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Interaction of Partially Purified Simian Virus 40 T Antigen with Circular Viral DNA Molecules

(nitrocellulose filter assay/column chromatography)

D. JESSEL, J. HUDSON, T. LANDAU, D. TENEN, AND D. M. LIVINGSTON

The Division of Medical Oncology, Sidney Farber Cancer Center, and the Departments of Medicine, Peter Bent Brigham Hospital, and Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT **Mixing chromatographic fractions containing simian virus 40 (SV40) T antigen with SV40 [^{3}H]-DNA I (double-stranded, circular, supercoiled) results in the conversion of the nucleic acid to a form that will bind to a nitrocellulose filter. Unlabeled SV40 DNA I successfully competes with this reaction. Under the conditions employed, the antigen-containing fractions bind a variety of circular, viral DNA molecules. Chromatography of the antigen in three systems reveals that the T immunoreactivity migrates with DNA binding activity. In a kinetic heat inactivation experiment, the antigenic reactivity disappears simultaneously with the DNA binding activity. The data indicate the presence of a discernible DNA binding activity in fractions containing T antigen and suggest that the T antigen is the DNA binding protein being measured.**

The simian virus 40 (SV40) T antigen was first detected by immunologic means in the nuclei of cells infected with this virus (1, 2). In infected, permissive cells, as determined by complement fixation analysis or immunofluorescence, it appears prior to the onset of viral DNA synthesis and then gradually disappears, while in virus-transformed cells it is continuously present (1–3). Although its function in the cell and its possible mode of action in relation to viral replication, viral gene expression, and/or transformation are yet unclear, some progress has been made in the chemical characterization of this molecule (4–8).

Recently, Carroll (5) and his colleagues have demonstrated that T antigen binds to columns of calf thymus DNA-cellulose at pH 6.2 (5). We have shown that T will bind to phosphocellulose and DEAE-cellulose but not to carboxymethylcellulose (6, 8). This differential chromatographic behavior on ionic absorbents suggested that T may have a relatively specific affinity for phosphate ligands and is not a basic protein. Both sets of results are compatible with the hypothesis that T may function, at least in part, by interacting with one or more species of nucleic acid.

In this report, we will describe a method for identifying a DNA binding activity in T-antigen-containing fractions which depends upon an interaction of this protein(s) with radioactive DNA. The assay depends upon the observation that unliganded, double-stranded DNA will not be efficiently trapped by a nitrocellulose filter except when bound to a protein that, itself, has affinity for the filter. This method was first employed in the assay of certain bacterial DNA binding proteins such as DNA-dependent RNA polymerase and the *lac* and λ repressors (9–11).

Abbreviations: SV40, simian virus 40, RF, replicative form; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

The present DNA binding assay has the advantage of being more rapid than the standard complement fixation test. Moreover, with the appropriate application, it may help shed some light on the biological function of T *in vivo*.

MATERIALS AND METHODS

Cells. T antigen is routinely extracted and purified from SV80, a continuous line of SV40-transformed human cells (12). Most recently, these cells were kindly and generously grown for us by the cell production facility of the Massachusetts Institute of Technology. They were grown in Dulbecco's modification of Eagle's minimal essential medium (DME) containing 10% fetal calf serum (Grand Island Biologicals, Grand Island, N.Y.) in glass, modular production roller bottles.

Virus. The small-plaque strain of SV40 (SVS) was employed throughout. Virus was propagated in primary African green monkey kidney cells. To prepare stocks, infection was performed at a multiplicity of 0.005–0.01 in DME containing 10% calf serum, gamma-globulin-free, (Gibco).

Preparation of Radioactive SV40 DNA. Double-stranded, covalently closed circular, supercoiled SV40 DNA I was isolated and purified from infected Vero cells 60 hr after infection with SVS (multiplicity of infection = 10) by the method of Hirt (13), followed by isopycnic sedimentation in CsCl/ethidium bromide gradients (14). SV40 DNA I was further purified by sedimentation in 5–20% neutral sucrose gradients, prepared in 1 mM Tris·HCl, pH 7.4, 1 mM EDTA. When analyzed at various times thereafter, each fraction appeared as a unimodal, 21S species.

