°C for 2 hr. It was again rinsed in water, blotted, air dried, and autoradiographed at −70°C with Dupont Cronex Lightning-Plus XL intensifying screens.

Diazo-transfers were erased by treatment with 2% $NaDodSO_4$/0.1 M 2-mercaptoethanol/50 mM phosphate buffer, pH 7.5, at 60°C for 30 min. This dissociates the IgG and *S. aureus* A protein from the diazo-paper, and thus the bound protein can be assayed again with antiserum after reequilibration with TENG-N buffer.

RESULTS

Electrophoretic Transfer of Proteins from $NaDodSO_4$/Polyacrylamide Gels to DBM-Paper. To establish optimal conditions for the transfer of proteins from gels in diazo-paper, we analyzed [^{35}S]methionine-labeled extracts of Ad2-infected and mock-infected KB cells. 1D gels were run in a 10–18% $NaDodSO_4$/polyacrylamide gradient with duplicate samples applied to each half. One half was dried and autoradiographed

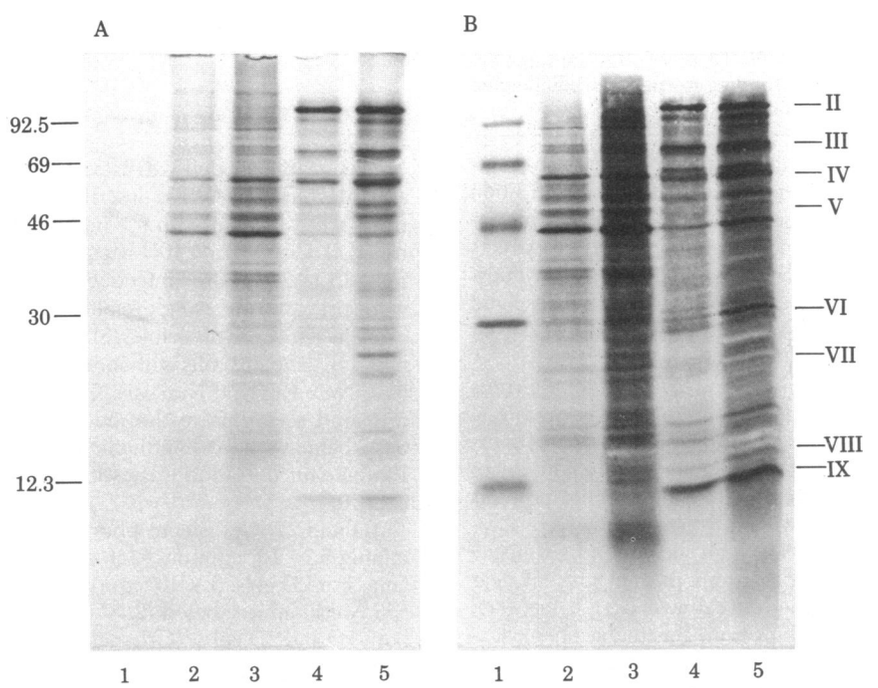

FIG. 1. Electrophoretic transfer of proteins from a 1D $NaDodSO_4$/polyacrylamide gel to DBM-paper. [^{35}S]Methionine-labeled polypeptides from Ad2-infected (lanes 4 and 5) and mock-infected (lanes 2 and 3) KB cells were separated by electrophoresis. (*A*) One half of the gel was autoradiographed directly. (*B*) Proteins from the other half were transferred electrophoretically to DBM-paper and autoradiographed. Lanes: 1, ^{14}C-labeled molecular weight marker proteins ($\times 10^{-3}$); 2 and 3, 0.5 and 1.0×10^6 cpm of mock-infected cell extract; 4 and 5, 0.5 and 1.0×10^6 cpm of late Ad2-infected cell extract. Roman numerals designate Ad2-specific proteins. Both the gel and the DBM-paper autoradiographs were exposed for 17 hr.

