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T Antigen of BK Papovavirus in Infected and Transformed Cells

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BK virus T antigen from BKV-transformed rat and hamster cells and from productively infected monkey cells has been examined by immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Forms of the antigen that migrate as proteins of 86,000 and 92,000 daltons have been identified. Both forms can be labeled by ^{32}P.

One consequence of the productive infection of human or simian cells by BK virus (BKV), a papovavirus of human origin, is the induction of a nuclear antigen (T) that reacts with sera from animals bearing BKV-induced tumors (19). Immunofluorescence studies with such antisera cannot distinguish the T antigen in cells undergoing a productive infection from T antigen in cell lines derived either from BKV-induced tumors or by in vitro cell transformation by BKV. Cells containing BKV T antigen also react with some simian virus 40 (SV40) tumor antisera (14, 17, 19, 20). However, it has not been conclusively demonstrated that SV40 and BKV tumor antisera react with the same component in BKV-infected cells. The importance of relatedness between T antigens of BKV and SV40 is emphasized by the finding that there is DNA sequence homology between BKV and SV40, but that the homologous regions of the genomes correspond to the part of SV40 DNA that codes for viral capsid proteins and not for T antigen (12, 16). Weak homology between the early regions has recently been detected (N. Newell, C. J. Lai, G. Khoury, and T. J. Kelly, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, S350, p. 337).

This report describes experiments in which electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) was used to analyze proteins immunoprecipitated from permissive cells infected with BKV and from several cell lines transformed by BKV. The results demonstrate that either BKV or SV40 T antisera can immunoprecipitate a polypeptide of 86,000 daltons from both BKV-infected monkey kidney (Vero) cells and BKV-transformed hamster cells. From a line of BKV-transformed rat cells, however, a polypeptide of 92,000 daltons is immunoprecipitated in addition to the 86,000-dalton polypeptide.

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MATERIALS AND METHODS

Cell cultures. Two continuous African green monkey kidney cell lines, Vero and CV-1 (American Type Culture Collection), were used to grow BK virus and SV40, respectively. BKT-2 is a line of hamster cells derived from a BKV-induced tumor as described previously (20). 2RE-BK is a cell line derived by BKV transformation in vitro of a rat embryo cell culture, obtained from Aimo Salmi. Two cloned derivatives of this cell line, C2 and C12, were also used in these experiments. H-50 cells, a hamster cell line transformed by SV40 (11), were obtained from Fred Rapp. Tumors were induced with H-50 cells in hamsters, and a cell line was established from one of the tumors and used in this study. All cell lines transformed by BKV or SV40 were tested and found positive for T antigen by immunofluorescence. BHK-21 (C13) cells (American Type Culture Collection) were used as T antigen-negative control cells.

All cell lines were passed with trypsinization and grown in plastic flasks (Falcon Plastics, Oxnard, Calif.) in Eagle minimum essential medium (MEM; Grand Island Biological Co., Grand Island, N.Y.) containing 5 to 10% fetal calf serum.

Viruses. The BKV strain used was originally isolated from a kidney transplant patient by Sylvia Gardner (7). A chronic type of infection was used for continued production of virus. A flask with a subconfluent layer of Vero cells was infected with BKV at about 0.01 mean tissue culture infective dose per cell. After 5 to 10 days, infected cells were detached by trypsinization and passed at a ratio of 1:3. After about another week, the medium which had a BKV hemagglutination titer of 1:128 was collected, and the cells were trypsinized and passed again. This procedure could be continued for about six passages.

A large-plaque strain of SV40 was propagated and titrated by plaque assay in CV-1 cells as described previously (4). For SV40 T-antigen experiments, Vero cells in plastic flasks were infected with SV40 (at a multiplicity of infection of 10 PFU/cell). After an

adsorption period of 4 h at 37°C, seed virus was washed off and medium was added. Infected cells were used for labeling 48 h after infection.