Other Viral DNA Molecules. Supercoiled λ [^{3}H]DNA was isolated from induced *Escherichia coli* AS2D [*thy*$^-$, *polA*$^-$ (λ *c*I857, *red* 113, γ5, S *am*^{7})] grown in Mg–casamino acid minimal media, by the method of Reuben *et al.* (15). The dye/density gradient purified DNA was repurified by neutral sucrose gradient sedimentation. The lysogen was the generous gift of Dr. Susan Gottesman. We are indebted to Drs. Gottesman and Malcolm Gefter for helpful discussions relevant to the λ DNA isolation and purification. Polyoma [^{3}H]DNA I was the generous gift of Drs. Lorne Sampayrac and Thomas Benjamin. Phage φX174 double-stranded, closed circular replicative form (RFI) [^{3}H]DNA was kindly given to us by Kirsten Koths.

Isolation and Purification of T Protein. The method is essentially that reported earlier for its isolation from SV80 nuclei (8) with the exception that a 60% saturated ammonium sulfate precipitation step was substituted for a 20–50% fractionation. The order of preparative steps was regularly: nuclear isolation, sonic nuclear rupture, isolation of a membrane-free nuclear extract, 60% $(NH_4)_2SO_4$ precipitation, dialysis, DEAE-cellulose chromatography, pooling of T immunoreactive fractions, concentration by precipitation with 60% ammonium sulfate and redissolving the pellet in 0.25 volumes of buffer, dialysis and agarose A-1.5m (Bio-Rad) chromatography.

Radioactive DNA Binding Assay. The assay is fundamentally that of Riggs *et al.* (10) and is carried out with either Schleicher and Schuell B-6, 0.45 μm or Millipore HAWP 0.45 μm nitrocellulose filters. The basic reaction mixture (0.25 ml) includes: 0.01 M Tris·HCl, pH 7.4, 0.1 mM manganese acetate, 0.1 mM dithiothreitol, 3% dimethylsulfoxide, 10 μg/ml of bovine serum albumin (BSA), SV40 [^{3}H]DNA I, and various amounts of T protein. After a 10-min incubation at room temperature, two separate aliquots (0.10 ml) are individually filtered at a rate of 1.2 ml/min. Each filter is then rinsed once with 0.10 ml of 0.01 M Tris·HCl, pH 7.4, 0.1 mM EDTA, 0.2 mM manganese acetate, 3% dimethylsulfoxide (FB buffer). Filters are routinely pre-treated for 30 min with 0.50 M NaOH, rinsed thoroughly with H_2O, and then soaked for at least 30 min in FB buffer to reduce background radioactivity (16).

Complement Fixation Test for T Antigen was carried out by the micromethod of Wasserman and Levine (17). Each reaction mixture (0.90 ml) includes 1.2 units of guinea pig complement (Gibco), 8×10^6 sheep erythrocytes, sensitized with 2 units of antiserum to sheep erythrocytes (Gibco). All dilutions and components of the reaction mixture were made up to 5 mM Veronal, pH 8.2; 0.14 M NaCl; 0.1% bovine serum albumin; 1 mM $MgCl_2$; 0.33 mM $CaCl_2$. Hamster anti-T antibody was kindly provided by Dr. Roger Wilsnack. We are indebted to Drs. David Howell and Jack Gruber of the Virus Cancer Program, National Cancer Institute for their assistance in obtaining this reagent. In the studies reported here, it was regularly used at a 1:70 dilution. Absorbance readings on final, clarified reaction mixtures were performed in 10 × 75 mm glass tubes at 413 nm in a Coleman Junior spectrophotometer.

Protein Concentration Measurements were performed by the method of Lowry *et al.* (18).

Buffers. A—0.05 M Tris·HCl, pH 7.8, 1 mM dithiothreitol, 20% (v/v) glycerol; B—same as A, but additionally, 0.10 M NaCl; C—same as A, but additionally, 0.40 M NaCl; D—same as A, but additionally, 0.60 M NaCl; E—same as A, but additionally, 0.90 M NaCl; F—0.05 M 2-(*N*-morpholino)-ethanesulfonic acid (Mes), pH 6.2, 1 mM dithiothreitol, 20% glycerol; G—0.03 M Imidazole, pH 6.8, 1 mM dithiothreitol, 20% glycerol; H—0.05 M Tris, pH 7.8, 1 mM dithiothreitol, 20% ethylene glycol.

