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New Properties of Simian Virus 40 Large T Antigen

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An 8,000-molecular-weight (8K) T antigen was found in all cells transformed by simian virus 40. The 8K T antigen was weakly labeled in vivo with [^{35}S]methionine or $^{32}P_i$. A deletion in the human papovavirus BK genome, in the region coding for the carboxy-terminal end of the large T antigen, reduced the size of the 8K T antigen. The last 80 amino acids of the large T antigen include the sequence Asp-Asp-Asp-Asp unique to the activation peptide of trypsinogen. Large T antigen bound diisopropyl fluorophosphate and was retained by D-phenylalanine coupled to Sepharose beads, an affinity adsorbent that can retain chymotrypsin. The large T antigen and the *recA* protein of *Escherichia coli*, a known protease, have several properties in common as well as several similar sequences. Antibodies against large T antigen interacted with native *recA* protein.

Simian virus 40 (SV40) codes for a multifunctional protein, large T antigen, which is involved in cell transformation and in viral DNA transcription and replication (7, 44). The entire DNA sequence forming the genome of SV40 has been determined (10, 28), and the amino acid sequence of large T antigen has been deduced (10, 27). The large T protein consists of 708 amino acids.

Naturally occuring or laboratory-created deletions within the last 80 amino acids of large T do not affect its ability to induce cell transformation (2, 6, 11, 32, 37, 49), suggesting that this part of large T is dispensable for this function (Fig. 1).

My colleagues and I noted that the sequence Asp-Asp-Asp-Asp present in SV40 large T, 72 amino acids before its carboxy-terminal end, is found in one other protein only: trypsinogen (9) (Fig. 1). This sequence is part of the activation peptide which is lost when trypsinogen is converted into trypsin (8) and plays a fundamental role in the activation process (1).

We searched for an 8,000-molecular-weight (8K) T protein related to the C-terminal end of large T and examined the ability of unpurified large T to interact with different active site inhibitors of proteases.

MATERIALS AND METHODS

Cells, chemicals, and affinity adsorbents. The SV40-transformed rat cell lines used in this study were A37, N31, and A65, induced by mutant tsA209, and 5051 and 5011, induced by wild-type (WT) virus (34). The human papovavirus BK (BKV)-transformed rat cell line was isolated as a dense focus after transfection of rat 3T3 cells with WT BKV. The carcinogen-transformed cell line was isolated as a dense focus after treatment of rat 3T3 cells with benzopyrene. All of the cells were grown at 33°C in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics. Unlabeled diisopropyl fluorophosphate (DFP) and iodoacetamide were purchased from Sigma Chemical Co. ^{3}H-labeled DFP (3 Ci/mmol), [^{35}S]methionine (1,000 Ci/mmol), and $^{32}P_i$ (0.20 Ci/mmol) were from Amersham. The ^{14}C-labeled protein markers were from New England Nuclear and Bethesda Research Laboratories. Plain Sepharose 4B beads and Sepharose beads coupled to protein A or lysine were from Pharmacia Fine Chemicals. Sepharose beads coupled to D-phenylalanine were from Pierce. Sepharose beads coupled to soybean trypsin inhibitor, lima bean trypsin inhibitor, or ovomucoid trypsin inhibitor were from P-L Biochemicals and Worthington Diagnostics. Solution C contained 30 mM Tris-hydrochloride (pH 8.2), 130 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM dextrose. All beads were washed and suspended in solution C (1.5 g/10 ml). Hamster anti-T serum was obtained from the National Cancer Institute through Peter Borchert. Rabbit anti-*recA* serum was a gift from Patrice Moreau, Laboratoire d'Enzymologie du Centre National de La Recherche Scientifique.

In vivo labeling with [^{35}S]methionine and $^{32}P_i$. Cells were washed twice and incubated for 1 h at 33°C in 2 ml of methionine-free or phosphate-free medium. Labeled methionine (100 μCi) or P_i (1 mCi) was then added, and the incubation was continued for 2 additional hours at 33°C. The cells were washed with phosphate-buffered saline and used as described below.

