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Purification of T Antigen from Nuclei of Simian Virus 40-induced Hamster Tumors

HARRISON M. LAZARUS, MICHAEL B. SPORN, JOSEPH M. SMITH, AND WILLIAM R. HENDERSON

National Cancer Institute, Bethesda, Maryland 20014

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We report here the extensive purification of the simian virus 40 (SV40) T antigen (virus-induced complement-fixing antigen, tumor neoantigen) from nuclei of transplanted, virus-free hamster tumors originally induced by SV40 virus. The cellular function of this protein or group of proteins is presently unknown. Purification of this material may lead to further definition of its role. Gilden et al. (Proc. Natl. Acad. Sci. U.S. **53**:684, 1965) have previously reported the preliminary purification of T antigen and its sedimentation pattern in sucrose density gradients.

SV40 fibrosarcomas were serially passaged in golden hamsters approximately every month in our laboratory by subcutaneous trocar or needle implantation. The principal tumor used in these studies was initiated by inoculation of suckling hamsters with SV40 virus, strain 777, and was in its 13th to 20th passage. The tumor has been shown to be free from virion antigen. When tumors were removed from the recipient animal between 3 and 4 weeks after transplantation, they usually weighed between 10 and 20 g and were generally free from necrosis. Tumors with even minimal necrosis were discarded.

The entire purification was performed at 0 to 4 C. Solutions used were as follows: Solution 1: sucrose, 0.32 M; $MgCl_2$, 0.004 M; potassium phosphate, *p*H 6.8, 0.001 M.

Solution 2: sucrose, 0.32 M; Triton N-101 (Rohm & Haas Co., Philadelphia, Pa.), 0.3%; $MgCl_2$, 0.001 M; adjusted with 0.001 M potassium phosphate to *p*H 6.2 to 6.4. Solution 3: NaCl, 0.1 M; potassium phosphate, *p*H 6.2, 0.005 M; $MgCl_2$, 0.002 M; dithiothreitol (DTT), 0.001 M. Solution 4: NaCl, 0.08 M; Na ethylenediaminetetraacetate, 0.02 M; tris(hydroxymethyl)aminomethane (Tris) chloride, *p*H 8.0, 0.02 M; DTT, 0.001 M. Solution 5: Tris chloride, *p*H 8.0, 0.1 M; DTT, 0.0003 M. Solution 6: $CdCl_2$, 0.004 M; Tris chloride, *p*H 8.0, 0.1 M. Solution 7: Tris chloride, *p*H 8.0, 0.05 M; DTT, 0.001 M. Nuclei were prepared from tumors as follows. A 15-g amount of tumor was finely minced and then homogenized with a loose pestle in a Dounce homogenizer in 40 ml of Solution 1. The homogenate was filtered through 103-mesh (approximately 200-μ openings) nylon monofilament screening cloth, gently rehomogenized with a tighter pestle, filtered through 230-mesh nylon (75-μ openings), and spun for 10 min at 1,300 $\times$ *g* (maximum). The supernatant fluid was discarded, and the crude nuclear pellet was gently homogenized in a Dounce homogenizer with 40 ml of Solution 2. The suspension was spun for 5 min at 900 $\times$ *g*; the pellet was saved and treated once more with Solution 2. The final nuclear pellet was washed once with Solution 1 and frozen at -80 C. Nuclei from 120 g of tumor are easily prepared in 4 hr.

T antigen was purified from nuclei as follows. The nuclei from 600 g of tumor were homogenized in 220 ml of Solution 3 and spun for 15 min at 13,000 $\times$ *g*. The supernatant fluids were discarded, and the pellets were extracted twice at *p*H 8.0 with a total of 250 ml of Solution 4. The combined *p*H 8.0 extracts were saved, and the nuclear residue was discarded. Dry ammonium sulfate was added to the *p*H 8.0 extract to bring it to 20% saturation, and the precipitate was discarded after removal by centrifugation. The supernatant fluid was then brought to 45% saturation; the precipitate was saved after centrifugation and dissolved in 160 ml of Solution 5. An equal volume of Solution 6 was then added slowly and the precipitate collected. The precipitate was dissolved in 60 to 120 ml of Solution 4 and was dialyzed for 3 hr against several changes of Solution 7. A small amount of material which had precipitated during dialysis was discarded after centrifugation. A measurement of the total protein of the preparation must be made at this point to determine the proper load for the subsequent ion-exchange column. A portion of the preparation, containing between 15 and 30 mg of protein, at a concentration of 0.3 to 1.0 mg/ml, was then applied to a 1 $\times$ 18 cm column of Whatman microgranular diethylaminoethyl (DEAE) cellulose, buffered with Solution 7. The column was washed with 30 ml of Solution 7 and then eluted with a linear salt gradient, with the mixing cham-

ber containing 50 ml of Solution 7 and the reservoir 50 ml of Solution 7 plus 0.4 M NaCl. Fractions of 8 ml were collected from the point when protein was first applied to the column. Final fractions were stored at −80 C or below.