Radioactivity labeling. [^{35}S]methionine was purchased from New England Nuclear (Boston, Mass.). Cultures of infected or transformed cells in 75-cm^2 or 150-cm^2 flasks were labeled at isotope concentrations of 25 to 100 μCi/ml. For labeling times of 4 h or less, MEM with 1/100 of normal methionine concentration was used. A complete medium was used for labeling for 8 to 16 h. Cultures for $^{32}PO_4$ labeling were rinsed with phosphate-free MEM and labeled with 50 to 100 μCi of ^{32}P phosphate (carrier-free, New England Nuclear) per ml in phosphate-free medium for 4 to 5 h. At the end of the labeling period, cells were detached by scraping with a rubber policeman in phosphate-buffered saline solution, pH 7.2 (PBS), and washed once with PBS. A low-speed centrifugation pellet (1,000 × *g*, 5 min) was used to make chromatin extracts.

Preparation of chromatin extracts. Chromatin was purified by the method of Hancock (8) with minor modifications. Briefly, the cell pellet, containing from 10^7 to 10^8 cells, was suspended in 30 ml of buffer A (0.1 M sucrose–0.2 mM sodium phosphate buffer, pH 6.8) with which it was washed three times by centrifugation (2,500 × *g*, 10 min, 4°C). The final cell pellet was resuspended in buffer A (1 ml), and cells were lysed by adding 4 ml of buffer B (0.5% Nonidet P-40–0.2 mM EDTA, pH 6.8). Lysate was layered on a 25-ml cushion of buffer A and centrifuged at 16,000 × *g* for 15 min at 4°C in a swinging-bucket rotor. The chromatin pellet was gently suspended, and centrifugation through buffer A was repeated.

T antigen was extracted from the chromatin pellet by ultrasonic treatment in the presence of 1 or 2 ml of extraction buffer consisting of 0.5 M LiCl–0.1% Triton X-100–1 mM EDTA–20 mM Tris-hydrochloride, pH 8.0. After centrifugation for 30 min at 20,000 × *g*, T antigen was immunoprecipitated from the supernatant fluid.

Immunoprecipitation. BKV T antiserum was a pool collected from hamsters carrying transplanted BKV-induced tumors. Two preparations of SV40 T antiserum were used; one was from the National Cancer Institute (batch 5-X-0856), the other one was a pool from hamsters carrying H-50 cell tumors. A serum pool from normal hamsters served as control. Antiserum against hamster immunoglobulin G (IgG) was produced in rabbits with hamster IgG purified by DEAE-cellulose chromatography as antigen. In the two-step immunoprecipitation procedure, 10 μl of an appropriate hamster serum was allowed to react with 0.5 to 1.5 ml of labeled chromatin extract overnight at 4°C. An equivalent amount of rabbit anti-hamster IgG was added, and the incubation was continued for 4 h. The immunoprecipitate was centrifuged through 5% (wt/vol) sucrose in the extraction buffer (3,000 × *g*, 10 min) and washed once with 2 mM Tris-hydrochloride buffer, pH 7.4.

In some experiments, *Staphylococcus aureus* protein A was used as an antibody adsorbent instead of the anti-IgG. The advantages of this procedure lie in the speed with which it can be carried out and the reduction in the amount of immunoglobulin in the final gel sample. The adsorbent was prepared as described by Kessler (10), using the Cowan 1 strain of *S. aureus* obtained from the American Type Culture Collection. Precipitation was done as follows: after adding 10 μl of either T antiserum or normal hamster serum to the chromatin extract and incubating at 4°C for 1 h, the bacterial adsorbent (200 μl of 10% wt/vol) was added and incubation continued at 4°C for 15 min. The adsorbent was then washed and prepared for electrophoresis as described above for the double-antibody method. After suspending the washed pellet in gel sample buffer and boiling for 5 min, the extract was centrifuged at 10,000 × *g* for 10 min, and the supernatant fluid was applied to the gel.

Gel electrophoresis and autoradiography. Immunoprecipitates were suspended in sample buffer containing SDS (3%), Tris-hydrochloride (0.06 M, pH 6.8), 2-mercaptoethanol (5%), glycerol (10%), and bromophenol blue (0.005%), and the solution was boiled for 10 min. Samples were analyzed by discontinuous PAGE. The buffer system of Laemmli (13) was employed in a slab gel apparatus similar to that described by Studier (21). A sample gel of 3% acrylamide and separating gels of 6.5, 7.5, or 10% were used.