Detection of the 8K T antigen. Cells (2×10^5) in a 25-cm^2 flask were labeled in vivo with ^{35}S or ^{32}P. Extraction and immunoprecipitation were done at 0 to 4°C. The cells attached on the substrate were extracted with 0.5 ml of solution C containing 0.5% Nonidet P-40, DFP (10^{-4} M), iodoacetamide (10 mM), and EDTA (10 mM) or EGTA (10 mM) (see the legend to Fig. 4). A portion of the extract was reacted with anti-T serum

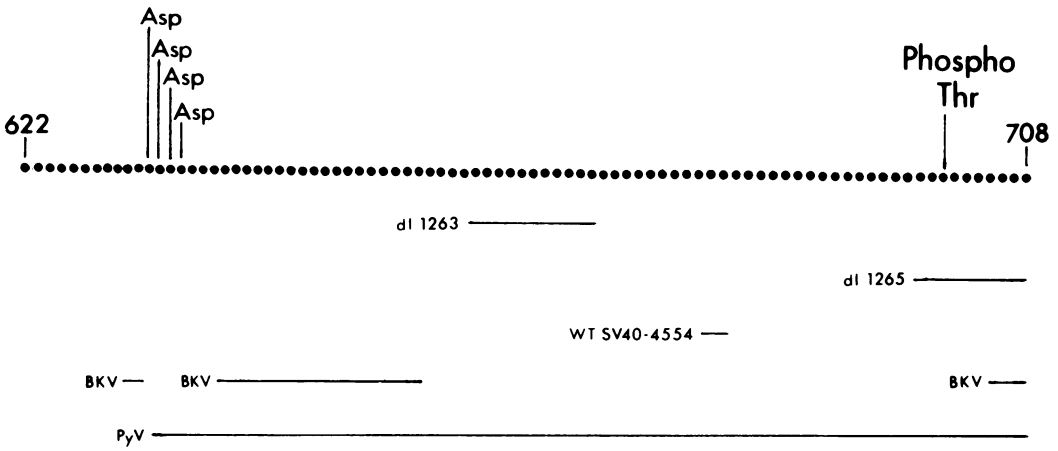

FIG. 1. Deletions in the C-terminal end of large T. The last 87 amino acids of SV40 large T (WT virus strain 776) are schematically represented in the upper part of the panel. The position of the Asp-Asp-Asp-Asp sequence is indicated. *dl*1263 and *dl*1265, SV40 mutants; strain 4554, another WT SV40; PyV, murine polyoma virus. Bars beside the strain designations indicate the amino acids which are deleted in that strain. All of the deletions are in frame.

(10 μl) and Sepharose beads coupled to protein A (50 μl); the mixture, contained in an Eppendorf tube, was rotated for 15 min. Another portion was reacted with nonimmune serum and Sepharose beads coupled to protein A. The beads were collected by brief centrifugation, washed five times with phosphate-buffered saline, and then used to prepare samples for electrophoresis.

Binding of ^{3}H-labeled DFP to unlabeled large T. Transformed 5051 cells (6×10^{7}) in a single 150-cm^{2} flask were collected by vigorous shaking in 30 ml of phosphate-buffered saline. All manipulations were done at 0 to 4°C. After being centrifuged, the cells were extracted in 3 ml of solution C containing 0.5% Nonidet P-40. The nuclei were collected, and the supernatant was supplemented with labeled DFP (10^{-6} M) and kept overnight at 4°C. The supernatant was then reacted with anti-T or nonimmune serum (100 μl) and Sepharose beads bearing protein A (250 μl). The beads were collected and washed extensively. In parallel, 2×10^{5} cells were labeled in vivo with [^{35}S]methionine, extracted, and processed under identical conditions with unlabeled DFP.

Binding of labeled large T to protease affinity adsorbents. Transformed 5051 cells (2×10^{6}) in a 25-cm^{2} flask were labeled in vivo with [^{35}S]methionine. All subsequent manipulations were done at 0 to 4°C. The cells attached on the substrate were swelled for 30 min in 0.9 ml of a hypotonic solution (10 mM Tris-hydrochloride [pH 8.2], 10 mM KCl). They were collected by vigorous shaking and ground in a Dounce homogenizer. The mixture was supplemented with 10× solution C (0.1 ml) and rotated for 30 min. The nuclei were collected by centrifugation, and the supernatant fraction containing large T was reacted with either a protease inhibitor coupled to Sepharose beads (50 μl, rotation for 15 min) or anti-T serum and protein A coupled to Sepharose beads. The beads were washed five times with solution C and used as described below.

