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Biochemistry

Tumor antigen(s) in cells productively infected by wild-type polyoma virus and mutant NG-18

(papovavirus/*hr-t* deletion/nontransforming mutant)

BRIAN S. SCHAFFHAUSEN, JONATHAN E. SILVER, AND THOMAS L. BENJAMIN

Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT When isolated by means of an anti-polyoma tumor (T) antiserum, the major product from mouse cells productively infected by wild-type polyoma virus is a polypeptide of 100,000 apparent molecular weight as judged by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. In cells infected by NG-18, an *hr-t* mutant carrying a deletion of about 150 base pairs in the early region of the viral DNA, a T antigen species appears that comigrates with that of the wild-type virus. Comparisons of peptides after partial proteolysis reveal no differences between mutant and wild-type products. Both wild-type and mutant 100,000 products can be labeled *in vivo* with [^{32}P]orthophosphate. An independent and more reliable estimate of the molecular weight of this protein using guanidine/Sepharose chromatography yields a value of 81,000 for both mutant and wild-type species. The apparent identity of wild-type and mutant products indicates that the deletion in NG-18 lies outside of the region encoding this major T antigen species.

Immunoprecipitates from wild-type infected cells show four bands in addition to the "100,000" band; these have apparent molecular weights of 63,000, 56,000, 36,000, and 22,000 by sodium dodecyl sulfate/polyacrylamide gel electrophoresis; the 56,000 and 36,000 species are phosphorylated. All four of these lower molecular weight bands are absent or drastically reduced in the immunoprecipitates from NG-18-infected cells.

Papovavirus tumor or T antigens are defined operationally as virus-specific products recognized by sera from animals bearing virus-induced tumors. Early demonstrations of the T antigens were by complement fixation with cell extracts (1, 2) or by nuclear immunofluorescence in both productively infected and transformed cells (3). Though usually referred to in the singular, "T antigen" should include all virus-coded (or virus-modified) products present in the tumor that the host recognizes as foreign. Two early polyoma gene functions, defined by the *hr-t* and *ts-a* complementation groups (4, 5), are essential for neoplastic transformation. Any corresponding gene products might be expected to be viral tumor antigens.

Recently attention has been directed toward molecular characterizations of the T antigens. Tegtmeyer and his colleagues carried out analytical studies by immunoprecipitation and sodium dodecyl sulfate (NaDodSO$_4$)/polyacrylamide gel electrophoresis of isotopically labeled materials from simian virus 40 (SV40) infected and transformed cells (6). They and others have shown the existence of a major product of 88,000–100,000 molecular weight (7–9). Livingston and his colleagues have extensively purified an immunoreactive protein of 93,000 from SV40-transformed human cells (10). This protein has specific SV40 DNA binding properties (11, 12). The major (~90,000) T antigen species is shown to be the product of the SV40 *tsA* gene by virtue of temperature sensitivity in both immunoreactivity and DNA-binding properties of the protein isolated from *tsA* mutant-infected cells (13). In addition to a 90,000 species, Prives *et al.* (14) and Greenblatt *et al.* (15) noted minor bands, including a 17,000–20,000 protein synthesized *in vivo* or *in vitro* and immunoprecipitated by antisera to SV40 T antigen.

Similar but less extensive work has been done with polyoma virus. Cuzin and coworkers have reported multiple forms of immunoreactive polypeptides (70,000–75,000, 85,000–90,000) in partially purified preparations of polyoma T antigen (16, 17). Analytical studies of labeled immunoprecipitates have shown a major reactive species of 100,000 and smaller minor components (18, 19). As with SV40, the *ts-a* gene affects the immune reactivity of T antigen as judged by immunofluorescence (20), complement fixation (21), and the disappearance of a ~100,000 band on NaDodSO$_4$ gels (18).

There are presently no data concerning the nature of any *hr-t* gene product. *hr-t* mutants, despite their altered growth properties and inability to transform cells, appear to be normal in T antigen induction on the basis of immunofluorescence (22) and complementation with mutants of the T antigen-defective *ts-a* class (4, 5). The protein coding capacity of the early region of the viral DNA is about 100,000 daltons in a single reading frame. The 100,000 protein, therefore, may be bifunctional, carrying out *hr-t* as well as *ts-a* function. Alternatively, the estimated molecular weight of this protein may be too high, leaving room for an additional polypeptide(s).