Electrophoresis was carried out at constant current of 25 mA for 10-cm gels or 15 mA for 20-cm gels. After electrophoresis, the gels were fixed and stained with Coomassie brilliant blue (5).

In ^{35}S experiments, destained gels were impregnated with 2,5-diphenyloxazole, vacuum dried, and exposed to Kodak RP Royal X-Omat film at −20°C as described by Bonner and Laskey (2). When ^{32}P was used for labeling, destained gels were vacuum dried and autoradiographed on the same type of film. Densitometer tracings of autoradiograms were done with a Joyce-Loebl densitometer.

Molecular weights were estimated with β-galactosidase (130,000), α-phosphorylase (94,000), and bovine serum albumin (68,000) as markers (2, 4).

Immunofluorescence staining. Cells were grown on cover slips, washed in PBS, air dried, and fixed in acetone for 10 min at room temperature. They were stained by an indirect immunofluorescent technique with the same hamster sera as for immunoprecipitation and fluorescein-conjugated anti-hamster IgG from the National Cancer Institute. A total of 400 to 600 cells was counted in Vero cell cultures infected with BKV to calculate the percentage of T antigen-positive cells.

RESULTS

BKV-transformed hamster cells. Because most chromatin-purification procedures call for salt concentrations high enough to remove a sizable portion of the chromosomal proteins and because SV40 T antigen is eluted from nuclei under conditions of physiological pH and salt concentrations (23), we purified chromatin as described by Hancock (8). Chromatin isolated by this method retains the nucleosome structure of native chromatin and contains the macromolecular components required for both RNA and DNA synthesis. For the experiment illustrated in Fig. 1 A, C, and D, a line of BKV-transformed

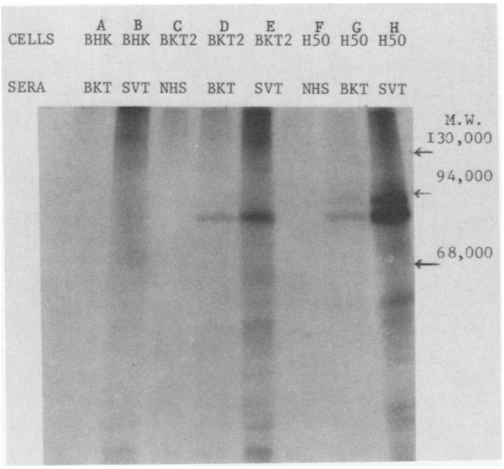

FIG. 1. *T antigens in BKV-transformed and SV40-transformed cells. BHK, BKT-2, and H-50 cells were labeled with $^{32}PO_4$ as described in the text. Chromatin was purified and eluted with extraction buffer. After immunoprecipitation and washing, the precipitate was dissolved in electrophoresis sample buffer and applied to gel. The 9-cm separating gel contained 10% acrylamide and was run for 1.5 times the length of time required for the dye to reach the bottom of the gel. After fixing, staining, destaining, and drying, the gel was exposed to film for 7 days. The sera were BKV T antiserum (BKT), SV40 T antiserum (SVT), or normal hamster serum (NHS) as indicated. SVT serum was obtained from National Cancer Institute. BKT serum was produced in this laboratory by injecting BKT-2 cells into hamsters.*

hamster cells, BKT-2, and control cells, BHK-21, were labeled with $^{32}PO_4$. Chromatin was purified, and the eluate, made with extraction buffer, was used for immunoprecipitation as described above.

The analysis revealed, in the material immunoprecipitated from BK-transformed cells by BKV T antiserum, a radioactive band (channel D) which was precipitated neither from transformed cells by control serum nor from control cells by BKV T antiserum (Fig. 1A and C). This band corresponds by definition to BKV T antigen. The electrophoretic mobility of this band is that of a protein of 86,000 daltons.