In vivo labeling and extraction of *recA* protein. Bacteria (strain GY4753) were grown in 10 ml of enriched M9 medium (23) to a density of 5×10^{8} cells per ml. [^{35}S]methionine was added to a final concentration of lysozyme, and lysed as described by Wickner et al. (47).

Elution of proteins bound to the Sepharose beads, electrophoresis, and autoradiography. The proteins retained by the beads were eluted with 100 μl of Laemmli buffer (17) without heating or with a buffer containing 0.1 M sodium phosphate (pH 7.2), 0.1% sodium dodecyl sulfate, and 6 M urea. They were separated by electrophoresis through 15% polyacrylamide–Tris–sodium dodecyl sulfate gels for 2 h, with Tris-glycine used as the running buffer. Alternatively, the proteins were separated on 15% polyacrylamide gels containing sodium phosphate, sodium dodecyl sulfate, and urea. Electrophoresis was for 6 h at 90 V, with sodium phosphate used as the running buffer. This procedure allows better separation of proteins with molecular weights less than 10K and is a modification of the method of Shapiro et al. (35) described by Bethesda Research Laboratories. The ^{3}H- or ^{35}S-labeled proteins in the gels were detected by fluorography at −70°C. The ^{32}P-labeled proteins were detected by autoradiography at −70°C with intensifying screens.

RESULTS

SV40 transformants all contained an 8K T antigen. Since the peptide extending from Asp 633 to Thr 708 (the last amino acid of large T) contains one methionine (Met 654), it seemed possible to look for an 8K protein related to T antigen by the usual immunoprecipitation procedure, in which proteins are labeled in vivo with [^{35}S]methionine. SV40-transformed cells were labeled with methionine and extracted at pH 8

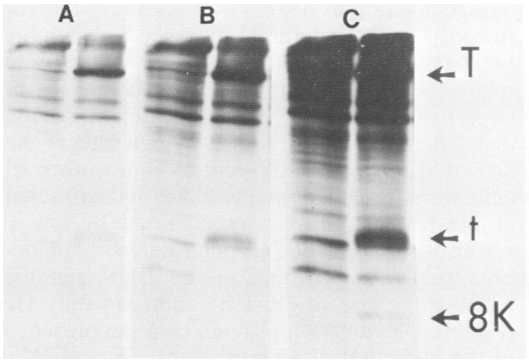

FIG. 2. Detection of the 8K T antigen. SV40-transformed rat cells (line A37) were labeled in vivo with [^{35}S]methionine and extracted as indicated in the text. The proteins were precipitated with nonimmune or anti-T serum. Each pair of tracks was exposed for (A) 1, (B) 3, or (C) 10 days. Left track, nonimmune serum; right track, anti-T serum. The positions of large T, small t, and 8K T antigens are indicated to the right.

with a variety of protease inhibitors in the extraction buffer (see above). DFP, iodoacetamide, and EDTA were used to inactivate serine, thiol, and metalloproteases active at pH 8 and to lessen the likelihood of protein degradation during extraction and immunoprecipitation. The precipitated proteins were separated by electrophoresis. The gels were used to expose X-ray films for 1 to 10 days. An 8K T protein was found (Fig. 2). The 8K protein, however, was not clearly detected unless the films were exposed for at least 10 days. On the other hand, large T and small t antigens were clearly detectable after 1 to 3 days. The size of the 8K protein was estimated by running the sample side by side with protein markers of the following molecular weights: 43K, 25K, 18K, 12K, 6K, and 3K. The 8K T protein was detected in four other SV40 transformants (Fig. 3). The 8K T antigen was labeled with eight times less [^{35}S]methionine than was the small t antigen. This was determined by cutting the gel slices containing the 8K T or small t antigen, eluting the proteins, and determining the radioactivity in a scintillation counter. The anti-T serum used contained an excess of antibodies against 8K T; a single immunoprecipitation was capable of removing all 8K T from the extract. The 8K protein was not present in normal rat 3T3 cells (Fig. 4).

