Rapid and Sensitive Quantitative Immunoassay for the Large Simian Virus 40 T Antigen

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A quantitative, enzyme-linked immunoabsorbent assay has been developed for the simian virus 40 large T antigen. When hamster anti-simian virus 40 tumor serum was used, this method permitted specific identification of large T antigen and its analog, the D2 hybrid protein, a molecule with the same C-terminal ~600 amino acids as large T antigen. The sensitivity limit of this test was 0.63 ng of protein. The slopes of the regression lines of the enzyme-linked immunosorbent assay titrations performed with highly purified D2 or simian virus 40 large T antigen and with crude extracts of simian virus 40-infected monkey and transformed human cells were identical. Thus, the curve generated with a purified protein, such as D2, can serve as a quantitative standard for the measurement of large T antigen in a wide variety of extracts. Furthermore, solutions containing high salt concentrations and buffers containing up to 0.1% Nonidet P-40 did not interfere with the assay, making it applicable to the measurement of large T antigen in a variety of chromatographic fractions. The enzyme-linked immunoabsorbent assay was three times more sensitive, was significantly faster to perform, and was quantitatively valid over a much broader large-T-antigen concentration range than the complement fixation test. As such, it should be useful in future studies of the structure and function of this protein.

A variety of qualitative and semiquantitative immunological assays have been used to identify the large T antigen and small t antigen of simian virus 40 (SV40) (3, 7, 30, 35). Individual shortcomings, such as lengthy incubation times, difficulties in quantitation and reproducibility, requirement for radioactive iodine, complement and erythrocytes, and interference by components of buffer solutions required for antigen stability or for chromatographic elution, have led to difficulties in the use of these methods. For example, the complement fixation assay has been the most commonly used assay for large T antigen during its purification. In its most sensitive form, this assay requires an incubation period of at least 12 h, leading to substantial delays during the purification process. In addition, large T antigen is unstable when present in dilute solution, and this property is manifested by time-related losses in immunoreactivity (36). Hence, a more rapid but equally sensitive assay might contribute to a higher yield of native large T antigen during its purification.

In principle, a quantitative enzyme-linked immunoabsorbent assay (ELISA) could provide a solution for these problems (9). Historically, such assays have resulted in the accurate detec-

tion of nanogram quantities of a variety of protein antigens after relatively short incubation periods (38). Such an assay for SV40 large T antigen is described here.

MATERIALS AND METHODS

T antigen sources and controls. All cells were grown on plastic surfaces at 37°C in Dulbecco modified Eagle medium supplemented with 10% calf serum in an atmosphere containing 10% CO₂. CV-1P cells (a strain of African green monkey kidney cells) were infected at 95% confluency with SV40 strain 777 (multiplicity of infection, 10) and harvested 48 h later together with sham-infected controls. To generate a whole cell extract, the cells were harvested and washed three times with cold phosphate-buffered saline (0.01 M NaPO₄, pH 7.4, 0.14 M NaCl), and suspended at 4°C in binding buffer B (0.03 M K₂HPO₄, pH 9.0, 0.15 M NaCl, 0.01% NaN₃, 1 mM dithiothreitol, 50 µg of phenylmethylsulfonyl fluoride per ml, 20 µg of tosyl-L-phenylchloromethyl ketone per ml). After three cycles of freezing and thawing, extracts were centrifuged at 12,000 × g and 4°C for 20 min, and the supernatant fluids were stored at -70°C. Nuclear extracts were prepared as described previously (19).

Escherichia coli RB113 transformed by pTR436 (PB113/pTR436) is a clone which synthesizes intact SV40 small t antigen (32). This clone was grown at 37°C in L-broth in parallel with RB113 transformed

by pBR322, and extracts of each were prepared by sonication as described previously (32).

SV40 large T antigen that was purified from SV80 cells by the method of Tenen et al. (36), as modified by Krieger et al. (19), contained 20 μg of protein per ml and was $\geq 90\%$ pure, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. D2 T antigen (100 $\mu\text{g}/\text{ml}$; $\sim 90\%$ pure) an analog of large T antigen, was isolated as described previously (37). A partially purified ($\sim 10\%$ pure) SV80 cell large T antigen fraction isolated after a modification of the first three steps of the standard method (19) was also utilized in some experiments.