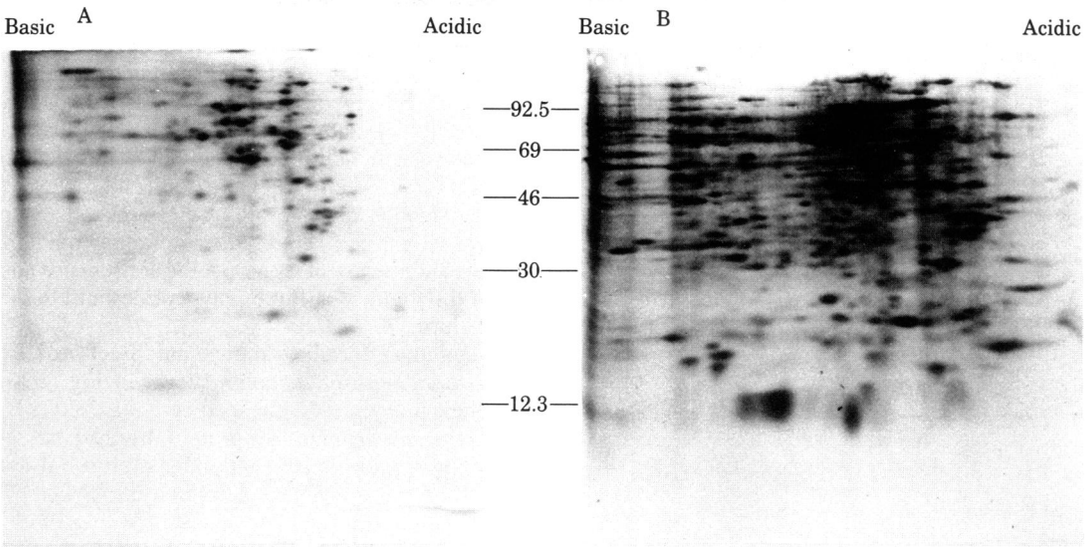

FIG. 2. Electrophoretic transfer of proteins from 2D NaDodSO$_4$/polyacrylamide gel (*A*) to DBM-paper (*B*). [^{35}S]Methionine-labeled polypeptides from KB cells were separated by 2D gel electrophoresis. Molecular weights ($\times 10^{-3}$) shown were determined by comparison with ^{14}C-labeled marker proteins in a parallel 1D gel. Both the gel and the DBM-paper autoradiographs were exposed for 7 days.

after electrophoresis. The other half was used for transfer of proteins to DBM-paper and the paper was then autoradiographed. We found that electrophoretic transfer of proteins from gels to DBM-paper was achieved efficiently in 25 mM sodium phosphate buffer at pH 6.5 with a constant voltage of 30 V (about 2.5 A) for 2 hr.

Proteins of all molecular weights were transferred to DBM-paper with excellent fidelity (Fig. 1). For example, note that in lane 5 of Fig. 1*B* the Ad2 hexon protein II (M_r 120,000) and protein IX (M_r 12,000) (13) are both clearly defined on the transfer. Autoradiographic analysis of DBM-paper is more sensitive than that of dried gels, as dramatically shown by comparison of the standard proteins in lanes 1 of Fig. 1 *A* and *B*.

For greater resolution, the same cell extracts were run on 2D O'Farrell gels and transferred as above; identical 2D gels were dried and autoradiographed or were used for transfer to DBM-paper which was then autoradiographed (Fig. 2). We obtained transfers of all proteins that were detected on the 2D gel, with excellent resolution.

The extent of electrophoretic transfer of ^{32}P-labeled proteins to DBM-paper depends on the concentration of protein in the gels and on the molecular weight (Table 1). By determination of the radionuclides in individual protein bands in gels with and without transfer and in bands bound to DBM paper, we found that, at the lowest protein input, 35–79% of the protein was transferred, depending upon the size of the protein; the least transfer occurred with proteins larger than 73,000 daltons. As the protein load increased, the transfer decreased; when 150 μg was loaded per lane, the transfer ranged from 18% to 30%. Extending the time up to 5 hr did not result in transfer of appreciably more proteins from the gel under our conditions.