Weak phosphorylation of 8K T antigen. Large T can be phosphorylated in vivo at several sites, including one threonine, located in the C-terminal end of large T at position 701, which can only be weakly phosphorylated (31) (Fig. 1). We therefore examined whether the 8K T antigen

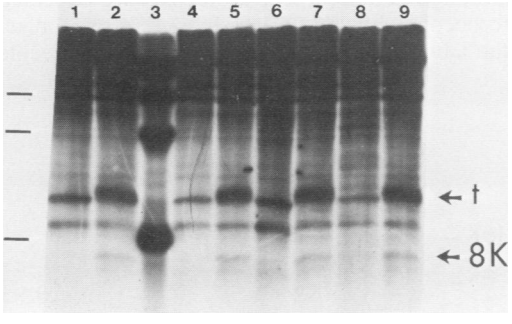

FIG. 3. Detection of 8K T in all cell lines transformed by SV40. Four other SV40 transformants (rat cell lines N31, A65, 5051, and 5011) were examined for the presence of 8K T as described in the legend to Fig. 2. Protein markers (46K, 30K, and 12K) are indicated to the left. Tracks: 1, A65 nonimmune serum; 2, A65 anti-T serum; 3, ^{14}C-labeled markers; 4, N31 nonimmune serum; 5, N31 anti-T serum; 6, 5051 nonimmune serum; 7, 5051 anti-T serum; 8, 5011 nonimmune serum; and 9, 5011 anti-T serum.

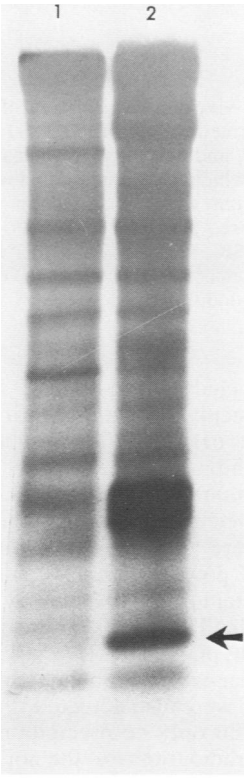

FIG. 4. The 8K protein is not present in normal rat 3T3 cells. ^{35}S-labeled extracts from normal (tracks 1) or SV40-transformed (track 2) cells were reacted with anti-T serum and protein A coupled to Sepharose beads. During extraction and immunoprecipitation, EGTA (10 mM) was added instead of EDTA.

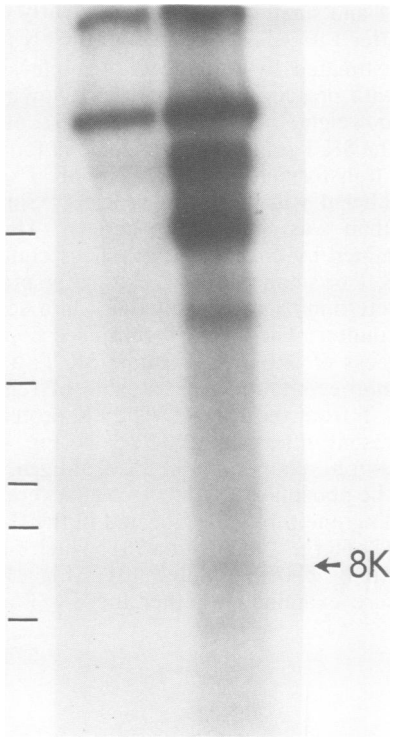

FIG. 5. In vivo phosphorylation of 8K T antigen. SV40-transformed rat cells (line 5011) were labeled in vivo with $^{32}P_i$ and extracted as indicated in the text. The proteins which were precipitated with nonimmune or anti-T serum were separated on 15% polyacrylamide–6 M urea gels. The positions of the markers (43K, 25K, 18K, 12K–14K, and 6K) are indicated to the left. The markers 12K and 14K do not separate under these conditions.

could be labeled in vivo with $^{32}P_i$. Transformed cells were incubated with $^{32}P_i$ for 2 h and then extracted at pH 8 in the presence of various protease inhibitors. The extract was reacted with nonimmune or anti-T serum and Sepharose beads coupled to protein A. The precipitated proteins were separated by electrophoresis through 15% polyacrylamide–6 M urea gels. The phosphoproteins were detected by autoradiography for 6 h. The 8K T antigen was weakly labeled with phosphate (Fig. 5). Three other low-molecular-weight phosphoproteins, 16K, 6K, and 4K, were also detected (Fig. 6). All four proteins could only be precipitated with anti-T serum; autoradiography of the nonimmune track for 60 h did not reveal any of them.