The experiments reported here were undertaken to determine the number and sizes of polyoma viral gene products made in productively infected cells and recognized by sera from rats bearing a syngeneic polyoma-induced tumor. An *hr-t* deletion mutant was used to look for alterations in the patterns of T antigens and to determine what polypeptides may be under direct or indirect control of the *hr-t* gene.

MATERIALS AND METHODS

Cells and Viruses. Primary baby mouse kidney cells (23) were infected at a multiplicity of infection of 10 plaque-forming units per cell with NG-18, an *hr-t* deletion mutant of polyoma virus (22), or with R4, a wild-type virus strain derived from NG-18 by marker rescue (24).

Isolation and Analysis of Immunoreactive Proteins. Our procedures are modifications of those of Tegtmeyer *et al.* (25), and Ahmed-Zadeh *et al.* (8). At 12–16 hr after infection, cells were labeled with either [^{35}S]methionine (20–50 μCi/ml; 400–900 Ci/mmol, Amersham) in methionine-free Dulbecco's modified Eagle's medium, or [^{32}P]orthophosphate (250 μCi/ml, carrier free) in phosphate-free medium. At 26 hr after infection, cells were washed twice with cold phosphate-buffered saline,

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Abbreviations: T antigen, tumor antigen; SV40, simian virus 40; NaDodSO$_4$, sodium dodecyl sulfate.

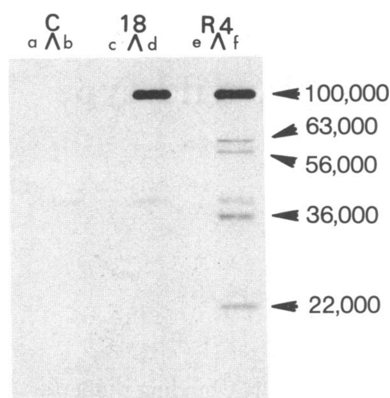

FIG. 1. [^{35}S]Methionine-labeled immunoreactive proteins separated by discontinuous buffer NaDodSO$_4$/polyacrylamide gel electrophoresis (12.5% acrylamide running gel). C, Mock-infected cells: a, preimmune serum; b, immune serum. 18, NG-18-infected cells: c, preimmune serum, d, immune serum. R4, R4-infected cells: e, preimmune serum; f, immune serum. The molecular weights on the right were calculated for the virus-specific polypeptides from standard curves.

rinsed with cold buffer containing 20 mM Tris·HCl at pH 9, 137 mM NaCl, 1 mM CaCl$_2$, and 0.5 mM MgCl$_2$, and lysed in the culture dishes (86-mm diameter) in 0.8 ml of the same rinsing buffer containing 1% (vol/vol) NP-40 detergent and 10% (vol/vol) glycerol. In mixing experiments, cells were harvested in the rinsing buffer, mixed, centrifuged and extracted with lysing buffer. After 20 min at 4°, the lysate was removed and spun at 10,000–12,000 rpm for 20 min in a Beckman J21 centrifuge. The supernatant was incubated with 10 μl of either preimmune or anti-T serum and 40 μl of settled protein A-Sepharose CL-4B (Pharmacia) as described by Schwyzer (26). Anti-T serum came from B/N rats inoculated with a syngeneic polyoma-induced tumor, and had a titer of greater than 1:200 by immunofluorescence on lytically infected cells (22). After 1 hr at 4° with constant vortex mixing, the Sepharose beads were pelleted by low-speed centrifugation and washed repeatedly with a buffer containing 100 mM Tris·HCl at pH 9, 500 mM LiCl, 1% (vol/vol) 2-mercaptoethanol. Immune complexes were eluted from the Sepharose beads with 30–50 μl of buffer containing 5% NaDodSO$_4$, 5% 2-mercaptoethanol, 0.03% bromophenol blue, 20% (vol/vol) glycerol and 100 mM Tris·HCl at pH 8. The following molecular weight standards were added to calibrate the gels: β-galactosidase (115,000), phosphorylase *a* (93,000), bovine serum albumin (69,000), gamma globulin heavy chain (53,000), ovalbumin (43,000), chymotrypsinogen (25,700), myoglobin (17,200), lysozyme (14,400). After heating at 85° for 5 min, the samples were run on 15.5-cm slab discontinuous buffer NaDodSO$_4$ polyacrylamide gels as described by Laemmli (27). The gels were stained with Coomassie Brilliant Blue, destained, prepared for fluorography as described by Bonner and Laskey (28), and exposed on Kodak XR-5 film.