Immunoautoradiographic Analysis of Ad2 Late and Virion Proteins Bound to DBM-Paper. Having established conditions for the transfer of proteins from NaDodSO$_4$/polyacrylamide gels to DBM-paper with a high degree of fidelity, we prepared equivalent imprints of unlabeled proteins from mock-infected and late Ad2-infected KB cell extracts for immunoautoradiographic analysis. DBM-paper transfers of 1D NaDodSO$_4$/polyacrylamide gels were incubated with anti-Ad2 virion antiserum and then with ^{125}I-labeled protein A (Fig. 3). No proteins were labeled in the mock-infected cell run. Among proteins from late Ad2-infected cells treated with anti-Ad2 virion antiserum and ^{125}I-protein A, seven viral protein bands were readily detected, and several others were faintly visible after a short exposure time. Those proteins intensely stained were from top to bottom virion protein II (hexon), III (penton), IV (fiber), V (core), pVI, VI (hexon-associated), and VIII (hexon-associated) (13). The DBM-paper transfer was incubated with NaDodSO$_4$ and 2-mercaptoethanol to remove ^{125}I-protein A and antibody; good erasure was achieved. When the erased paper was given a second reaction with antiserum to Ad2 virions, excellent staining with antibody was obtained.

Fig. 4*A* is an autoradiograph of a 2D gel of a [^{35}S]methionine-labeled late Ad2-infected cell extract. Fig. 4*B* shows an immunoautoradiograph made by electrophoretically transferring unlabeled proteins from an identical gel to DBM-paper, treating with anti-Ad2 virion antiserum, and labeling with ^{125}I-labeled protein A. Fig. 4*C* is a 2D gel autoradiograph of [^{35}S]methionine-labeled Ad2 virion proteins for comparison. In Fig. 4*B*, the prin-

Table 1. Electrophoretic transfer of proteins to DBM-paper as a function of size and concentration of protein

Molecular weight $\times 10^{-3}$	% transfer* by protein concentration on gel			
	1.2 μg	6 μg	30 μg	150 μg
11–19	52	63	46	30
20–26	52	50	44	27
31–37	79	78	52	21
45–50	66	51	38	26
73–80	35	30	26	18

^{32}P-Labeled KB cell proteins were resolved by 1D NaDodSO$_4$/polyacrylamide gel electrophoresis and electrophoretically transferred to DBM-paper; 1×10^7 cpm was applied to each lane and the protein load was varied by addition of unlabeled KB cell extract. Bands detected in autoradiographs were cut from parallel gels before and after transfer and from DBM-paper, and their radioactivity was determined. Recovery of radioactivity in gels after transfer to DBM-paper was $\geq$80%, for proteins larger than 26,000 daltons, of that in bands from untransferred gels. For smaller proteins, recovery was 44–73%, some loss occurring during transfer or during the 2-hr wash after transfer in the protocol used for immunoautoradiography.

* Determined by comparing the cpm on DBM-paper with the cpm in matching bands in untransferred gels. Four or more bands were used for each determination. The protein concentration is shown as μg per lane.