BKV 8K T antigen is truncated. The large T coded by BKV is structurally and functionally very similar to SV40 large T and has a C-terminal end 15 amino acids shorter than its SV40 counterpart (32, 49) (Fig. 1). This is due to a large deletion in BKV which is responsible for the only major structural difference between the two versions of large T (32, 49). In an attempt to relate the 8K T antigen to the C-terminal end of large T, we examined whether the deletion in the distal part of the BKV early region affects the size of 8K T. Both BKV- and SV40-transformed cells were labeled in vivo with $^{32}P_i$ and extracted at pH 8. The proteins precipitated by anti-T serum were separated through polyacrylamide-urea gels. The deletion in the BKV genome reduced the size of 8K T by approximately 1K (Fig. 6). The deletion also affected the presence of the two other phosphoproteins, 6K and 4K.

DFP bound to SV40 large T. DFP binds covalently to the active site of serine proteases, and inhibition of a protease by DFP is considered diagnosis for it to be classified as a serine protease (16). We examined the ability of DFP to bind in vitro to large T. SV40-transformed cells were extracted at pH 8 without prior labeling or use of protease inhibitors. The extract was then supplemented with ^{3}H-labeled DFP and kept overnight at 4°C. The proteins immunoprecipitated with anti-T or nonimmune serum were separated by electrophoresis and detected by fluorography. Parallel cultures were labeled with [^{35}S]methionine in vivo and processed under identical conditions with unlabeled DFP. DFP bound to large T (Fig. 7). Large T labeled with [^{35}S]methionine incorporated in vivo was detected after a 1-day exposure period. On the other hand, large T, initially unlabeled, which bound ^{3}H-labeled DFP in vitro was detected after 6 months. This length of time was expected for the following reasons. Large T contains 21 methionine residues and was labeled in vivo after a period of starvation in methionine-free medium, although not all large T molecules are expected to become labeled during the period of a pulse; the labeled methionine used had a high specific

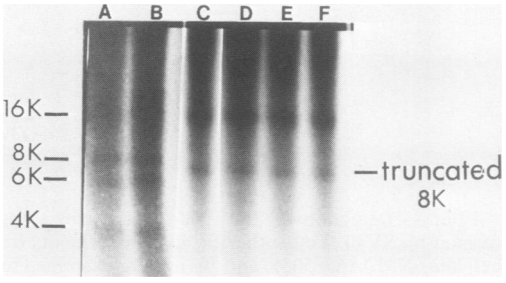

FIG. 6. Effect of a deletion in the BKV genome on size of 8K T antigen. SV40 or BKV-transformed cells were labeled in vivo with $^{32}P_i$. The proteins immunoprecipitated by anti-T serum were separated as indicated in the legend to Fig. 5. Tracks: A and B, SV40-transformed cells; C–F, BKV-transformed cells.

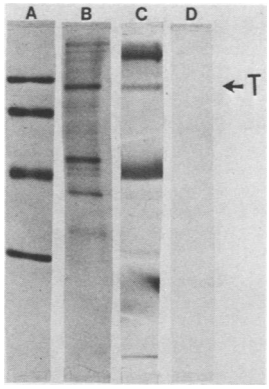

FIG. 7. Binding of ^{3}H-labeled DFP to unlabeled large T. SV40-transformed cells (line 5051) were extracted without labeling as indicated in the text. The extract was supplemented with [^{3}H]DFP and left overnight at 4°C before the proteins were precipitated with anti-T or nonimmune serum. In parallel, cells were labeled in vivo with [^{35}S]methionine and processed under identical conditions. The panel shows four tracks of the same gel: A, ^{14}C-labeled markers (92K, 68K, 46K, and 30K); B, large T labeled with [^{35}S]methionine incorporated in vivo, gel exposed for 1 day; C, large T labeled with [^{3}H]DFP captured in vitro, gel exposed for 180 days; D, extract supplemented with [^{3}H]DFP and precipitated with nonimmune serum, gel exposed for 180 days.