Exclusion Gel Chromatography. A Sepharose 4B (Pharmacia) column, 1.5 × 85 cm, equilibrated with 6 M guanidine·hydrochloride/0.1 M 2-mercaptoethanol, pH 6.8, was used as described by Fish et al. (29) to estimate the molecular weights of immunoreactive species labeled with ^{35}S or ^{3}H. Blue dextran, phenol red, and standard proteins (5 mg each of phosphorylase *a*, human gamma globulin, cytochrome *c*, as indicated) and guanidine·hydrochloride were added to the labeled samples. The sample mixtures were lyophilized and dissociated in a solution of 6 M guanidine·hydrochloride, 0.1 M 2-mercaptoethanol, 0.1 M Tris·HCl, pH 9, for 18–24 hr. About 10^5 cpm was applied to the column, and 20-drop fractions were collected. Standards were measured spectrophotometrically or turbidimetrically as described (29). The radioactivity of a 100- to 200-μl aliquot of each sample was determined in 5 ml of distilled water and 12.5 ml of Aquasol (New England Nuclear) in a Beckman LS300 scintillation counter.

Partial Proteolytic Digestion. Partial enzymatic digests of the 100,000 bands have been analyzed in a manner similar to that described by Cleveland *et al.* (30). Immunoreactive proteins were separated on cylindrical gels (1.5 × 100 mm) containing 5% acrylamide in the running gel. These gels were placed horizontally on 15-cm slab gels that had a 15% acrylamide running gel and a 1.5-cm spacer gel of 3% acrylamide. Two milliliters of a solution of 0.125 M Tris·HCl, pH 6.8/0.01 M EDTA/20% (vol/vol) glycerol containing bovine serum albumin at 25 μg/ml and either chymotrypsin (Worthington) or *Staphylococcus aureus* V8 protease (Miles) at 10 μg/ml were layered over the horizontal cylindrical gel. Electrophoresis was carried out at 50 V for 3 hr and then at 100 V until the bromophenol blue was about 2 cm from the bottom. The gels were then stained and fluorographed.

RESULTS

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis Analysis of [^{35}S]Labeled Immunoprecipitates. Anti-polyoma T serum was incubated with NP-40 extracts of infected cells labeled with [^{35}S]methionine. The immunoreactive proteins were collected and run on discontinuous buffer NaDodSO$_4$ gels. To help determine which virus-specific products may be related to the *hr-t* function, we have used wild-type and mutant strains differing by a substantial deletion. NG-18 contains a deletion of approximately 150 base pairs in endonuclease *Hpa* II fragment 4 located in the 5′ part of the early region (22). The wild-type strain R4 has been isolated from NG-18 by marker rescue using wild-type *Hpa* II fragment 4 (24). The genomes of these two viruses should therefore be identical except for sequences contained within or near the deletion.

Fig. 1 shows results from mock-infected cells, cells infected by NG-18, and cells infected by R4. The patterns obtained with immune serum are shown on the right side of each pair. The wild-type extract shows a major band of apparent molecular weight 100,000 and at least four bands of lower molecular weight (63,000, 56,000, 36,000, 22,000) not observed in extracts from control cells or with pre-immune serum. Additional faint virus-specific bands can be seen on longer exposures. The *hr-t* mutant shows one virus-specific band at 100,000. All extracts treated with immune serum show a band at about 40,000 that is not virus-specific.

The 100,000 band has been identified by Ito *et al.* as the product of the *ts-a* gene because it is temperature sensitive in pulse–chase experiments using *ts-a* mutants (18). We have carried out similar pulse–chase experiments, using a different *ts-a* class mutant and a different serum, and have confirmed this result (data not shown).