Absorption of antisera. Pooled hamster anti-SV40 tumor sera (79 x-141) were obtained from the National Institutes of Health. SV40-free, human lymphoblast lines LAZ-221, SB, and CEM were grown at 37°C in spinner cultures in RPMI 1640 medium containing 10% fetal calf serum. LAZ-221 and CEM extracts were used interchangeably for preabsorption of the antisera. Cell pellets (150 μl) were washed in phosphate-buffered saline, quick-frozen in a dry ice-acetone bath, lyophilized, and stored at -20°C . Before use, pellets were rehydrated in water and washed twice with phosphate-buffered saline. To absorb an antiserum, 1 ml of serum diluted 10-fold in buffer A (0.05 M Tris-hydrochloride, pH 7.8, 1% bovine serum albumin [BSA], 1 mM MgCl_2 , 0.01% NaN_3) was added to the rehydrated cell pellet, and the suspension was agitated continuously for 16 h at 4°C . Before use in some experiments, the sera were further absorbed with *E. coli* grown and extracted as described previously (32). These absorptions involved the addition of human cell-absorbed serum (~ 1 ml) to a 150- μl lyophilized bacterial extract pellet and another 16 h of incubation at 4°C . After centrifugation, 200- μl portions of the absorbed serum were added to 300- μl polystyrene wells (covered Microtest II; Falcon Plastics, Oxnard, Calif.) previously coated for 2 h with 10% BSA in water. After 2 h of continuous agitation at room temperature, absorbed sera were stored at -20°C , and these sera could be frozen and thawed (at room temperature) repeatedly without significant loss of activity.

Preparation of immunoglobulin G-alkaline phosphatase conjugates. A 2-mg portion of rabbit anti-hamster immunoglobulin G (Cappel Laboratories, Downingtown, Pa.) was coupled to 1 mg of calf intestine alkaline phosphatase (type VII; Sigma Chemical Co., St. Louis, Mo.) by the method of Avrameas et al. (1) in the presence of 0.05% glutaraldehyde. After the final dialysis and centrifugation, this conjugate was diluted with an equal volume of twice-concentrated buffer A. When stored in the dark at 4°C , this conjugate was stable for more than 9 months but it was rapidly inactivated after one freezing (at -23°C) and thawing (at 4°C).

ELISA for large T antigen. Covered Microtest II round-bottomed polystyrene plates (96 wells; Falcon Plastics) were used throughout. Each plate was chilled to 4°C before use. A 25- μl portion of a test solution diluted in buffer B (0.03 M PO_4 , pH 9.0, 0.15 M NaCl, 0.01% NaN_3 , 1 mM dithiothreitol) was added to each well. The plate was then agitated continuously for 30 min at 4°C . The wells were then washed with coating buffer (0.05 M Tris-hydrochloride, pH 7.8, 10% BSA,

0.01% NaN_3) without allowing flow from one well to another, and the wash buffer was then removed by inverting the plate and shaking it vigorously. The wells were refilled with buffer C and agitated for an additional 1 h at 4°C ; the plate was then inverted and shaken to clear the wells of fluid.

To each well was added 50 μl of immune hamster serum (standard dilution, 1:200) or preimmune hamster serum (same dilution as immune serum) diluted in buffer A. The plate was agitated for 1 h at 4°C , inverted, and shaken. Each well was washed once with cold wash buffer W (8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4, 0.15 M NaCl, 2.7 mM KCl, 0.05% Tween 20, 0.01% NaN_3), and the plate was then immediately inverted and shaken. From this point on, operations were carried out at room temperature. A 50- μl portion of a 1:150 dilution of the immunoglobulin G-enzyme conjugate (diluted in buffer A) was added to each test well. After the plate was agitated for 30 min, it was inverted and shaken. Wells were washed twice for 3 min with high-salt wash buffer (buffer W, but containing 1.1 M NaCl) and once with buffer W; the plate was inverted, and the wells were shaken free of fluid. Then, 200 μl of *p*-nitrophenylphosphate (2 mg/ml; in 0.05 M Tris-hydrochloride [pH 9.8]–1 mM MgCl_2) was added to each well. Plates were incubated at 37°C for 2 h. The reaction was stopped by adding 50 μl of 3 M NaOH. Controls for nonspecific enzyme activity included wells coated with buffer B only and antigen-coated wells reacted with nonimmune serum. The optical density of each solution at 400 nm was determined with a Zeiss PMQII spectrophotometer.