activity (1,000 Ci/mmol). On the other hand, one molecule of protease binds only one molecule of DFP; the labeled DFP had a specific activity of 3 Ci/mmol, and ^{3}H has approximately 10 times less energy than ^{35}S, although this difference is reduced in fluorography. Finally, only part of the large T molecules were expected to interact with DFP. To compensate for the enormous difference between the two labels, unlabeled large T to be interacted with ^{3}H-labeled DFP was 300 times more abundant than was ^{35}S-labeled large T (see above). Two additional proteins, 130K and 48K, were also labeled with DFP and precipitated by anti-T serum. Those proteins may be related to the ones previously described (4, 19, 22). If ^{3}H labeling was caused by a slow but nonspecific binding of DFP, the pattern of ^{3}H-labeled proteins would have followed the pattern of ^{35}S-labeled proteins. The length of the experiment has prevented so far the determination of the kinetics of the large T-DFP interaction.

Binding of large T to a proteolytic enzyme affinity adsorbent. Protease inhibitors coupled to Sepharose beads are used to partially purify proteases present in highly heterogeneous protein mixtures. We examined the ability of protease affinity adsorbents to capture large T present in crude cellular extracts. Large T synthesized in SV40-transformed cells was labeled in vivo with [^{35}S]methionine and extracted at pH 8 without using detergent or protease inhibitors. A portion of the extract was reacted with D-phenylalanine coupled to Sepharose beads; at pH 8, this adsorbent attracts a variety of serine and metalloproteases. Another portion was reacted with anti-T serum and protein A coupled to Sepharose beads (see above). The proteins bound to the beads were separated by electrophoresis. The protease affinity adsorbent efficiently captured large T from the crude extract (Fig. 8). To further identify the protein captured by the protease affinity adsorbent as large T, extracts from carcinogen- or SV40-transformed cells were reacted in parallel with D-phenylalanine coupled to Sepharose beads (Fig. 9). Large T did not bind to plain Sepharose beads or to Sepharose beads bearing any of the following: lysine, soybean trypsin inhibitor, lima bean trypsin inhibitor, or ovomucoid trypsin inhibitor (Fig. 10). Since D-phenylalanine binds reversibly to large T and the binding to DFP is irreversible,

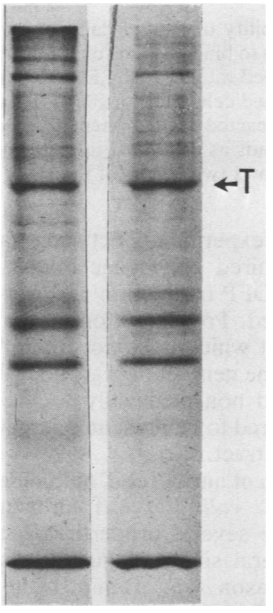

FIG. 8. Binding of labeled large T to a protease affinity adsorbent. SV40-transformed cells (line 5051) were labeled in vivo with [^{35}S]methionine. They were extracted without detergent as indicated in the text. A portion of the extract was reacted with D-phenylalanine coupled to Sepharose beads; another portion was interacted with anti-T serum and protein A coupled to Sepharose beads. The beads were washed extensively before the bound proteins were eluted and separated by electrophoresis. Left track, Large T captured by the protease affinity adsorbent; right track, large T immunoprecipitated.

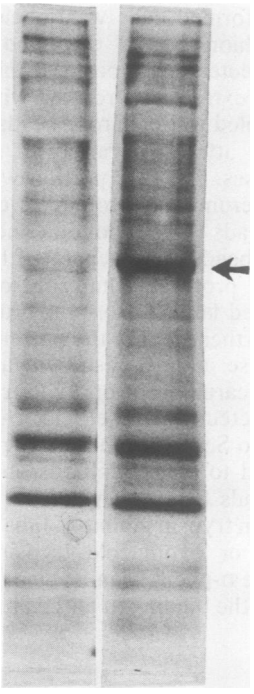

FIG. 9. Ability of D-phenylalanine coupled to Sepharose beads to bind proteins extracted from carcinogen-transformed rat 3T3 cells. Extracts from carcinogen-transformed cells (left) or SV40-transformed cells (right) were reacted with D-phenylalanine coupled to Sepharose beads as described in the legend to Fig. 8. The figure shows two tracks of the same gel.

competition experiments between those two inhibitors required knowledge of the kinetics of the large T-DFP interaction and were therefore not attempted. Preadsorption of the extract on an adsorbent which does not retain large T did not reduce the general background; the proteins which bound nonspecifically to the Sepharose beads appeared to be present in large amounts in the crude extract.