No alteration was seen in the migration of the 100,000 protein coded for by the *hr-t* deletion mutant. To look more closely for a deletion in the protein, immunoprecipitates were electrophoresed on a 5% acrylamide gel through a distance of 12 cm (Fig. 2). The major band resolves into a doublet for both mutant and wild-type products, as we have noted previously (19). The apparent molecular weights on 5% gels are also ~100,000. The same value has been obtained on 7.5% and 10% gels. No indication of a deletion in the *ar-t* mutant 100,000 product is observed, but it should be noted that a deletion of less than 1000 molecular weight would not have been detected.

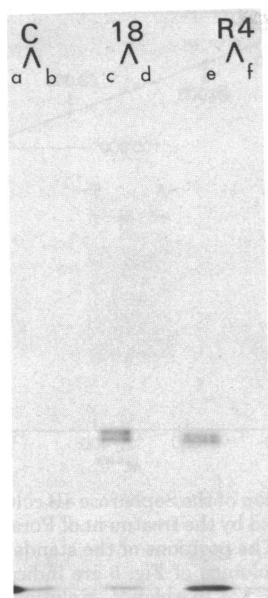

FIG. 2. [^{35}S]Methionine-labeled immunoreactive proteins separated by discontinuous buffer NaDodSO$_4$/polyacrylamide gel (5% acrylamide running gel). C, Mock-infected cells: a, immune serum; b, preimmune serum. 18, NG-18-infected cells: c, immune serum; d, preimmune serum. R4, R4-infected cells: e, immune serum; f, preimmune serum. Only the 100,000 and 63,000 species of Fig. 1 are seen because the other polypeptides have migrated to the bottom of the gel.

Relatedness of Mutant and Wild-Type 100,000 Products by Partial Peptide Analysis. Using a procedure described by Cleveland *et al.* (30), we have compared the patterns of partial proteolytic digestion of mutant and wild-type major T antigen products. Fig. 3 shows a comparison of cleavage products with two different enzymes. Because the extent of digestion cannot be precisely controlled in these experiments, the presence or absence of bands is more significant than their relative intensities. On this basis, the patterns are highly similar, if not identical, with the two viruses.

Tests for Modifications of Mutant and Wild-Type 100,000 Proteins. Though apparently the same size and similar in their proteolytic cleavage patterns, the mutant and wild-type 100,000 species may be differentially modified. The *tsA* protein of SV40 contains phosphoserine (31). To examine phosphorylation of the polyoma T antigens, immunoprecipitates prepared from ^{32}P-labeled infected cells were run on 12.5% gels. The major 100,000 band of both mutant and wild-type virus is phosphorylated (Fig. 4). (The greater intensity of the NG-18 band is due to the loading of twice as much material as for wild type.) A longer exposure of the same gel shows 56,000 and 36,000 bands to be phosphorylated in wild-type extracts. Both mutant and wild-type ^{32}P-labeled 100,000 proteins show the same split on 5% gels (not shown) as was seen with ^{35}S-labeled material (Fig. 2).

A partial characterization of the phosphate linkages in the wild-type 100,000 protein was made after elution from NaDodSO$_4$ gels. The phosphate was found to be alkali labile (1 M NaOH, 100°, 15 min), but stable to treatment with acid (0.1 M HCl, 60°, 10 min) or hydroxylamine (1 M NH$_2$OH, 37°, 20 min). These results are consistent with the presence of phosphoserine or phosphothreonine. It should be noted, however, that, after elution from the gel, only about 50% of the phosphate was acid-precipitable. This might result from hydrolysis of phosphoserine or phosphothreonine during extraction or might reflect some other type of labile phosphorylation.