RESULTS

Properties of the Filter-Binding Assay. Agarose-purified SV40 T protein was mixed in increasing concentrations with a fixed amount (130 pM) of SV40 [^{3}H]DNA I and each reaction mixture was then filtered through a nitrocellulose disc (Fig. 1). Under these conditions, increasing amounts of radioactivity are trapped by the filter. A saturation value of 100% of the input counts occurs at ≥ 15 μl (≥0.45 μg) of the T protein fraction. Importantly, ^{125}I-labeled agarose-purified antigen binds to the filter under identical conditions. In fact, the standard binding conditions were determined using maximal ^{125}I-labeled T filter binding (65%) as the endpoint. Previously boiled, nonradioactive antigen was substituted for untreated material, and, under these conditions, a 95% reduction in DNA binding activity was noted (Fig. 1). Native and boiled ^{125}I-labeled material bind to an equivalent degree (65%) to nitrocellulose filters under conditions identical to those used in the assay of DNA binding activity. Thus, the reduction in DNA trapping by the filter after antigen boiling is not due to a reduction in the absorption of the DNA binding protein to the filter.

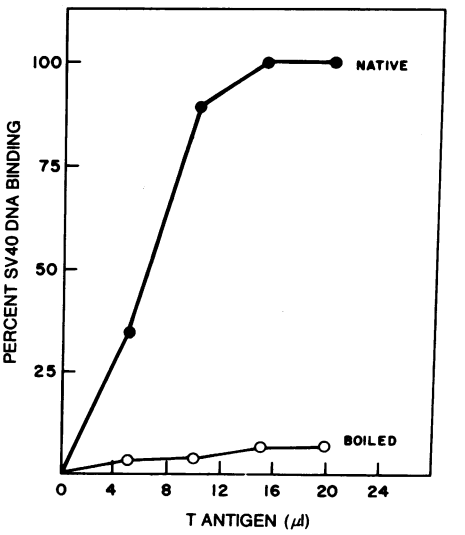

FIG. 1. Interaction between SV40 [^{3}H]DNA I and SV40 T protein. The assay was carried out as described in *Materials and Methods*. Incubation was for 10 min at room temperature. The antigen fraction employed had been purified through the DEAE-cellulose and agarose A-1.5m steps (see *Materials and Methods*), and its protein concentration was 0.03 mg/ml. This was the most highly purified preparation of T antigen available to us at this time. Where indicated, antigen boiled at 100° for 10 min and then chilled on ice was employed. The specific activity of the SV40 [^{3}H]DNA I used was 5×10^3 cpm/μg, and 500 cpm were added per reaction mixture. The background was 45 cpm.

Unlabeled SV40 DNA I competes effectively with radioactive SV40 DNA I in the binding assay (Fig. 2). Importantly, when the input molar ratio of unlabeled to tritiated DNA is 1 (0.15 μg of unlabeled SV40 DNA I), binding of the latter is reduced by 50%. In contrast, adding a large excess of unlabeled DNA to the reaction mixture after completion of the T–SV40 [^{3}H]DNA reaction does not lead to a reduction in SV40 [^{3}H]DNA binding. This result rules out the possibility that, under these conditions, unlabeled DNA is competing with a T–SV40 [^{3}H]DNA complex for binding to the filter.

The relative ability of agarose-purified T antigen to bind SV40 DNA and each of three other circular, viral DNA species was tested (Fig. 3). In each experiment, the concentrations of the two DNA species being evaluated were identical, and the T antigen input was varied. Polyoma DNA I,

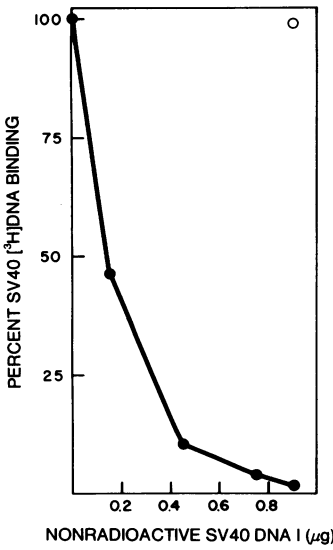

FIG. 2. Effect of nonradioactive SV40 DNA I upon binding of SV40 [³H]DNA to T antigen. The assay and specific activity of the input SV40 DNA I are identical to what was employed in the Fig. 1 experiments. Each reaction mixture contained 800 cpm (0.16 μg) of DNA, and the background was 50 cpm. Where indicated, nonradioactive SV40 component I DNA was added prior to addition of radioactive DNA (●——●), and the reaction was initiated by addition of 0.45 μg of T purified on DEAE-cellulose and agarose. As a control, in one duplicate set of assays, nonradioactive DNA was added to the reaction mixture after completion of the incubation and immediately prior to filtration (○).