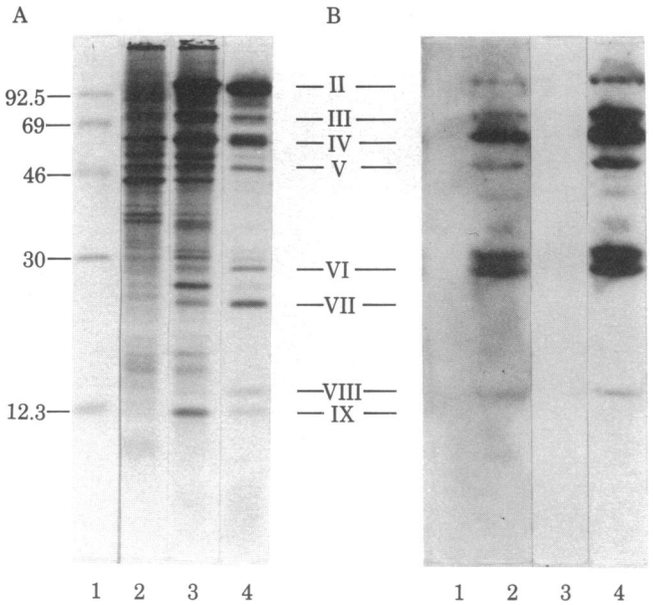

FIG. 3. Immunoautoradiographic detection of Ad2 late proteins transferred to DBM-paper. (*A*) Autoradiograph of [^{35}S]methionine-labeled Ad2-infected (lane 3) and mock-infected (lane 2) KB cell and Ad2-virion proteins (lane 4) resolved on a 10–18% NaDodSO$_4$/polyacrylamide gel. The ^{14}C-labeled molecular weight markers ($\times 10^{-3}$) are shown in lane 1. (*B*) Immunoautoradiograph of Ad2-infected (lanes 2–4) and mock-infected (lane 1) KB cell proteins electrophoretically transferred to DBM-paper and treated with anti-Ad2 virion antiserum and ^{125}I-labeled protein A. Lane 3 is an autoradiograph of lane 2 "erased"—i.e., after treatment with NaDodSO$_4$ and 2-mercaptoethanol. Lane 4 is an immunoautoradiograph of lane 3 after a second reaction of the DBM-paper transfer with anti-Ad2 virion antiserum and ^{125}I-labeled protein A. The Roman numerals refer to Ad2 specific proteins.

cipal virus-related proteins seen in the immunoautoradiograph are II at the top, III in the band at 85,000, IV at 62,000, several viral-related proteins discernible in a broad band centered at 62,000, and several proteins at about 50,000, one of which is probably V. Lighter spots of smaller Ad virion proteins are also detectable. In addition, other spots which may represent pVI and pVII are seen. The increased resolution of 2D gels allows the detection of many more virus-related polypeptides than were seen in the 1D immunoautoradiograph (Fig. 3). Peptide maps and the use of monospecific antisera are needed to establish the identity of these polypeptides and to determine relationships.

Immunoautoradiographic Analysis of Ad2-Encoded DBP. The immunoautoradiograph shown in Fig. 4*D* illustrates the detection of the Ad2-encoded early M_r 73,000 DBP by monospecific antiserum to that protein. Because the same DBM-paper was used as for Fig. 4*B* (after erasure), it is apparent that the DBP spot was not labeled when anti-virion serum was used.

1D immunoautoradiographs can be used to follow the production of DBP and antigenically related components. Ad2 DBP was detected early during infection after cells had been released from a CHX block but while DNA synthesis was being inhibited by AraC (Fig. 5*A*, lane 2). Impressive levels of DBP were detected by 18 hr. Several smaller bands at 41,000–48,000 were present. The immunoradiographic technique showed these to be related to 73,000-dalton DBP, confirming the relationship suggested by tryptic peptide analyses (14). The ease of direct analysis of the cell extracts by immunoautoradiography to detect specific virus-related components is an advantage. In addition, normal cell proteins do not interfere. The smaller components are present in lower concentrations than the 73,000-dalton component and may be protease digestion products. These could play a biological role.