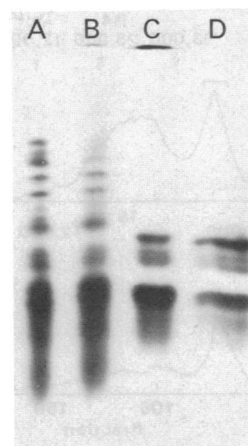

FIG. 3. Partial proteolytic digests of 100,000 bands from NG-18- and R4-infected cells. Proteins were separated on a cylindrical NaDodSO$_4$/polyacrylamide gel and digested during slab gel electrophoresis (15% acrylamide running gel). A, Chymotryptic digest of the NG-18 100,000 species. B, Chymotryptic digest of the R4 100,000 species. C, *Staphylococcus aureus* V8 protease digest of the NG-18 100,000 species. D, *Staphylococcus aureus* V8 protease digest of the R4 100,000 species. The autoradiograms of the slab gels of the chymotryptic and V8 proteolytic digests were cut along the lanes of the 100,000 bands and placed together for photographing.

Attempts to label T antigens in infected cells with a mixture of [^{14}C]glucosamine (1.7 μCi/ml; 0.2 Ci/mmol), and [^{3}H]fucose (8.3 μCi/ml; 10 Ci/mmol) from 13 to 23 hr after infection gave negative results.

Guanidine Column Chromatography. In view of the difficulty in reconciling the apparent molecular weight of 100,000 for the major T antigen species with the absence of an effect on its size by the deletion in NG-18, an independent estimate of molecular weight was made by molecular sieve chromatography in the presence of 6 M guanidine·hydrochloride. Samples of NG-18 and R4 immunoprecipitates shown in Fig. 1 were dissociated in 6 M guanidine·hydrochloride/0.1 M 2-mercaptoethanol and applied to an 85-cm column of Sepharose 4B. The elution profiles are shown in Fig. 5.

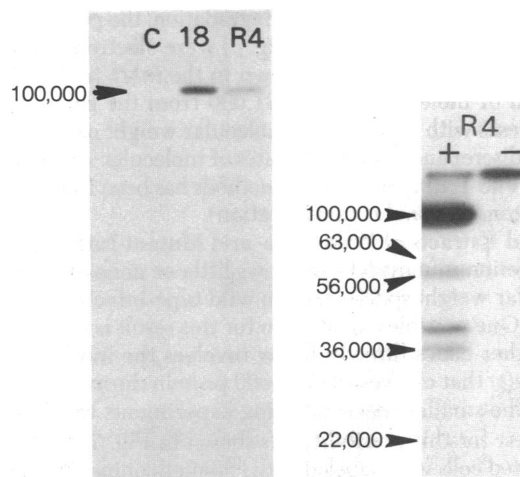

FIG. 4. ^{32}P-Labeled immunoreactive proteins separated by discontinuous buffer NaDodSO$_4$/polyacrylamide gel electrophoresis (12.5% running gel). (*Left*) C, Mock-infected cells; 18, NG-18-infected cells; R4, R4-infected cells; all with immune serum. Two-hour exposure. (*Right*) R4-infected cells reacted with immune serum (+) or preimmune serum (−). Two-day exposure. Arrows indicate positions of virus-specific bands in ^{35}S-labeled immunoprecipitates (see Fig. 1).

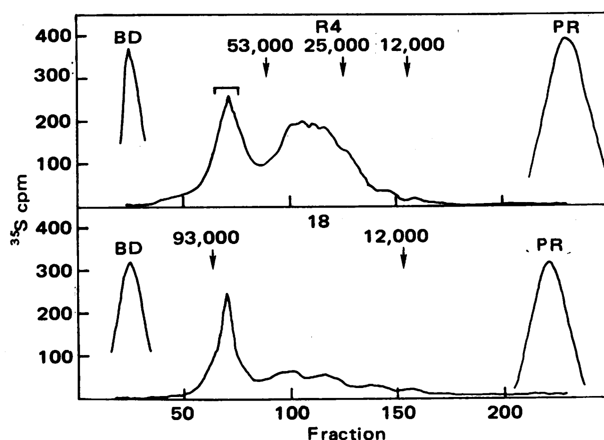

FIG. 5. Exclusion gel chromatography. ^{35}S-Labeled immunoreactive proteins were chromatographed on Sepharose 4B in 6 M guanidine·hydrochloride along with unlabeled standard proteins. (*Upper*) Elution profile of ^{35}S-labeled immunoreactive proteins from R4-infected cells. The elution positions of human gamma globulin heavy chain (53,000), light chain (25,000), and cytochrome *c* (12,000) are indicated by the arrows. The bracket indicates the fractions pooled for electrophoresis. BD is blue dextran; PR is phenol red. (*Lower*) Elution profile of ^{35}S-labeled immunoreactive proteins from NG-18-infected cells. The elution positions of phosphorylase *a* (93,000) and cytochrome *c* (12,000) are indicated by the arrows.