Titer of T antigen was measured by a microcomplement-fixation test (J. L. Sever, J. Immunol. **88**:320, 1962) with 2.0 units of complement and fixation overnight at 2 to 4 C. Microtiter plates were kept in trays of crushed ice when the antigens, antibodies, and complement were being dispensed. Antigens were serially diluted in Veronal-buffered saline containing 0.3% gelatin, to give a final gelatin concentration of 0.1% during fixation. T antigen was measured on fractions from DEAE columns without further concentration. Several immune sera were used: (i) pooled ascites fluid from hamsters bearing transplanted SV40 tumors, with a titer of 1:320 versus 4 to 8 units of homologous antigen (Flow Laboratories, Rockville, Md.); (ii) pooled blood serum from hamsters bearing transplanted SV40 tumors, with a titer of 1:256 versus 4 to 8 units of homologous antigen and a titer of less than 1:16 versus the Fortner isoantigen; (iii) and (iv) sera from hamsters bearing either adenovirus 12- or polyoma virus-induced transplantable tumors, with titers of 1:80 versus homologous antigens (Flow Laboratories). In all complement-fixation tests, assays with both 4 and 8 units of antibody were made. Protein was measured by the method of Lowry et al. (J. Biol. Chem. **193**:265, 1951). In order to determine whether a nuclear exoribonuclease, found in SV40 tumors as well as Ehrlich ascites tumors, might be part of the T antigen complex, assays were also performed for this enzyme on fractions from the DEAE column. The enzyme degrades single-stranded ribonucleic acids to 5'-mononucleotides at *p*H 7.0 to 8.0 (H. M. Lazarus and M. B. Sporn, Proc. Natl. Acad. Sci. U.S. **57**:1386, 1967).

TABLE 1. *Purification of SV40 T antigen*

Fraction	Total T antigen (units)	Total protein (mg)	Specific activity (antigen units/mg)
Nuclei	7,000	1,940	3.6
Extract, *p*H 8.0	8,000	176	45
Ammonium sulfate, 20 to 45% precipitate	10,000	149	67
$CdCl_2$ precipitate	7,600	74	103
DEAE, peak tube[a]	1,000	1.4	710

[a] Of protein from $CdCl_2$ precipitate, 15 mg containing 1,280 units of T antigen was applied to the DEAE column.

The results of a typical purification of T antigen are shown in Table 1 and Fig. 1; the procedure has been repeated three times on three separate preparations of tumor nuclei. Specific activities of peak tubes from DEAE columns have ranged from 350 to 700 units of antigen per mg of protein; thus, the T antigen has been purified 100- to 200-fold over its concentration in the nucleus. If eluting gradients are steep, and relatively large amounts of T antigen applied to columns, recovery of activity from DEAE columns is essentially quantitative. Purified material is completely stable at −80 C for 1 week, and fractions with a titer of 1:64 still had a titer of 1:16 after 3 months

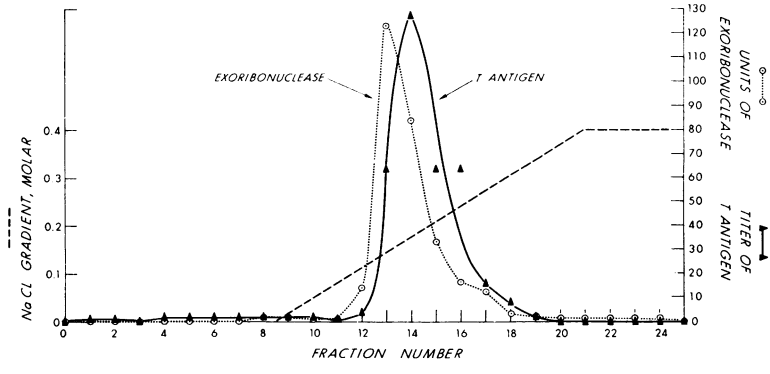

FIG. 1. *Chromatography of T antigen and nuclear exoribonuclease on DEAE cellulose. Of protein from $CdCl_2$ precipitate, 15 mg, containing 1,280 units of T antigen and 275 units of exoribonuclease, was applied to the column and eluted as described in the text. Exoribonuclease was assayed by incubating fractions with polyadenylic acid for 30 min at 37 C and then measuring acid-soluble product. A unit is defined as the amount of enzyme which produces 1 μmole of 5'-adenosine monophosphate per hr.*

at −80 C. T antigen titers were identical whether measured with antiserum 1 or 2. No cross reaction was seen with either adeno or polyoma tumor antisera.

Figure 1 also shows that the profile of exoribonuclease activity eluted from DEAE columns is separate from the profile of T antigen activity; this finding was constant in all three preparations of T antigen and shows that the bulk of T antigen activity is distinct from the nuclear exoribonuclease.

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