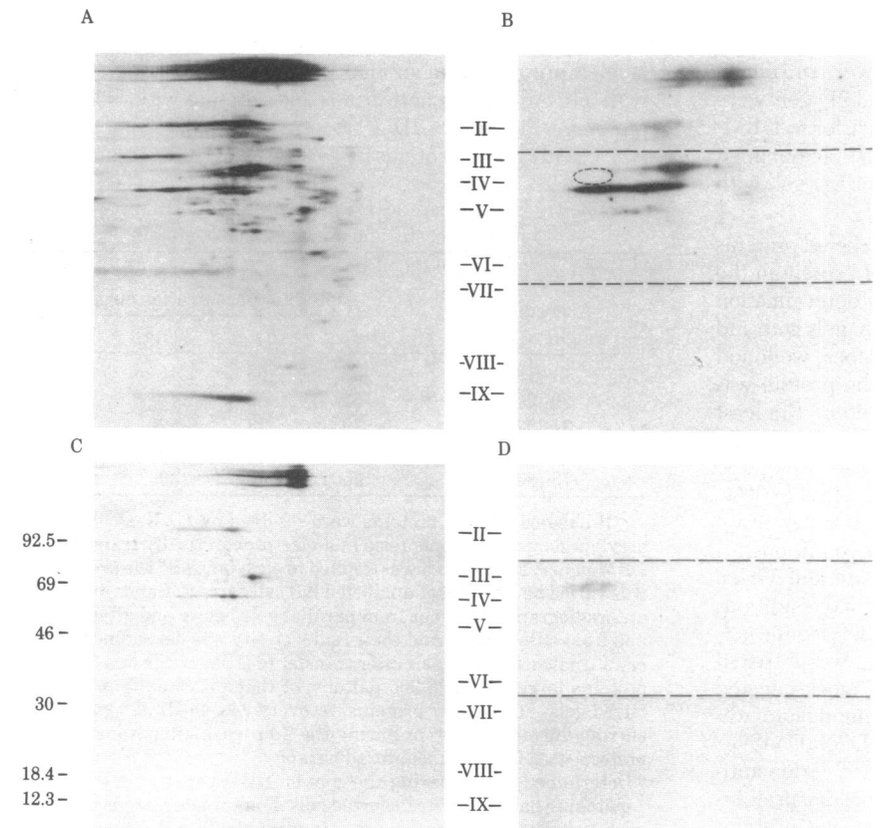

FIG. 4. Immunoautoradiographic detection of Ad2 proteins transferred from a 2D gel to DBM-paper. (*A*) 2D gel autoradiograph of [^{35}S]methionine-labeled late Ad2-infected KB cell proteins. (*B*) 2D immunoautoradiograph of late Ad2-infected KB cell proteins electrophoretically transferred to DBM-paper and treated with anti-Ad2 virion antiserum and ^{125}I-labeled protein A. (*C*) 2D gel autoradiograph of purified [^{35}S]methionine-labeled Ad2 virion. The Roman numerals refer to Ad2 specific proteins. Molecular weights were determined by comparison with ^{14}C-labeled marker proteins in a parallel 1D gel. (*D*) 2D immunoautoradiograph of *B* after erasure and second treatment with anti-73,000-dalton DBP antiserum. The portion of paper between the dotted bands was used for the second reaction; only one spot was labeled. This area in the original reaction with antivirion antiserum was not labeled (indicated by dotted oval).

