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Purification of Simian Virus 40 Large T Antigen by Immunoaffinity Chromatography

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Simian virus 40 large T antigen from lytically infected cells has been purified to near homogeneity by immunochromatography of the cell extract on a protein A-Sepharose-monoclonal antibody column. The resulting T antigen retains biochemical activity; i.e., it hydrolyzes ATP and binds to simian virus 40 DNA at the origin of replication.

The large T antigen of simian virus 40 (SV40), the product of the viral A gene, is a 708-amino acid regulatory protein that is involved in initiation of viral DNA replication, regulation of viral gene expression, and transformation of cells to tumorigenicity (11). The biochemically defined activities of T antigen include an ATPase activity yielding ADP and orthophosphate (10) binding to the SV40 origin of replication (9), and promotion of the replication of SV40 origin containing DNA in vitro (1, 4a). As part of our analysis of SV40 T-antigen mutants, we have developed a rapid method for the purification of biochemically active T antigen to near homogeneity by immunochromatography. In this report, we describe the purification procedure and some of the activities of the purified T antigen.

The source of T antigen was BSC-40 monkey kidney cells (2) infected with the SV40 mutant cs 1085 (3). This mutant has a deletion within its T-antigen binding site I. As a result of defective autoregulation, cs 1085-infected cells contain ca. 5 to 10 times the amount of T antigen found in wild-type SV40-infected cells (3). BSC-40 cells were propagated as described previously and infected at 37°C at a multiplicity of infection of 10 to 20. (In a typical experiment, ca. 10^8 cells were used.) After 48 h at 37°C, the medium was removed, the monolayer was rinsed twice with ice-cold phosphate-buffered saline, and the cells were scraped into phosphate-buffered saline and pelleted by centrifugation. All subsequent steps were carried out at 0 to 4°C. After two washings with phosphate-buffered saline, the cells were suspended in 10 packed cell volumes of a solution containing 20 mM Tris-hydrochloride (pH 9.0), 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 1.0% Nonidet P-40 (NP40), and 200 μg of phenylmethylsulfonylfluoride per ml. The cell lysate was incubated for 10 min, and the nuclei were removed by centrifugation at 2,000 rpm for 10 min. The nuclear supernate was clarified by centrifugation at 20,000 rpm for 30 min in a SS34 rotor. One-half volume of a solution containing 100 mM Tris-hydrochloride (pH 6.8), 1 mM EDTA, 1 mM DTT, 10% glycerol, and 1% NP40 was added to the supernate, yielding a final pH of 8.0. This preparation will be referred to as the cell extract.

For immunoaffinity chromatography, a matrix was prepared by covalently linking purified anti-T monoclonal antibody PAb 419, which recognizes the amino end of T antigen (4), to protein A-Sepharose beads (Pharmacia Fine Chemicals, Inc.) (10 mg of IgG/ml of Sepharose) by the method of Schneider et al. (8). The antibody-linked beads were rinsed in a solution containing 20 mM Tris-hydrochloride (pH 8.0), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% NP40, and 10% glycerol and then added to the cell extract (100 to 200 μl of packed bead volume per 15 ml of extract); the mixture was