Other assays. The complement fixation assay for SV40 large T antigen (36) was performed as previously described. Usually, antibody, antigen, and complement (1.2 U) were incubated at 4°C for 16 h. Protein determinations were performed by the method of Esen (10), with the following modifications. Triplicate 5- μl samples were spotted onto squares of Whatman 3MM filter paper (1 by 1 cm), which were dried and stained with 0.1% filtered Coomassie brilliant blue for 30 min. The paper strips were destained in three changes of 5% acetic acid–7.5% methanol (40 min each). After drying, the protein-specific stain was eluted into 1 ml of 1% sodium dodecyl sulfate by incubation at 68°C for 30 min or at 25°C for 2 h. Background, nonspecific staining was determined with filters spotted with buffer alone. The optical density of each sample was measured at 585 nm. BSA served as a quantitative standard in these assays.

RESULTS

Specificity of the SV40 large-T-antigen ELISA. Samples containing SV40 large T antigen and relevant controls were tested in duplicate by the ELISA with both pooled hamster anti-T antigen serum and preimmune hamster serum (Table 1). Sera were preabsorbed with a whole cell extract of the SV40-free, human T-lymphoblast cell line CEM and, in some cases, with an extract of *E. coli* RB113.

A nuclear extract of SV40-infected monkey cells (CV1-P) yielded a positive reaction with

TABLE 1. *Specificity of the ELISA performed with hamster anti-SV40 tumor serum*

Solution	Absorbance at 400 nm		Ratio ^a	Amt of protein added (μ g)
	Im-mune serum	Preim-mune serum		
SV80 extract	2.020	0.490	4.05	16.80
	2.410	0.605		
SB extract	0.410	0.490	0.85	13.20
	0.450	0.535		
Heated SV80 extract ^b	0.500	0.410	1.22	16.80
	0.515	0.425		
SV40-infected CV1-P extract	2.115	0.445	4.52	8.40
	2.025	0.470		
CV1-P extract	0.375	0.445	0.92	13.20
	0.400	0.400		
Standard ^c	2.065	0.420	5.52	0.72
	2.465	0.400		
D2 T antigen ^d	2.445	0.435	5.55	0.03
	2.185	0.400		
RB113/pTR436 extract	0.405	0.400	1.09	6.00
	0.460	0.390		
RB113/pBR322 extract	0.445	0.400	1.09	6.90
	0.445	0.410		
Buffer B	0.355	0.370	1.00	None
	0.370	0.355		

^a Ratio of immune serum absorbance at 400 nm to preimmune serum absorbance at 400 nm.

^b Heated at 56°C for 10 min.

^c Large T antigen partially purified from SV80 cells (see text).

^d Highly purified D2 T antigen diluted in binding buffer containing 0.01% BSA.

anti-T antigen serum, whereas mock-infected monkey cells were unreactive. A nuclear extract of human cells stably transformed by SV40 (SV80 cells) reacted strongly with immune serum and not with preimmune serum, whereas comparable amounts of a human lymphoblast (SB) whole cell extract not containing SV40 showed no reactivity. Heating the SV80 extract for 10 min at 56°C resulted in a marked loss of immunoreactivity. Large T antigen, partially purified from SV80 cells, was also reactive in a specific fashion, as was the D2 hybrid T antigen extensively purified from Ad²⁺ D2-infected HeLa cells (15, 37). When tested with preimmune serum, all antigen extracts yielded results which were not significantly different from the results obtained with wells coated with binding buffer only. Ratios of immune serum optical density at 400 nm to preimmune serum optical density at 400 nm reflected the specificity of the ELISA for large T antigen (Table 1). In view of the values obtained, ratios of more than 1.1 were indicative of a significant and specific immunological reaction. Moreover, in no case was an apparent positive reaction with a crude cell ex-

tract due simply to nonspecific effects of a high protein input, since extracts of T-antigen-free cells assayed in comparable amounts yielded negative results.