To compare T antigen from cells transformed by BKV to T antigen from cell lines transformed by SV40, chromatin extracts of H-50 cells were immunoprecipitated and analyzed by SDS-PAGE. Both BKV and SV40 T antisera precipitated specifically two polypeptides from SV40-transformed H-50 cells (Fig. 1G and H). One of the polypeptides had the same molecular weight as the T antigen from BKT-2A cells, i.e., 86,000 daltons; the other one had an apparent molecular weight of 92,000. Both forms were phosphorylated as indicated by their incorporation of ^{32}P. SV40 T antiserum also immunoprecipitates from BKT-2 cells material which migrates as an 86,000-dalton protein (Fig. 1E).

Productive infection. Since it has been reported that the molecular weight of SV40 T antigen in transformed cells is greater than in cells undergoing productive infection by about 10,000 (1, 3), we looked for such a difference in cells infected or transformed by BKV. Vero cells were chronically infected with BKV, as described above, and monitored for appearance of T antigen by immunofluorescence with BKV T antiserum. At the time when cells were used for labeling experiments, the number of T antigen-positive cells was 8 to 9%. The amount of T antigen in each positive Vero cell was, however, greater than in BKT-2 cells as judged by the intensity of immunofluorescent staining with the same antiserum. Radioactive labeling of BK-infected cells, chromatin extraction, immunoprecipitation, and PAGE were carried out as described above. The resulting autoradiogram (Fig. 2C, D, and E) reveals a single T antigen band of molecular weight 86,000. This band comigrates with that found in the BKT-2A cells (Fig. 2B). In contrast to the results with SV40, no significant difference in molecular weight was detected between the BKV T antigen from cells undergoing productive infection and from BKV-transformed hamster cells.

BKV-transformed rat cells. We decided to look at a line of BKV-transformed rat cells (2RE-BK) to see whether BKV T antigen in these cells had the same molecular weight as that in the BKV-transformed hamster cells. When immunoprecipitation and SDS-PAGE analyses were applied to labeled extracts of these cells, the results shown in Fig. 2A and 3 were obtained. Two bands are clearly visible which are absent from control preparations (Fig. 3). The lower band from the BKV-transformed rat cells has an apparent molecular weight of 86,000 and comigrates with T antigen from BKT-2 cells. The second protein has a molecular weight of 92,000, and it contains less $^{32}PO_4$ radioactivity than the 86,000-dalton protein.

In addition to the T antigen bands already mentioned, in some experiments (e.g., see Fig. 5) we also observed a broad band at the 55,000-dalton position. This band has been observed in both the transformed and nontransformed rat and hamster cells with BKV T and SV40 T antisera and with control hamster sera with both $^{32}PO_4$ and [^{35}S]methionine-labeled material. It did not always appear when the double-antibody immunoprecipitation method was used. With the protein A bacterial-adsorbent technique, among the large number of non-T antigen bands found in the gel is a disproportionately broad

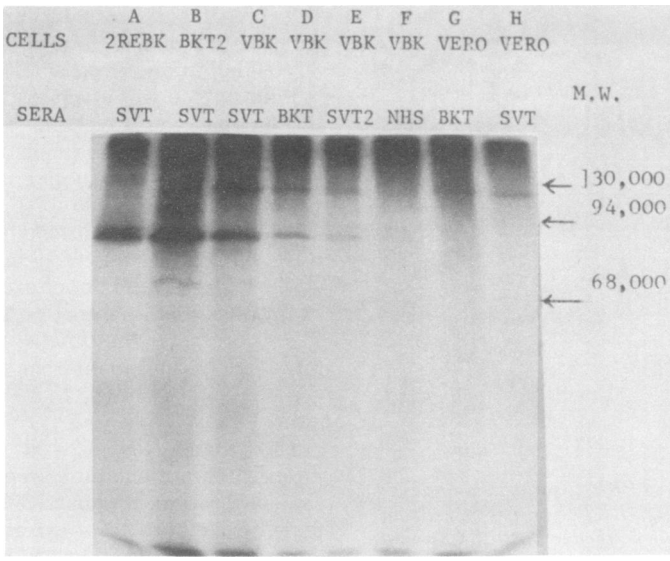

FIG. 2. *T antigen in cells undergoing productive infection by BKV. 2RE-BK, BKT-2, and Vero cells and BKV-infected Vero cells were labeled with $^{32}PO_4$ and extracted as described in the text. In this case the immunoprecipitates were applied to a 7.5% polyacryalmide gel, which was run until dye reached the bottom.*

band at the 55,000-dalton position. This band can be seen in all slots of the gel shown in Fig. 4. It was not eliminated by the use of protease inhibitors including phenylmethylsulfonyl fluoride (PMSF) and L-1-(tosylamide-2-phenyl)ethyl-chloromethyl ketone (TPCK) (6, 15).