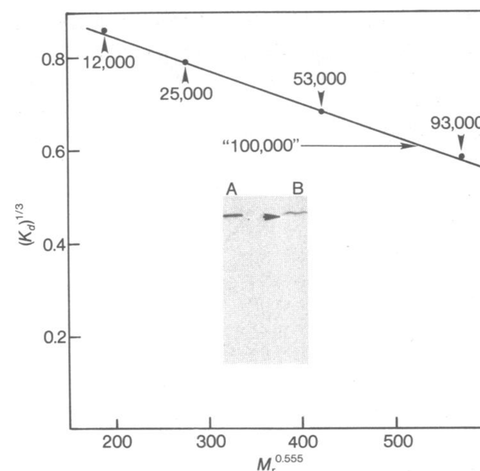

FIG. 6. Calibration of the Sepharose 4B column. The standards of Fig. 5 were analyzed by the treatment of Porath (32) as described by Fish *et al.* (29). The positions of the standard proteins and the major peak of the columns of Fig. 5 are indicated by the arrows. $K_d = (V_e - V_0)/(V_i - V_0)$, in which V_e = elution volume of the peak, V_i = column volume, and V_0 = void volume. M_r is the molecular weight. (*Inset*) Electrophoresis of the protein peak from the guanidine/Sepharose column on discontinuous buffer $NaDodSO_4$ gels (12.5% running gel). Lane A, "100,000" T antigen marker run directly on $NaDodSO_4$ gels. Lane B, electrophoresis of material eluting from the guanidine/Sepharose column in the position indicated by the bracket in Fig. 5. The material from the column was dialyzed against 0.1% $NaDodSO_4$ and 1 mM 2-mercaptoethanol, lyophilized, and resuspended in a sample buffer for $NaDodSO_4$/polyacrylamide gel electrophoresis. The arrow in lane B indicates the position of the phosphorylase *a* marker (93,000).

Both R4 and NG-18 show a major peak eluting near fraction 70 after the phosphorylase *a* marker (93,000). This peak corresponds to the major band of "100,000" observed on $NaDodSO_4$ gels. For the wild type, another broad region of radioactivity is seen eluting after the major peak, representing a combination of the lower molecular weight bands observed on the gels. As expected, the NG-18 pattern contains much less radioactivity in this region (see Fig. 1). Cochromatography of extracts of R4 labeled with ^{3}H and NG-18 labeled with ^{35}S showed a coincident elution profile for the major peak (data not shown).

After calibration of the columns, the molecular weight is estimated to be 81,000 for both the wild-type and the NG-18 major species (see Fig. 6), i.e., about 20% smaller than estimated on $NaDodSO_4$ gels. To rule out degradation, the peak fractions (indicated by the brackets in Fig. 5) were electrophoresed on a 12.5% $NaDodSO_4$ gel. As shown in the *inset* in Fig. 6, this material of molecular weight 81,000 from the column electrophoreses with an apparent molecular weight of 100,000. A similar discrepancy in the estimates of molecular weight of the major SV40 T antigen by these methods has been found (D. M. Livingston, personal communication).

Mixed Extracts of Wild-Type- and Mutant-Infected Cells. The deletion mutant NG-18 shows little or none of the lower molecular weight species seen in wild type-infected cells (see Fig. 1). One possible explanation for this result is that the *hr-t* gene either codes directly for or involves the induction of a protease(s) that cleaves the 100,000 protein during extraction to give the smaller species. Mixing experiments were carried out to test for this possibility. As shown in Fig. 7, when NG-18-infected cells were labeled with [^{35}S]methionine, then mixed prior to extraction with unlabeled wild type-infected cells, the pattern was the same as with the unmixed NG-18 control. The converse experiment, in which wild-type-infected cells were labeled and extracted in the presence of unlabeled mutant-infected cells, shows the same pattern as found in wild-type-infected cells alone. Therefore, the minor bands do not arise or disappear during extraction. However, these results do not preclude that the smaller species may arise by proteolytic processing *in vivo*.