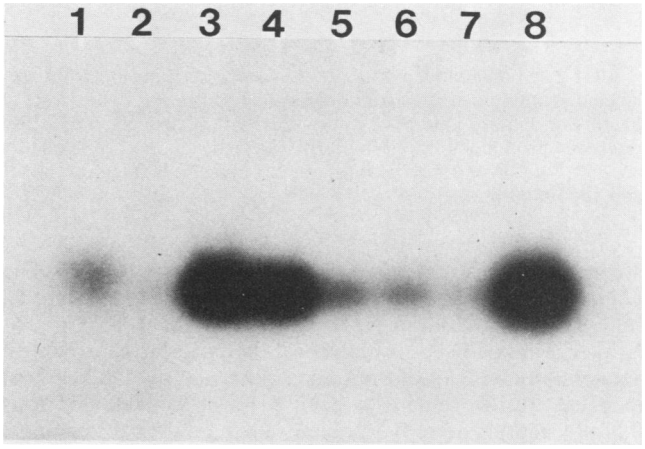

FIG. 1. Assay of the immunoaffinity column eluate for T antigen. An origin-containing fragment of SV40 DNA (*Hin*fI to *Hpa*II) labeled at the *Hin*fI end by incorporating ^{32}P-nucleotide was incubated at 0°C for 30 min with 5 μl of a given column fraction (see below) in a total volume of 100 μl containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [pH 7.3]), 10 mM NaCl, 0.1 mM EDTA, 2 mM DTT, 0.05% NP40, and 100 μg of bovine serum albumin per ml. At the end of the binding reaction, 10 μl of PAb 419 monoclonal anti-T antibody (30 μg of immunoglobulin G per ml) was added; after 30 min at 0°C, 10 μl of rabbit anti-mouse immunoglobulin serum (Miles-Yeda) was added, and incubation was continued for 30 min, after which the complex was absorbed to staph A protein (Pansorbin; Calbiochem-Behring). The precipitate was collected and washed three times with a solution of 10 mM Tris-hydrochloride (pH 8.0), 150 mM NaCl, and 0.5% NP40. The precipitated [^{32}P]DNA was then counted and electrophoresed in 2% agarose gel, and the dried gel was exposed to X-ray film. The figure shows such an autoradiogram. Lane 1, input DNA fragment, not precipitated; lane 2, no T antigen; lane 3, T antigen purified by sequential column chromatography; lane 4, 5 μl of cell extract; lane 5, 5 μl of pooled column flowthrough; lane 6, 5 μl of pooled pH 8 eluate, lane 7, 5 μl of pooled pH 9 eluate; lane 8, 5 μl of pooled pH 11 eluate.

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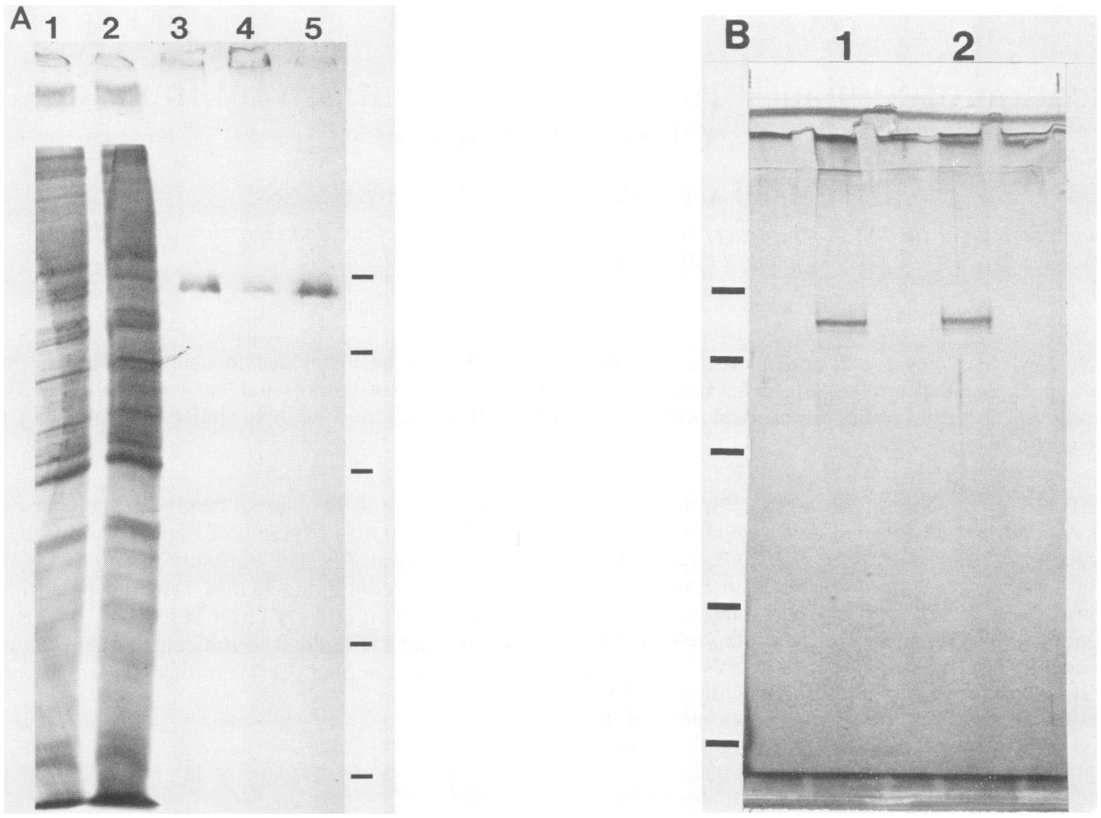

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunochromatographically purified T antigen. (A) [^{35}S]methionine-labeled protein detected by autoradiography. Lane 1, cell extract applied to immunoaffinity column; lane 2, column flowthrough; lane 3, pH 11 column eluate; lane 4, hydroxylapatite flowthrough; lane 5, hydroxylapatite column eluate. Indicated at the right are the positions of reference proteins of (top to bottom) 97, 68, 43, 26, and 18 kilodaltons. (B) Silver stain of hydroxylapatite column eluate. Lane 1, preparation no. 1, 1.7 μg; lane 2, preparation no. 2, 2.0 μg. Indicated on the left are the positions of reference proteins of (top to bottom) 97, 68, 43, 26, and 18 kilodaltons.