DISCUSSION

The results presented in this study demonstrate that BKV T antigen is a component of the cellular chromatin purified by a low-salt technique and that it can be labeled by $^{32}PO_4$ as well as [^{35}S]methionine. In a BK-transformed hamster cell line, we observed a single high-molecular-weight (86,000) form of T antigen. An SV40-transformed hamster cell line, however, produced both an 86,000 and 92,000-dalton form. Since these results, initially observed with $^{32}PO_4$-labeled material, were confirmed by the use of [^{35}S]methionine as label (Fig. 4), the absence of a 92,000-dalton band from the BKV-transformed hamster cells labeled with $^{32}PO_4$ cannot be due to a difference in phosphorylation ability between BKV- and SV40-transformed hamster cells.

Comparison of T antigen from BKT-2 cells with T antigen from BKV-infected Vero cells did not reveal the size difference that might be expected by analogy with SV40 (1, 3). Both the transformed and permissively infected cells contained the 86,000-dalton form. No higher-molecular-weight form was revealed even when the autoradiograms were grossly overexposed. Since

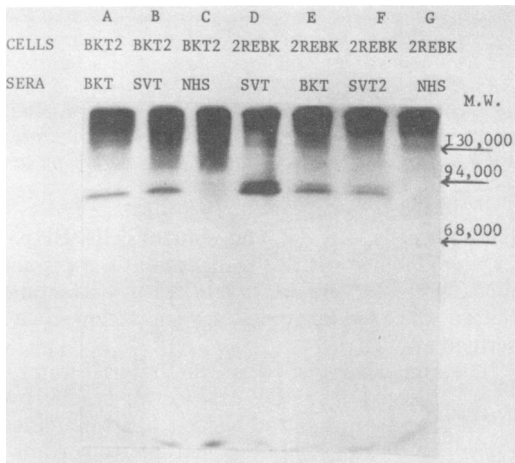

FIG. 3. *T antigen in BKV-transformed rat cells. 2RE-BK and BKT-2 cells were labeled and extracted as in Fig. 1. Sera are as in Fig. 1 except SVT-2 is an SV40 T antiserum produced in this laboratory by injecting H-50 cells into hamsters. Gel electrophoresis as in Fig. 2.*

it seemed possible that the size of BKV T antigen might be an accidental consequence of the cell type in which it is made and that this is not related to the cell's ability to permit productive infection, we examined a line of BKV-transformed rat cells, 2RE-BK (22). In this cell line we observed production of not only the 86,000-dalton species but also a 92,000-dalton form (Fig. 5). Both these forms were precipitated by BKV T antiserum and by SV40 T antisera from sev-