To determine whether the ELISA performed under these conditions detected both large T antigen and small t antigen, extracts containing small t antigen, but not large T antigen, were tested. *E. coli* RB113 transformed by pTR436 is a bacterium which synthesizes immunoreactive SV40 small t antigen but does not synthesize large T antigen (32). When an extract of this organism was assayed in parallel with an extract of RB113 transformed by pBR322 (an organism which lacks any SV40 DNA), negative results were obtained (Table 1). When 5- and 10-fold greater amounts of protein from each extract were tested (Table 2), negative results were also obtained. In contrast, as noted previously, a strong positive reaction was detected with an SV80 nuclear extract, which contained predominantly large T antigen. Since there are approximately 5,000 molecules of immunoreactive small t antigen per pTR436/RB113 cell (32), we estimated that there were nearly 11 times more small-t-antigen molecules added to an assay well when the input was 60 μ g of protein than there were large-T-antigen molecules in 16.8 μ g of SV80 lysate (Table 2). In contrast, this lot of antiserum could immunoprecipitate both [³⁵S]-methionine-labeled large T antigen and small t antigen from SV80 cells when added in an undiluted state (Fig. 1). To determine whether more antiserum would result in a positive small-t-antigen reaction, we compared the reactivities of purified D2 protein and the RB113/pTR436 extract at increasing levels of anti-T antigen antibody (up to 1 order of magnitude more than the level used to obtain the ELISA results shown in Table 1). Once again, the D2 protein reacted, and the RB113/pTR436 extract did not (Fig. 2). In this case, approximately 2.5 times more small

TABLE 2. *Reactivities of various large-T-antigen- and small-t-antigen-containing extracts with hamster anti-tumor serum, as determined by ELISA*

Extract	Serum ^a	Antigen(s) present	Amt of protein added (μ g)	Absorbance at 400 nm
SV80	T	Large T	16.8	1.68
SV80	Pre	Large T	16.8	0.47
pTR436	T	Small t	6	0.41
pTR436	Pre	Small t	6	0.40
pTR436	T	Small t	30	0.47
pTR436	Pre	Small t	30	0.43
pTR436	T	Small t	60	0.48
pTR436	Pre	Small t	60	0.39

^a T, Hamster anti-SV40 tumor serum; Pre, preimmune hamster serum.

FIG. 1. Immunoprecipitation of [35 S]methionine-labeled extracts of SV80 cells with hamster anti-SV40 tumor serum. A crude extract (lane B) and a partially purified extract (lane A) of [35 S]methionine-labeled SV80 cells were immunoprecipitated with the hamster anti-tumor serum (79 $\times$ 127) used in all of these experiments. The dissolved immunoprecipitates were analyzed by sodium dodecyl sulfate gel electrophoresis and autoradiography as described previously (19). The markers T and t indicate large T antigen and small t antigen, respectively.

t antigen than D2 large T antigen should have been present in the wells. We were not able to measure the reactivity of either antigen at antibody dilutions of less than 1:20 because of increases in nonspecific background reactivity. No inhibition or alteration of the D2 T antigen or

FIG. 2. ELISA of solutions containing large T antigen and small t antigen at varying antibody concentrations. Symbols: $\diamond$, 12 μ g (25 μ l) of RB113/pTR436 extract; $\bullet$, 12 μ g of RB113/pBR322 extract; Δ , 10 ng of purified D2 protein plus 12 μ g of RB113/pBR322 extract; $\blacktriangle$, 10 ng of D2 protein in 0.01% BSA. Preparations were assayed by the ELISA, as described in the text at varying hamster anti-T and preimmune hamster serum concentrations. The values for optical density at 400 nm shown are the differences between immune and preimmune wells at each antibody dilution.