DISCUSSION AND SUMMARY

The reaction of anti-polyoma tumor (T) antiserum with extracts of cells productively infected by polyoma virus yields a major T antigen species with a molecular weight estimated at 100,000

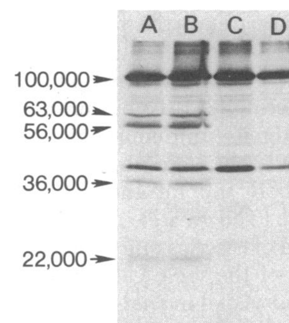

FIG. 7. [^{35}S]Methionine-labeled immunoreactive proteins from cells mixed prior to extraction. Separation is by $NaDodSO_4$/polyacrylamide gel electrophoresis (12.5% acrylamide running gel). A, [^{35}S]Methionine-labeled R4-infected cells alone. B, [^{35}S]Methionine-labeled R4-infected cells mixed with an equal number of unlabeled NG-18-infected cells prior to extraction. C, [^{35}S]Methionine-labeled NG-18-infected cells mixed with an equal number of unlabeled R4-infected cells prior to extraction. D, [^{35}S]Methionine labeled NG-18-infected cells alone. Procedures were as described in *Materials and Methods* with the following modifications. Cells were washed with rinsing buffer at pH 6.85, harvested by gentle scraping with a rubber policeman, mixed, lysed in 100 µl per dish of extraction buffer made 2% in NP-40, pH 6.85, diluted to 0.8 ml per dish with extraction buffer at pH 9, and incubated for 20 min at 0° prior to immunoprecipitation.

by gel electrophoresis and 81,000 by molecular sieve chromatography. $NaDodSO_4$ gel electrophoresis is subject to occurrence of artifacts from protein charge, protein–protein interaction, abnormal binding of $NaDodSO_4$, and protein conformation (33, 34). The guanidine method has a firm theoretical background that depends on the ability of 6 M guanidine hydrochloride to convert any protein to a random chain polymer. This conversion occurs for most proteins (35). We consider the value of 81,000 to be a more reliable estimate of the true molecular weight.

The *hr-t* deletion mutant NG-18 produces a polypeptide that behaves identically to the major wild-type species in both the electrophoretic and chromatographic procedures. Further evidence for the similarity of the wild-type and mutant "100,000" proteins comes from the essentially identical patterns of partial proteolytic cleavage and from the phosphorylation of both proteins. The presence of normal major T antigen species in NG-18 infections is consistent with the previous results of immunofluorescence (22) and of complementation with *ts-a* mutants (24).

The value of 81,000 for the molecular weight of this protein is also more reasonable in terms of the mapping results. A protein of this size could be encoded entirely "downstream," i.e., in the 3′ direction of transcription, from the deletion in NG-18. The results would also be consistent with any of various messenger RNA "splicing" mechanisms, in which part of the sequences in the protein are encoded "upstream," as long as the region of the deletion itself is removed from the messenger RNA. Splicing of early RNAs of SV40, involving the loss of sequences corresponding roughly to those of the *hr-t* gene in polyoma virus, has recently been observed (A. Berk and P. Sharp, personal communication).

A striking and unexpected manifestation of the deletion in NG-18 is the absence of, or sharp reduction in, the four smaller polypeptides that also react with anti-T sera. It is not possible from these results to surmise which, if any, of these polypeptides may be involved in the *hr-t* function. If the deletion in NG-18 lies within a coding region and does not alter the reading frame, then a new polypeptide would be expected, about 5000 molecular weight smaller than the corresponding wild-type protein. The fluorograms do not reveal consistently any such species, although new and faint bands in the lower molecular weight region of the gels sometimes appear in longer exposures. Further experiments, employing additional deletion as well as nondeletion *hr-t* mutants, may provide a definitive answer. Determination of possible peptide relatedness among the large and smaller polypeptides and of their locations within the cell will obviously be important in understanding their significance.

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