Interaction of anti-large T antibodies with *recA* protein of *E. coli*. Large T antigen and *recA* protein have several properties in common as well as several similar sequences (see below). For this reason we examined the ability of antibodies directed against large T to interact with native *recA* protein. A polyclonal anti-T serum contained some antibodies that bound to *recA* protein (Fig. 11).

DISCUSSION

This paper reports that SV40 large T antigen was able to bind DFP, a general inhibitor of serine proteases, and could be specifically retained by Sepharose beads bearing D-phenylalanine but not other protease inhibitors. The paper

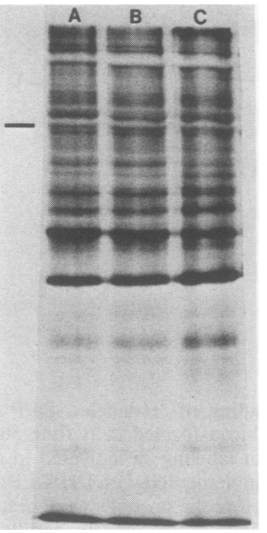

FIG. 10. Ability of other protease inhibitors to bind to large T. Extracts from SV40-transformed cells (line 5051) containing large T labeled in vivo with [^{35}S]methionine were reacted as described in the legend to Fig. 8 with plain Sepharose beads (A), Sepharose beads bearing lysine (B), or Sepharose beads bearing soybean trypsin inhibitor (C).

also reports the existence, in all cells transformed by SV40, of an 8K T antigen, related to the C-terminal end of large T, which contains a sequence unique to the activation peptide of trypsinogen. These observations suggest a possible functional relationship between large T antigen and the serine proteases.

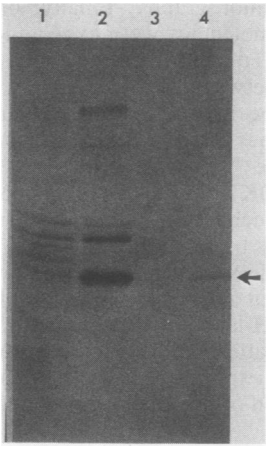

FIG. 11. Immunoprecipitation of native *recA* protein with anti-large T antibodies. *recA* protein was labeled in vivo with [^{35}S]methionine. Samples of a crude bacterial extract containing *recA* were reacted with protein A coupled to Sepharose beads and one of the following sera: 1, rabbit nonimmune; 2, rabbit anti-*recA*; 3, hamster nonimmune; or 4, hamster anti-T.