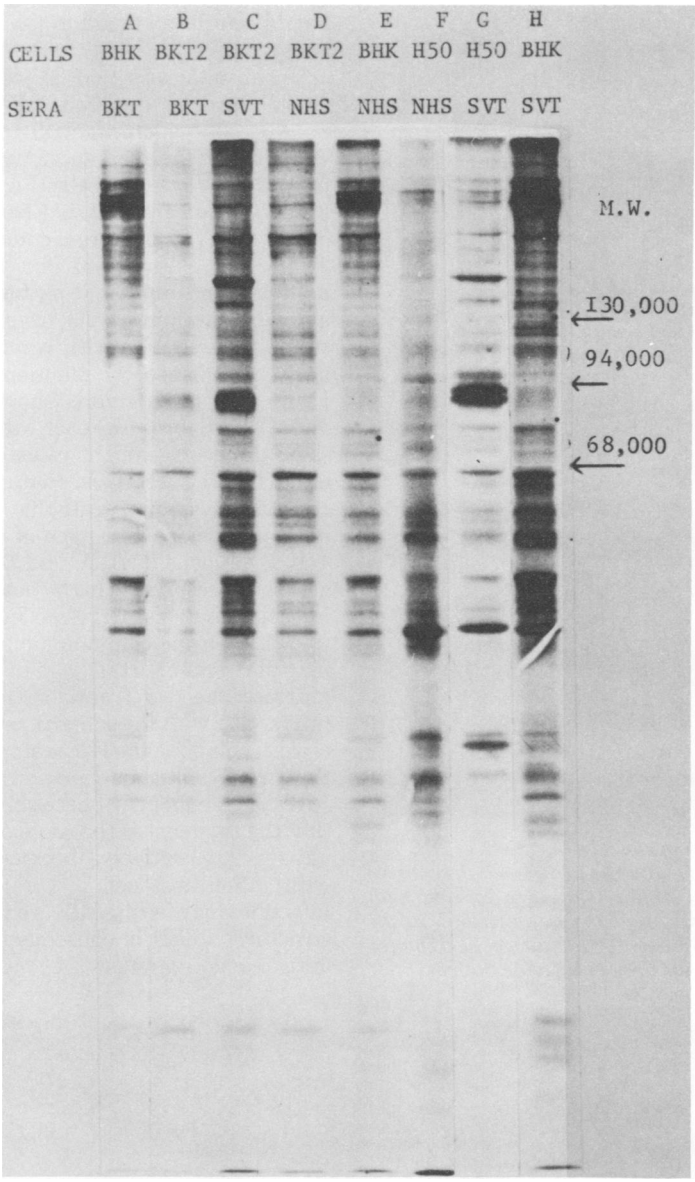

FIG. 4. *T antigen prepared by antibody-adsorbent method. BHK, BKT-2, and H-50 cells were labeled with [^{35}S]methionine as described. Immunoprecipitation was done by the S. aureus protein A antibody-adsorbent technique described in the text. The 15-cm, 7.5% polyacrylamide gel was run until dye reached the bottom.*

eral different animals but not by control sera. Both forms could be labeled by either [^{35}S]methionine or $^{32}PO_4$.

One possible explanation for the occurrence of two molecular weight forms of T antigen is that the 2RE-BK line contains two populations of cells, one producing high- and the other producing low-molecular-weight forms of T antigen. To test this possibility, we examined two clones isolated from 2RE-BK cells (Fig. 5). Both antigens found in the parental line also occur in the clones. Because the low-molecular-weight forms of T antigen might be artifacts produced during extraction, experiments were done to examine this possibility. Inclusion of a protease inhibitor, PMSF (3 mg/ml) (15), in the buffers had no noticeable effect on the pattern of bands produced. When TPCK, another protease inhibitor (6), was included in the buffers (1 mM) instead of PMSF, the only effect observed was an apparent decrease in the amount of radioactivity precipitated. We observed, however, the same

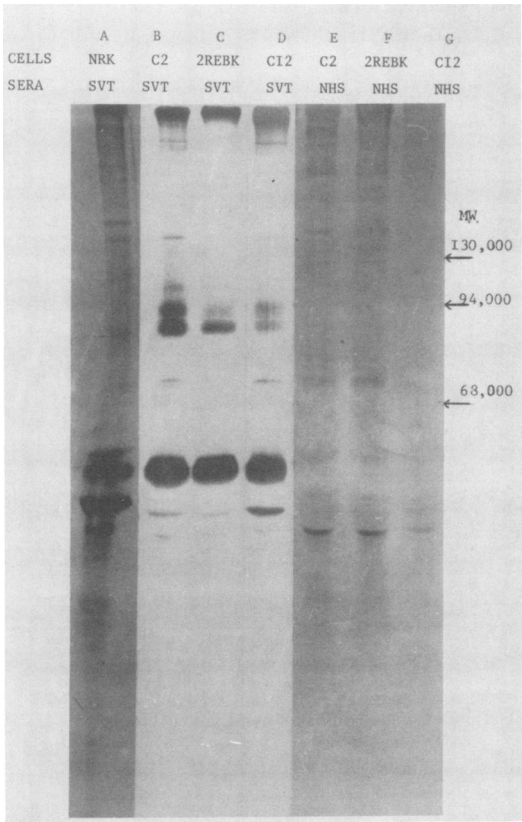

FIG. 5. *T antigen in cloned derivatives of 2RE-BK cells. NRK, 2RE-BK, and the two cloned derivatives of 2RE-BK cells, C2 and C12, were labeled with [^{35}S]methionine and extracted as described in the text. The 15-cm, 7.5% polyacrylamide gel was run for 1.25 the time taken for dye to reach the bottom.*

band patterns as found in the absence of inhibitors.