stirred gently overnight and packed into a small column. The column was washed with 2 ml of a solution containing 50 mM Tris-hydrochloride (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 1% NP40, followed sequentially by 2 ml of the same solution at pH 9.0 and 2 ml at pH 11.0. The eluate at each pH was collected in four 0.5-ml fractions directly into neutralizing buffer containing 2 ml of 50 mM Tris-hydrochloride (pH 8.0), 1 mM EDTA, 1 mM DTT, and 10% glycerol. The same antibody-Sepharose beads could be used repeatedly after washing with the loading buffer.

T antigen present in the various column fractions was assayed by its ability to bind a ^{32}P-labeled SV40 DNA fragment containing the replication origin (6). Figure 1 shows the results of a typical immunochromatographic fractionation; binding activity eluted at pH 11. The T antigen was further concentrated by pooling the active fractions and applying them to a 1-ml column of hydroxylapatite (Bio-Rad Laboratories) equilibrated with a solution containing 50 mM potassium phosphate (pH 7.0), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 10% glycerol. After the column was washed with the above solution, T antigen was eluted with the same solution containing 300 mM potassium phosphate (pH 8.0). The fractions showing specific DNA-binding activity were pooled, dialyzed against a solution containing 10 mM Tris-hydrochloride (pH 8.0), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 50% glycerol, and then stored at −20°C. DNA-binding activity appeared stable for over 12 months.

The purity of the T-antigen preparation was determined by examination of samples of the hydroxylapatite fractions by electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels (Fig. 2). Figure 2A shows the results with [^{35}S]methionine-labeled protein. A single pass through the immunoaffinity column eliminated nearly all of the labeled cellular proteins. A silver-stained gel showed in each of two preparations a major band of protein of ca. 85 kilodaltons (the size of large T antigen) and minor protein bands immediately below the 85-kilodalton band which might be cleavage products since their amount varied from one preparation to another. We occasionally observed a ^{35}S-labeled protein with the mobility of SV40 small T antigen in the antibody column eluate, but small T antigen was not detected by silver staining. Thus, the immunochromatographic method yields electrophoretically nearly homogeneous large T antigen.

To learn whether T antigen purified by immunochromatography retains its specific biochemical activities, we determined its ATPase activity and its sites of binding to SV40 DNA. The T-antigen preparation (3.3 μg of T antigen per ml, 1 μM ATP, 5 mM $MgCl_2$; pH 7.3) split ATP at a rate of 110 pmol/h per μg of protein. The site of binding of the purified protein to an SV40 DNA fragment containing the origin of replication was localized by DNase footprinting. From the autoradiogram shown in Fig. 3A, we conclude that the purified T antigen binds specifically to sites I and II at the replication origin, as previously defined (9). When the auto-