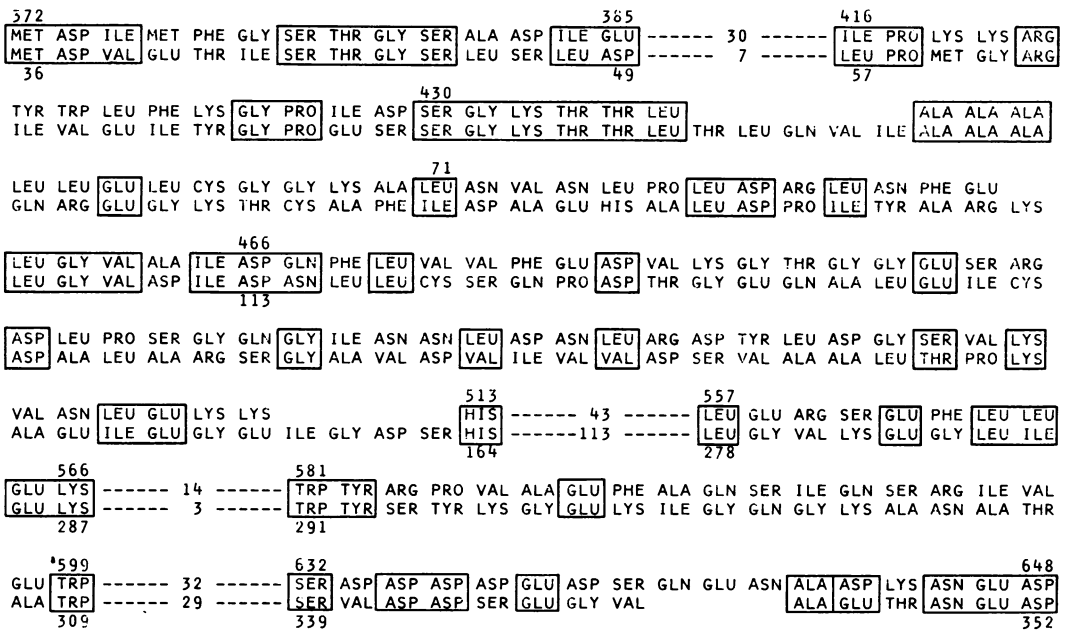

FIG. 12. Amino acid sequence 372 to 648 of SV40 large T (top line) compared with the sequence 36 to 352 of *recA* protein (bottom line). Serine 430, aspartic 466, and histidine 513 of SV40 large T, or serine 579, aspartic 615, and histidine 662 of polyoma virus large T, are equivalent to serine 71, aspartic 113, and histidine 164 of the *recA* protein. Histidine 513 is the only histidine conserved in the C-terminal half of large T and the only histidine in the *recA* protein after aspartic 113.

The data do not exclude the possibility that the protein capable of interacting with DFP and D-phenylalanine is a protein associated with large T and of the same molecular weight. Moreover, since DFP can also interact with certain esterases and phosphatases, it is possible that large T is an esterase or a phosphatase. The following aspects, however, appear to favor the protease-large T relationship.

In all trypsin-like serine proteases, the active serine occurs in the tripeptide Asp-Ser-Gly (9). SV40 large T contains 46 serine residues. Only eight serines can be found conserved when SV40 large T is compared to its two other versions coded by polyoma virus and BKV (10, 11, 27, 32, 37, 49). The conserved serines are at positions 147, 152, 207, 219, 430, 504, 571, and 608, and they occur in the following tripeptides: Leu-Ser-His, Phe-Ser-Asn, Val-Ser-Ala, Phe-Ser-Phe, Asp-Ser-Gly, Gly-Ser-Val, Gln-Ser-Gly, and Phe-Ser-Leu. The tripeptide in which serine 430 occurs is identical to the tripeptide in which the active serine of trypsin-like proteases is present. Serine 430 is the only serine residue conserved between positions 273 and 446, where 12 out of 13 temperature-sensitive mutants have their mutation site (18, 33). It is actually the only serine conserved in a region of 284 amino acids.

The large T antigen (708 amino acids) and *recA* protein (353 amino acids) of *E. coli* (14, 30) have a number of properties in common. Besides having a protease activity (29), *recA* protein, like large T, induces mutations (26, 41, 48); initiates DNA synthesis when conditions are suboptimal for cell growth (20, 44), has a role in the excision of integrated viral DNAs (3, 5), regulates the expression of its own gene (21, 44), activates and regulates the expression of other genes (15, 38, 44), interacts with double- and single-stranded DNA (36, 39, 44, 45), has an ATPase activity (7, 12, 25, 29) and a DNA unwinding activity (13, 46), and can be present as a monomer and a tetramer (7, 25). Moreover, large T and *recA* protein have several similar sequences, the most notable being approximately 100 amino acids long (Fig. 12). The stretches of maximum homology may be required for the protease or ATPase activities of both proteins.

We have avoided so far a direct demonstration that SV40 large T has a proteolytic activity for the following reasons. (i) Large T has been purified by using phenylmethylsulfonyl fluoride, tosyl lysine chloromethyl ketone, and tosyl phenyl chloromethyl ketone as supplements in all buffers throughout the purification process (39, 40, 42, 43). They are all inhibitors of serine proteases, which bind covalently to the active site, added to prevent total degradation of large T during purification. Large T purified in the

presence of phenylmethylsulfonyl fluoride cannot be used directly to demonstrate a serine protease activity. (ii) A direct demonstration that large T has a protease activity requires the isolation or determination of its potential substrates. Such substrates may have no precedents; trypsinogen, for example, is the only known substrate for enteropeptidase, and protease *recA* degrades only specific proteins, e.g., the λ repressor or *lexA* protein. (iii) It is still not possible to isolate pure large T antigen; activities due to closely associated contaminants have been reported (7). We are presently attempting to overcome these difficulties.

ADDENDUM IN PROOF

We recently found that the *recA* protein of *E. coli* is also able to bind DFP.

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