Our results show that SV40 T antiserum specifically precipitates from BKV-infected cells a polypeptide which, in SDS-PAGE, comigrates with BKV T antigen and SV40 T antigen from productively infected cells (Fig. 6). Reciprocally, a polypeptide precipitated from SV40-infected cells by BKV T antiserum comigrates with SV40 T antigen. Both SV40 and BKV T antisera produce the same band pattern from all the T antigen-containing cells examined. These results, in agreement with what has been previously established by immunofluorescence (14, 17, 19, 20), furthermore, show that both BKV and SV40 T antisera react with material of the same molecular weight in cells that contain T antigen of either virus. Peptide mapping may conclusively demonstrate that both sera can immunoprecipitate T antigen of either virus.

If T antigens of BKV and SV40 share amino acid sequences coded for by heterologous regions in their DNAs as suggested in previous reports (6, 12), then the common amino acid sequences may play an important role in the function of T antigen. The two T antigens might function by interacting with the same or very similar cellular or viral components. For example, it may be that the two T antigens function by binding to a particular sequence of bases in DNA (9, 18), and that the sequence is the same or similar for both viruses. Alternatively, interaction of T antigen with other host cell or viral proteins during infection may stringently require a particular structure, which is consequently an accessible antigenic determinant.

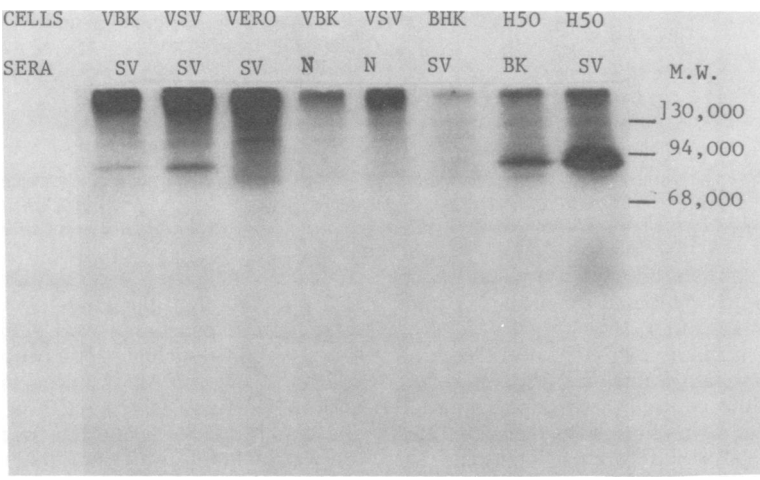

FIG. 6. *BKV and SV40 T antigens from productively infected cells and from SV40-transformed cells. Vero cells, Vero cells infected with SV40 or BKV, BHK cells, and H-50 cells were labeled with $^{32}PO_4$ and extracted as described in the text. After immunoprecipitation with SV40 antiserum (SV), BKV T antiserum (BK), or normal hamster serum (N), gel electrophoresis was carried out as described in Fig. 2.*

An alternative explanation for an antigenic structure common to T antigens of the two viruses could be the existence of a particular post-translational modification on the polypeptide. The possibility of such a modification is emphasized by the finding that both T antigens contain phosphorus (22). We do not yet know to which amino acid residues of BKV T antigen the phosphorus is attached, whether it is directly esterified to amino acids, or is attached to other moieties such as nucleotides or poly ADP ribose. It is conceivable that a modification directly involving phosphorus or perhaps another kind of modification—e.g., glycosylation or acetylation—is responsible for the common antigenic properties.

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