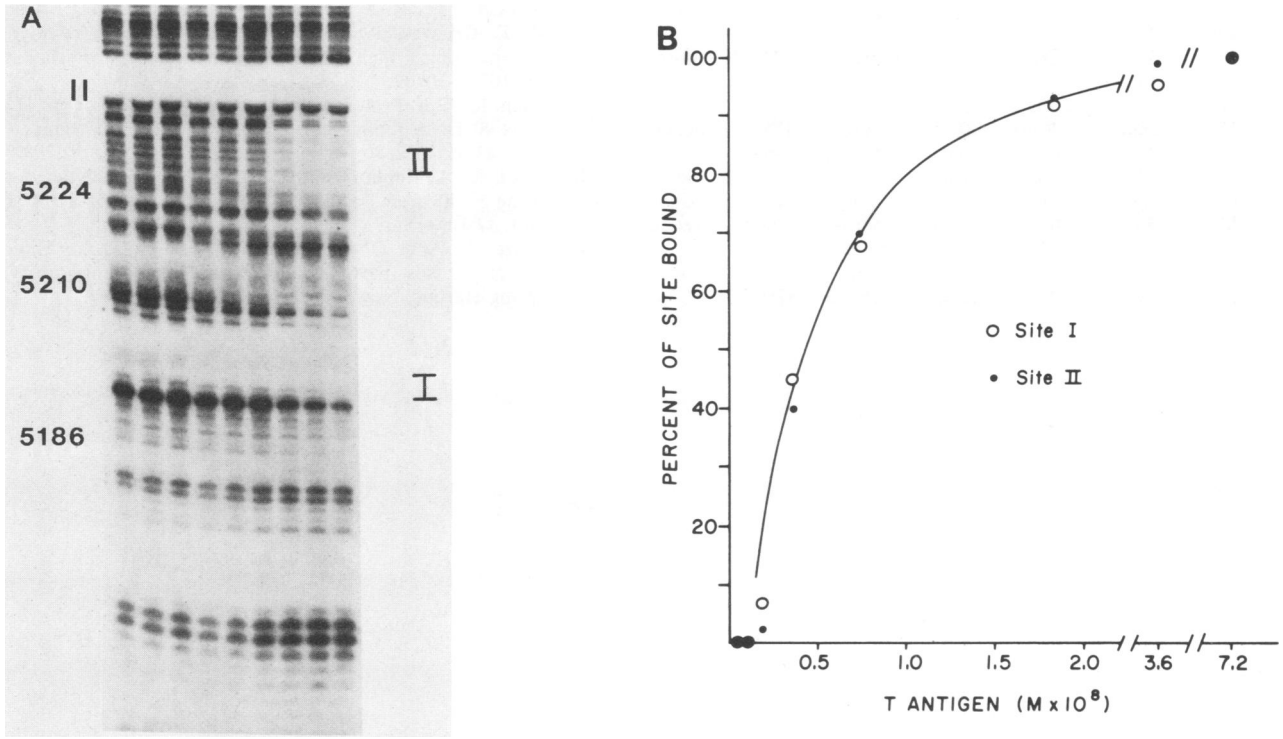

FIG. 3. Binding of purified T antigen to the SV40 origin of replication: DNase footprint. The origin-containing fragment of SV40 DNA between *Hin*fI and *Sph*I was labeled at the *Hin*fI end by incorporating ^{32}P-nucleotide. A 20-pmol sample of the fragment was incubated at 37°C for 30 min with increasing amounts of purified T antigen in a solution containing final concentrations of 10 mM HEPES (pH 7.3), 10 mM NaCl, 0.1 mM EDTA, 2 mM DTT, 0.05% NP40, 100 μg of bovine serum albumin per ml, and 5% glycerol in a total volume of 100 μl. The samples were then chilled in ice; 5 μl of 100 mM $MgCl_2$ was added, and then pancreatic DNase was added to give a final concentration previously determined to yield appropriate cleavage (from 0.4 to 1 μg/ml). After 15 min at 0°C the digestion was stopped by addition of EDTA to 20 mM followed by phenol extraction. The DNA was extracted once with chloroform-isoamyl alcohol, precipitated twice with ethanol, washed with 95% ethanol, vacuum dried, and dissolved in 10 mM NaOH–1 mM EDTA–80% formamide. After being heated to 90°C for 2 min the DNA was electrophoresed in an 8% sequencing gel (7), fixed, dried, and autoradiographed. (A) Autoradiogram of DNase products. T antigen concentration increases from left to right: 0, 0.033, 0.066, 0.165, 0.33, 0.66, 1.65, 3.3, and 6.6 μg/ml. The nucleotide positions shown at the left were taken from a prior experiment in which the same end-labeled fragment was subjected to base-specific cleavage (5). Sites I and II are indicated. (B) T antigen binding curves. Percentage of site bound was calculated from densitometry measurements of the appropriate bands in the autoradiogram shown in (A); for each sample, the binding site values were normalized to the density of bands remote from the binding sites.

radiographic bands representing sites I and II were quantitated and binding curves were generated, each site showed binding to T antigen with similar affinity (Fig. 3B); half-maximal binding occurred at a T-antigen concentration of 4×10^{-9} M. This value is similar to that estimated with standard preparations of T antigen (about 50% pure) isolated by sequential column chromatography (unpublished data). It should be noted that under the conditions of the binding experiments, the relative affinity of T antigen for sites I and II varied with temperature. At 0°C, the affinity for site I exceeded that for site II, whereas at 37°C, the affinities were similar. We also note that the immunochromatographically purified T antigen is active in promoting the replication of SV40 *ori*- containing plasmids in vitro, as recently demonstrated by Li and Kelly (4a). Finally, we tested purified T antigen for DNA nicking and topoisomerase activity on form I SV40 DNA. Neither activity was detectable, indicating that the purified T antigen is not contaminated with endonuclease or topoisomerase, nor are these activities intrinsic to T antigen.

In the experiments described in this report, lytically infected cells were used as the source of T antigen, and the final product was shown to be active in all of the known T-antigen functions. The procedure has also been used to purify active T antigen from SV40-transformed cells as well as from cells infected with various T-antigen mutants. The efficiency and simplicity of the method allow for the purification of T antigens from small numbers of cells and thus will facilitate in vitro analysis of T antigens produced by SV40 mutants.

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