SV40 large-T-antigen (data not shown) reactivity was observed when either purified protein was mixed with a pBR322/RB113 extract. Hence, the bacterial extract did not suppress the binding of large T antigen to plastic, the reactivity of this antigen with antibody, or the enzymatic reaction with substrate. Therefore, under the conditions used in these experiments, the ELISA appears to be functionally large T antigen specific and does not measure small t antigen.

Buffer effects on large-T-antigen reactivity as determined by ELISA. In an effort to establish a variety of permissive conditions for our assay, purified D2 protein was assayed by the ELISA in the presence and absence of reagents frequently present in large-T-antigen solution (36). The reactivity of the protein was not influenced appreciably by 2 M sodium chloride, 1 M ammonium sulfate, 50% glycerol, or a pH of 7 (compared with pH 9 of the standard binding buffer or buffer B). However, 2% Nonidet P-40 totally eliminated detection of large T antigen by the ELISA. When the Nonidet P-40 effect was studied further, inhibition was negligible at detergent concentrations up to 0.1% but increased in a semilogarithmic fashion at higher concentrations (data not shown). The inhibitory effect of higher detergent concentrations was probably due to blockade of large-T-antigen binding to plastic, a reaction which, for most proteins, may depend upon hydrophobic interactions (29). Finally, the effect of protein concentration on the binding of antigen to microti-

ter wells was studied by adsorbing 0.1 μg of highly purified D2 protein to wells in the presence of binding buffer containing 0 to 1.5 mg of BSA per ml. A constant level of reactivity was observed at BSA concentrations of 25 to 800 $\mu\text{g}/\text{ml}$, but reactivity fell off significantly at higher and lower BSA concentrations (data not shown). Thus, highly purified large T antigen should be "protected" with BSA in the range noted above in order to avoid underestimating large-T-antigen content.

Comparing the results of the ELISA and the complement fixation test. Different dilutions of a crude SV80 cell nuclear extract were tested for antigen by both the ELISA and the complement fixation assay. Determinations were made at antigen dilutions chosen to encompass the previously estimated limits of detectability of the complement fixation test (Fig. 3). The ELISA was clearly positive with 0.12 μl of extract, whereas the complement fixation assay required 0.4 μl . Thus, under these conditions the ELISA was more sensitive than the complement fixation test by approximately threefold. More importantly, the ELISA results fell on a predictable line with a broad, reproducible linear component (see below). In contrast, the complement fixation test yielded false low determinations at higher extract concentrations due to a "zone" effect, and the linear segment of the complement fixation titration curve was only present over a twofold range of protein input, thus limiting the working range for quantitation. The ELISA and the complement fixation test were also compared during an analysis of the DEAE-cellulose column fractions generated by the purification of large T antigen from SV80 cells. Figure 4 shows the results of assaying 2- μl portions of the

FIG. 3. Comparative assay of a large-T-antigen-containing extract by the ELISA ($\square$) and the complement fixation test ($\bullet$). A crude SV80 cell nuclear extract (5.6 mg/ml) prepared as described in the text was diluted in binding buffer. Duplicate 25- μl samples were assayed by the methods described in the text.

FIG. 4. Assay of large-T-antigen-containing chromatographic fractions by the ELISA and the complement fixation test. DEAE-cellulose chromatography of $(\text{NH}_4)_2\text{SO}_4$ -fractionated nuclear large T antigen from SV80 cells was performed as described previously (19, 36). Samples (2 μl) from the fractions were assayed by the ELISA and by the complement fixation test as described in the text.