λ supercoils, and φX174 RFI were all bound by the antigen fraction. From a general inspection of the curves, it can be seen that the antigen binds λ and SV40 equally well. It appears to have slightly greater affinity for φX and a somewhat lower affinity for polyoma than for SV40 DNA. While these differences are quantitatively small, they are reproducible under

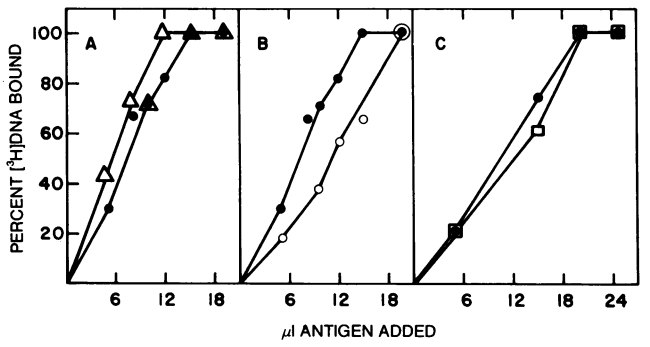

FIG. 3. SV40 T antigen binding to various radioactive DNA species. The assay was performed as described in *Materials and Methods*, using DEAE-cellulose- and agarose-purified antigen fractions (0.033 μg/ml). φX174 RFI [³H]DNA, SV40 [³H]DNA I and polyoma [³H]DNA I were all used in equimolar amounts (15 pM) in parallel assays (A,B). In an analogous experiment, λ [³H]DNA supercoils and SV40 [³H]DNA I were compared, each at a concentration of 32 pM (C). The specific activities were as follows: SV40 DNA I, 4×10^4 cpm/μg; polyoma DNA I, 4×10^4 cpm/μg; φX174 RFI DNA, 6×10^4 cpm/μg; and λ DNA, 5×10^3 cpm/μg. φX174 RFI (△), SV40 DNA I (●) polyoma DNA I (○), λ supercoils (□).

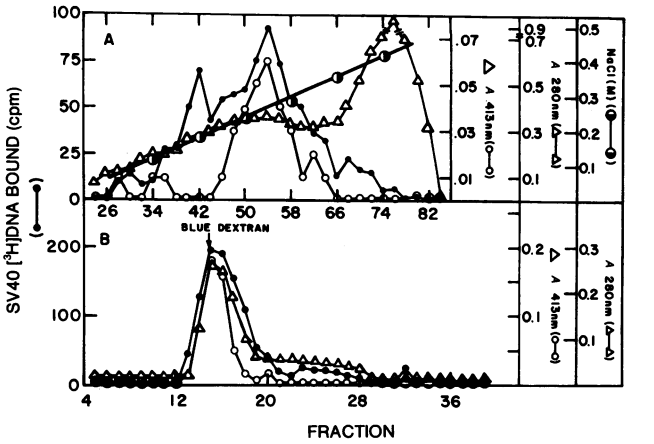

FIG. 4. DEAE and agarose A-1.5m chromatography of SV40 T antigen. (A) Washed SV80 cells (66 g) were disrupted in 120 ml of buffer and the nuclei isolated and further extracted up to the $(NH_4)_2SO_4$ step as described previously (4). At that point, the clarified nuclear sonicate was brought to 60% saturation with solid $(NH_4)_2SO_4$ at 0°. When maximal precipitation had occurred (20 min after completion of salt addition), the precipitate was pelleted and the supernatant discarded. The former was then dissolved in 20 ml of Buffer A and dialyzed against two changes of 100 volumes of Buffer A, each change lasting 90 min. This material was then applied to a (2.8 × 11 cm) column of DEAE-cellulose (DE 52) equilibrated in Buffer A. The column was then washed with 2 column volumes of Buffer A and then eluted with a 250 ml linear gradient between Buffers A and C. The column was then further eluted with 60 ml of Buffer D and additional fractions were collected. Fraction volume was 4 ml. Five-tenths microliter of the indicated fractions was tested in the complement fixation assay, and 0.5 μl in the filter binding assay. SV40 [³H]DNA I (800 cpm; 0.15 μg) was employed in each reaction mixture. The background was 60 cpm. The immunoreactivity unit in this and all subsequent experiments is ΔA 413 nm (17). (B) Fractions (48–58) from the DEAE-cellulose column (Fig. 4A) were pooled; total volume = 44 ml; and solid $(NH_4)_2SO_4$ was added over a 10-min period to a final saturation of 60% at 0°. After an additional 10 min, the resulting precipitate was pelleted by centrifugation, and the supernatant was discarded. The former was dissolved in 5.5 ml of Buffer A and then dialyzed against 100 volumes of this buffer for 3 hr with one change at 90 min (Fraction I). This material (2.0 ml; 2 mg of protein) was applied to a 1.2 × 35 cm column of agarose A-1.5m equilibrated in Buffer G. Fractions (0.78–0.80 ml) were collected, and 1.5 μl were tested in the complement fixation assay and 1.0 μl in the binding assay. SV40 [³H]DNA (600 cpm; 0.12 μg) was added to each reaction mixture. The background was 42 cpm.