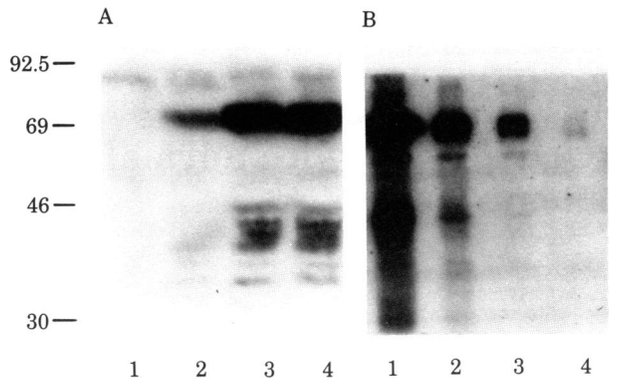

FIG. 5. Immunoautoradiographic detection of DBP in Ad2 KB cell extracts transferred to DBM-paper. (*A*) Proteins electrophoretically transferred from $NaDodSO_4$/polyacrylamide gel to DBM-paper and treated with anti-73,000-dalton DBP antiserum and ^{125}I-labeled protein A. Lanes: 1, mock infected; 2–4, Ad2 infected (2, early; 3, 18 hr; 4 24 hr). Molecular weights ($\times 10^{-3}$) indicated were determined by ^{14}C-labeled marker proteins. (*B*) Proteins harvested from cells at 26 hr, electrophoretically transferred from a gel to DBM-paper, and treated with anti-73,000-dalton DBP antiserum and ^{125}I-labeled protein A. The amounts of protein in the cell extract on the gel before transfer were: lane 1, 30 μg; lane 2, 6 μg; lane 3, 1.2 μg; lane 4, 0.24 μg.

Sensitivity of Immunoautoradiographic Analysis. To assess the sensitivity of this method, we used bovine serum albumin and rabbit anti-bovine serum albumin. From 1 to 10 ng could be detected on DBM-paper by immunoautoradiography. A further test of the sensitivity made use of a series of dilutions of Ad2-infected KB cell extracts and specific anti-73,000-dalton DBP antiserum. Fig. 5*B* shows the detection of DBP when as little as 240 ng of total cell protein was applied to the gel. The amount of Ad2-specific DBP present in KB cells is about 0.5–1% of the total protein (11). Thus, about 2 ng of DBP was detected. Because one can load 600 times this much cellular protein on a 1D gel and get good definition, and even more on a 2D gel, this technique allows one to detect a specific protein present in a cell extract at a level of 0.001% of the total protein content.

DISCUSSION

The analysis of complex biological mixtures for specific proteins present in small amounts is important in many laboratory and clinical investigations. We present in this report a system to resolve such proteins and to identify them by probing with specific antibodies and other reagents.

To evaluate this system, we analyzed Ad2-specific late proteins and DBP in KB cells. Proteins were separated by conventional 1D $NaDodSO_4$/polyacrylamide gel electrophoresis or O'Farrell type 2D gel electrophoresis using 10–18% slab gels because these systems give good resolution and are applicable to proteins of a wide range of molecular weights. Electrophoresis for 2 hr was used to transfer the proteins from gels to DBM-paper, to which they are efficiently bound. The transfer and binding is a function of concentration of protein as well as size. With increased protein load, the efficiency of transfer is reduced from 35–79% to 18–30%. Our procedure is an improvement over several recently described procedures. For example, one procedure requires overnight incubation to get 11–16% transfer with a load of only 7 ng of each of several proteins (3). Another procedure, using nitrocellulose (15), requires 36–48 hr. These proteins are not covalently bound. We were able to transfer both large and small proteins from gels to DBM-paper with excellent fidelity. Autoradiographic analysis of the thin DBM-paper transfers is more sensitive than that of the original gels. We have used DPT-paper in similar experiments and obtained similar results.

This system for the detection and identification of proteins is very sensitive. As little as 1 ng of protein present at a concentration of 1 part in 100,000 of cell proteins can be detected. In addition, the background is very low, unlike that found with nitrocellulose (15). Finally, and of great advantage, is that the DBM-protein imprints can be erased and reused (Fig. 3*B*) (3). Thus, the protein transfer can be used with different antibody probes to detect various specific proteins.

An interesting potential application would be the screening of cells for transformation proteins. Anti-tumor cell antiserum might be used to probe for unknown transforming proteins. In addition to the advantage that labeled antigen is not required, purified antigen need not be used to make the antiserum. Protein imprints may be valuable for various additional studies. For example, proteins that specifically bind to various types of ligands can be identified by incubation of radiolabeled molecules such as DNA and RNA fragments or hormones with diazo-protein transfers.

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