fractions by both methods. The results of the two assays were comparable; both defined a single major antigen peak that eluted at approximately 0.2 M NaCl. The minor peaks identified by the complement fixation test between fractions 18 and 30 were the result of anti-complementary activity, since they were also observed in the absence of added hamster antibody. The peak between fractions 58 and 64 was defined better by the ELISA than by the complement fixation test; this peak was the result of a true anti-T antigen-dependent immunological reaction and was reproducible. Similar ELISA and complement fixation test results have also been obtained in each of the four other steps of SV80 large-T-antigen purification (ammonium sulfate fractionation, QAE-Sephadex chromatography, heparin-Sepharose chromatography, and glycerol gradient sedimentation) (Bradley and Livingston, unpublished data). Moreover, the time required to assay each set of chromatographic fractions from the purification of SV80 cell large T antigen has been reduced from the 16 h required for the complement fixation test to 2.5 to 3 h. Specifically, when sufficiently large amounts of each fraction were tested, a yellow color was observed after a 15-min incubation of the bound immune complex with substrate at 37°C. Therefore, we have substituted the ELISA technique for the complement fixation test during routine large-T-antigen purification, with major savings in time.

Construction of a standard large-T-antigen titration curve. Experiments were performed to determine whether the ELISA for large T antigen could be used quantitatively.

First, the reproducibility of the titration results was assessed. In these experiments, 10 replicate samples of four dilutions of partially purified large T antigen from SV80 cells were tested. A positive response was observed in immune serum wells but not in preimmune serum wells, and the level of reactivity rose about background in a linear fashion (Fig. 5A). The optical density change specifically attributable to large-T-antigen immunoreactivity was determined by subtracting the preimmune control results at each point. A least-squares analysis of these values (Y) versus the standard dilution (X) was performed, yielding the equation $Y = 0.1429 + 0.1023X$ ($R^2 = 0.9813$; standard error of the slope, 0.002). This equation fit the data well, and the four dilutions explained more than 98% of the variation in optical density, as implied by the

FIG. 5. Construction of ELISA standard curve. (A) Replicates containing 2.5, 7.5, 12.5, and 17.5 μ l of a partially purified SV80 large-T-antigen fraction (0.24 mg/ml; ELISA standard) were tested by the ELISA as described in the text, using immune ($\bullet$) or preimmune ($\circ$) serum. Replicate optical density values of each aliquot are displayed vertically and to the right. (B) After the average of the results of the preimmune serum reactions at each dilution was subtracted from the results of the immune serum reactions, a regression line was established by the least-squares method. The regression equation was $Y = 0.1023X + 0.1429$. (C) The regression line slope could be reproduced on another day (dotted line). The average immune serum result minus the average preimmune serum result at each dilution on day 2 is indicated by a diamond. The regression equation for the dotted line data was $Y = 0.0976X + 0.0565$.

high R^2 value. The regression line of this straight-line segment of the standard curve is shown in Fig. 5B. Using the same technique, we generated another set of standard curve data with the same reagents on another day. The regression analysis of Y and X for these data gave the equation $Y = 0.0565 + 0.0976X$ ($R^2 = 0.9606$; standard error of the slope, 0.004). This second regression equation, together with the average optical densities of the four dilution points, was plotted with the regression line from the first experiment (Fig. 5C). The difference in the two slopes was not statistically significant. Thus, in the range defined here, the standard curve yielded reproducible results from day to day.

A requirement for the use of such a standard curve for quantitation of large T antigen in other solutions is that all of the regression lines resulting from the titrations of various large-T-antigen-containing solutions should be parallel, implying comparability of antigenic determinants active in the large-T-antigen-specific reaction. Four dilutions shown in preliminary experiments to give responses in the range of a previously defined titration curve (Fig. 5B) were chosen for a crude SV80 cell extract, a crude extract of SV40-infected CV1-P cells, and highly purified D2 T antigen. Eight replicates of each dilution were assayed (Fig. 6A). The relevant regression equations are given in the legend to Fig. 6. We found no significant differences in the slopes of the various titration curves. The range of D2 protein concentration assayed over the course of the titration was 11 to 81 ng (Fig. 6B). Since the protein was at least 90% pure (as assessed by sodium dodecyl sulfate gel electrophoresis) and the slopes of the various regression lines generated in the experiments shown in Fig. 5 and 6 were superimposable, the data in Fig. 6B can serve as a quantitative standard for measuring either D2 protein or native large T antigen in various fractions, both crude and highly purified. To confirm that this approach applies to the quantitation of highly purified large T antigen, we assayed a $\geq 90\%$ pure solution of large T antigen from SV80 cells (isolated as described above) by the ELISA technique under standard conditions. From these data, we calculated that 1 μ l of highly purified large T antigen contained 20.3 ng of antigen. The total protein concentration in this fraction was also measured and was approximately 20 μ g/ml (10). Thus, the D2 T-antigen standard curve is valid for measurements of highly purified native large T antigen.