these conditions. Moreover, agarose-purified antigen binds SV40 DNA I and full length linear molecules generated by cleavage with either *Eco*RI (19) or *Hpa*II (20) restriction endonucleases equally well (Jessel, D. and Livingston, D., unpublished observations). Employing the method of Riggs *et al.* (10), we have calculated a binding constant for the protein–SV40 DNA I interaction of approximately 7 pM.

Tracking SV40 T Protein by DNA Binding and Immunologic Assays. Shown in Fig. 4 are the results of T protein chromatography on DEAE-cellulose (Fig. 4A). Following elution with a linear salt gradient, a major species of immunoreactive material appeared at approximately 0.20 M NaCl. When the column fractions were simultaneously tested for their ability to bind SV40 [³H]DNA I, two major peaks of binding activity

appeared, the second coinciding with the major immunoreactive species.

The major DEAE-cellulose-purified immunoreactive species was pooled, concentrated, dialyzed, and applied to an agarose A-1.5m column. A single, immunoreactive species appeared in the void volume (Fig. 4B). Similarly, a single major species of DNA binding activity also appeared in the void volume, while some DNA binding activity was also noted in the included volume of the column. Radioiodination of the DEAE-cellulose and agarose-purified material has been achieved by the lactoperoxidase method (21). The labeled product has been chromatographed on Sephadex G-150 and agarose A-1.5m, and a single radioactive species is detected in the void volume of the column. In addition, analytical gel electrophoresis experiments performed in urea (on protein heated at 65° for 15 min but not boiled) reveal the presence of one major protein species.

As a test of the association of T immunoreactivity and DNA binding activity the material pooled from the DEAE-cellulose column was applied to a phosphocellulose (P-11) column equilibrated in Mes buffer, pH 6.2. Under these conditions, little or no immunoreactive material washed through the column. Application of a linear gradient from 0.1 to 0.9 M NaCl at pH 7.8 led to elution of two immunoreactive species, one at 0.40 M NaCl and a second at 0.60 M NaCl (Fig. 5). We have reported such a phenomenon previously (5, 7). Two peaks of DNA binding activity also appeared, and each cotracked with a peak of T antigen immunoreactivity. The lack of constancy in the ratio between ΔA 413 nm and cpm [^{3}H]DNA bound across each T antigen peak is most likely a function of the quantitatively different nature of the two assays. The complement fixation assay here yields a sigmoid titration curve, while the DNA binding assay is essentially linear, under the conditions employed. Moreover, sequential DEAE-cellulose, agarose, and phosphocellulose steps were attempted and found to be unsuccessful due to the lability of both the antigenic reactivity and DNA binding function when the gel filtration step preceded phosphocellulose chromatography.

Quantitative heat inactivation studies were performed with the DEAE-cellulose- and agarose-purified material. As shown in Fig. 6, heating this fraction at 37° for up to 4 hr led to a simultaneous and parallel reduction in T immunoreactivity and DNA binding activity, each measured under limiting conditions.