Sensitivity limit of the assay. To determine the sensitivity limit of the assay, we assayed six replicates of six different amounts (0.312, 0.625,

FIG. 6. Quantitation of large T antigen. (A) Purified D2 T antigen (5 µg/ml) (Δ) and crude lysates of SV40-infected CV1-P cells ($\odot$) and SV80 cells ($\square$) were assayed by the ELISA, as described in the text. The regression line for the partially purified large-T-antigen titration shown in Fig. 5B is also plotted. Using least-squares analyses, we detected no statistically significant difference in regression between the prior large-T-antigen standard curve and these three titration curves. The regression equations for the various titrations are as follows: SV80 extract (7.5, 5.0, 3.0, and 1.5 µl), $Y = 0.9955 + 0.1095X$ ($R^2 = 0.9401$; standard error of the slope, 0.012); extract of SV40-infected CV1-P cells (7.5, 5.0, 3.0, and 1.5 µl), $Y = 0.8701 + 0.1176X$ ($R^2 = 0.9054$; standard error of the slope, 0.007); purified D2 T antigen (13.5, 8.5, 4.5, and 1.5 µl), $Y = 0.3077 + 0.1005X$ ($R^2 = 0.9591$; standard error of the slope, 0.004); standard (17.5, 12.5, 7.5, and 2.5 µl), $Y = 0.1429 + 0.1023X$ ($R^2 = 0.9913$; standard error of the slope, 0.002) (see Fig. 5B). (B) Calibrated D2 T-antigen standard curve calculated from the data in (A). (C) Quantitation of large T antigen in 5 µl of SV80 cell nuclear extract ($\square$) and 5 µl of lytically infected CV1-P nuclear extract ($\odot$). The concentrations of large T antigen in the solutions were 11.6 and 10.4 µg/ml, respectively.

1.25, 2.5, 5.0, and 10 ng) of purified D2 T antigen diluted in buffer B containing 0.01% BSA (Table 3). Six replicates of each dilution were also tested in the presence of preimmune serum, and the optical density differences between paired sets of wells were determined for each quantity of D2 T antigen tested. The difference between the anti-T antigen serum and preimmune serum wells at each point was analyzed statistically by the pooled *t* test for equality of means. The results indicated that at T-antigen protein inputs of ≥ 0.625 ng, the difference between the means of the anti-T antigen serum and preimmune serum wells was highly statistically significant.

Relative concentration of large T antigen in crude extracts. The approximate steady-state concentrations of immunoreactive large T antigen in fresh crude SV80 and SV40-infected monkey cell nuclear extracts were measured by the ELISA, using a calibrated D2 protein standard curve (Fig. 6C). The SV80 and infected monkey cell nuclear extracts contained 11.6 and 10.4 µg of large T antigen per ml, respectively. The protein concentrations of these extracts were 5.6 and 2.8 mg/ml, respectively, indicating that approximately 0.2 and 0.4% of the protein present in these extracts was large T antigen. These data are consistent with previous results that indicated that SV80 cells contained quantities of large T antigen comparable to the quantities present in lytic infections (16).