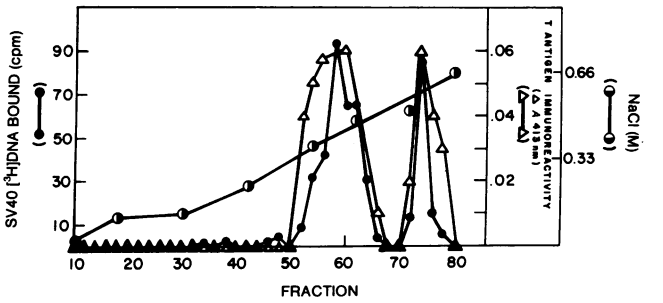

FIG. 5. Phosphocellulose chromatography of SV40 T antigen. Three milliliters (3 mg of protein) of pooled DEAE-cellulose-purified material (fractions 48–58; Fig. 4A) were dialyzed for 2½ hr against two changes of 100 volumes of Buffer G and then applied to a 4 ml column of phosphocellulose (P-11, Whatman, Piscataway, N.J.) equilibrated in Buffer F. The column was then washed with 12 ml of Buffer F and then eluted with a 40 ml linear gradient between Buffers B and E, followed by 15 ml of Buffer E. Fractions of 1.0 ml were collected. Aliquots (0.01 ml) of the indicated fractions were tested in the DNA binding assay, and 3 μl were tested in the immunoassay. Each reaction mixture contained 600 cpm (0.12 μg) of SV40 DNA I. The background was 70 cpm.

DISCUSSION

T antigen cotracks with a DNA binding activity on three types of columns: DEAE-cellulose, agarose A-1.5m, and phosphocellulose. These results suggest that the T antigen itself may be the DNA binding protein being detected. In further support of such a suggestion is the observation (Fig. 6) that the heat inactivation kinetics of the DNA binding activity and T antigen immunoreactivity parallel each other. However, we wish to indicate the possibility that the DNA binding protein present in these fractions might be a non-T protein which happens to cochromatograph with it and has identical heat lability.

The data represented in Fig. 3 clearly indicate that the T antigen containing fraction does not recognize DNA sequences uniquely present in SV40 DNA. There are several possible interpretations of such results. First, it is possible that under these conditions, the DNA binding species does not recognize specific nucleotide sequences at all. Alternatively, the antigen may more closely resemble certain DNA-dependent RNA polymerases than either the *lac* or λ repressor and, under optimal conditions, may bind to specific sequences that are common to DNAs from several species (9). Specific binding, then, may occur to a limited set of critical sequences within the SV40 genome. Such sequences may be common to a wide variety of DNA molecules. Alternatively, the antigen may, under appropriate conditions, bind to a unique sequence analogous to the λ or *lac* operator (22, 23) and, therefore, present only in SV40 DNA. If this is true, we have not yet

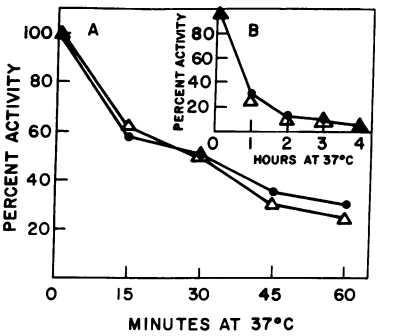

FIG. 6. Heat inactivation of partially purified antigen. A solution of T antigen (1 mg/ml) purified through the DEAE-cellulose and agarose steps was incubated at 37° in a water bath. Prior to heating and at indicated intervals thereafter, aliquots were removed and tested simultaneously in the DNA binding assay and the complement fixation test under standard conditions. Increasing concentrations of each aliquot were assayed by both methods, and portions of the titration curves where the binding or complement fixation responses are directly proportional to the amount of protein added were defined in each instance. The data reported along the ordinates are specific activities derived from the results of each assay. SV40 DNA I (500 cpm; 0.012 μg) was added to each binding assay reaction mixture. The data in the major and inset segments of the figure are from separate experiments. (●) DNA binding activity; (△) T antigen immunoreactivity.

established the correct conditions for demonstrating the specific effect. In this instance, the presently observed binding activity of the antigen would, at least superficially, resemble the non-operator DNA binding demonstrated by the *lac* repressor (24).

If, as seems likely, the T antigen is the DNA binding protein being observed, then after preliminary purification steps, a filter binding assay using [^{3}H]DNA may be the first quantitative test that measures some aspect of the function of this protein. It would have the additional advantage of being more rapid than the currently employed complement fixation test.

Note Added in Proof. Subsequent to the preparation of this paper, we have learned of the development of a similar T antigen–DNA filter binding assay by Spillman *et al.* (25). Moreover, Reed *et al.* have obtained results suggesting that SV40 T antigen can bind to the origin of replication on SV40 DNA (26).

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