DISCUSSION

Our results indicate that the SV40 large T antigen can be detected accurately with a simple, rapid ELISA. This test is applicable to large T

TABLE 3. Sensitivity limit of the ELISA^a

Amt of D2 T antigen added (ng)	Absorbance at 400 nm		<i>P</i>
	Immune serum	Preimmune serum	
0.0	0.355	0.370	
0.312	0.365	0.381	0.417
0.625	0.410	0.392	0.003
1.25	0.425	0.410	<0.0001
2.5	0.445	0.410	<0.0001
5.0	0.535	0.405	<0.0001
10.0	0.885	0.407	<0.0001

^a Six replicates of each sample of purified D2 T-antigen protein were assayed in parallel with anti-T-antigen serum. Six additional replicates were assayed simultaneously with preimmune serum. The averages of the assay results are shown. These data were also analyzed by the pooled *t* test for equality of means.

antigen from a variety of sources in various states of purity and is highly sensitive, having a detection limit of 0.625 ng. It can use a common source of anti-T antigen antibody, hamster anti-SV40 tumor serum. In preliminary experiments, it has also been used with rabbit antibody raised against sodium dodecyl sulfate gel-purified large T antigen and yielded comparable results (Bradley, Kilton, and Livingston, unpublished data).

D2 hybrid T-antigen protein and large T antigen differ by the absence and presence, respectively, of an N-terminal 82-amino acid, SV40-encoded sequence (15, 28). However, these two proteins reacted equivalently in repeated ELISA titrations. Thus, it is clear that the antibodies used in these experiments primarily recognize sequences encoded by SV40 DNA between 0.53 and 0.17 map units. In agreement with this ob-

servation is the finding that small t antigen failed to react significantly at any concentration of antibody or antigen tested. This was the case even when equivalent numbers of small t antigen and large T antigen molecules were assayed in parallel. Although the small t antigen fractions tested were from *E. coli*, the failure of small t antigen to react with antibody was probably not a result of inhibitory effects conferred by other bacterial proteins, since mixing an extract of *E. coli* RB113 transformed by pBR322 (the parent of pTR436) with either D2 T antigen or SV80 cells failed to inhibit the immunoreactivity of either protein. Despite the failure of small t antigen to react in the ELISA, the pooled hamster sera used here did precipitate in vivo-labeled small t antigens from SV80 cells (Fig. 1) and from RB113/pTR436 (data not shown). Thus, either the anti-t-antigen antibodies are of sufficiently low affinity, concentration, or both that small-t-antigen reactivity does not register in the ELISA (but does in the immunoprecipitation test) and/or small t antigen binds poorly to plastic under the conditions of the assay.

Our results were not dependent upon the presence of the 56,000-dalton nonviral T antigen(s) (6, 18, 21, 22, 25). This was clear from the observation that purified D2 and SV80 large-T-antigen proteins and a crude SV80 cell extract which contained this protein(s) all yielded titration curves with identical slopes.

To insure the absolute large-T-antigen specificity of the assay, it was necessary to preabsorb the anti-tumor sera used with uninfected mammalian cell extracts. To reduce a low level of nonspecific serum adherence to BSA-coated wells, BSA-coated polystyrene preabsorption was also used. Conceivably, the first absorption step can be eliminated when monospecific antibody or monoclonal antibody or both are used in the assay.

Most of the studies described here involved a 2-h incubation with substrate, making the total time for each assay from initiation to data collection approximately 5.5 h, compared with 16 h for the complement fixation test. The ELISA time has been successfully reduced to 2.5 to 3 h during large-T-antigen purification by testing ≥ 50 -ng samples of antigen or increasing the antibody concentration or both. Thus, the ELISA is much quicker to perform than the complement fixation test, which until recently has been a standard method. Furthermore, under the conditions used, the ELISA was three times more sensitive, yielded a much broader linear titration segment, and was not susceptible to zoning phenomena in antigen excess.

Although this particular ELISA is specific for the products of the large-T-antigen cistron, it

should be applicable to small t antigen, with certain modifications. If small t antigen binds poorly to polystyrene, it may be possible to identify other strata to which it binds efficiently. Alternatively, the assay might be performed by incubating small t antigen with anti-t-antigen serum adsorbed to plastic, reacting the bound small t antigen with the anti-t-antigen from a second species, and then reacting the second anti-t-antigen with a suitable anti-immunoglobulin G-enzyme conjugate. If the problem with the detection of small t antigen is the relatively low affinity of the sera used, other sera with higher affinities for small t antigen could be tried.

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