Properties of Simian Virus 40 Small t Antigen Overproduced in Bacteria

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We constructed a series of bacterial plasmids which contained the *Escherichia coli lac* promoter fused to a simian virus 40 restriction fragment coding for small t antigen. These plasmids expressed different levels of intact viral protein depending on the length of the constructed ribosome binding site. Small t antigen synthesized by the most efficient producer, HP1, constituted 0.5 to 1% of the total cellular protein. On the basis of extensive characterization by immunoprecipitation, gel electrophoresis, isoelectric focusing, tryptic fingerprint analysis, and chromatographic properties, this plasmid-encoded protein was virtually identical to authentic simian virus 40 small t antigen. Partial purification of the HP1-encoded and authentic small t antigens revealed the presence of both monomeric and multimeric forms.

Two proteins are expressed by simian virus 40 (SV40) early after infection of either permissive monkey cells or nonpermissive rodent cells. One of these polypeptides, large tumor (T) antigen, is a 90,000- to 100,000-dalton nuclear protein encoded by the viral A gene (56). The SV40 large T antigen has been extensively characterized and shown to be involved in the initiation of viral DNA replication (52), regulation of viral transcription (10, 39), and induction of neoplastic transformation (49, 53). In contrast, small tumor (t) antigen, a 15,000- to 20,000-dalton cytoplasmic protein encoded by the SV40 F gene, was only recently detected and plays an as yet undefined role in the viral life cycle (11, 37, 45).

S1 mapping (2) and sequence analysis (38) of the SV40 early mRNA's revealed two transcripts which could be distinguished by the size and location of their intervening sequences. Although these mRNA's share their initial 81 codons, the coding sequence for the carboxy terminus of small t antigen is spliced out from the primary transcript for large T antigen (38). In agreement with these observations, both tumor antigens share some tryptic peptides (28, 46) as well as their N-terminal amino acid sequence (36). Furthermore, deletion mutants lacking sequences between 0.54 and 0.59 map units on the conventional SV40 genomic map produce a truncated small t polypeptide, but retain the ability to synthesize a wild-type large T antigen (11, 15, 45).

These F gene deletion mutants have been used to study the role of small t antigen in productive and nonproductive infections, but the results were variable and, in certain cases,

difficult to interpret. Some of the F gene deletion mutants appear to be partially defective for lytic growth on monkey cells (57). In addition, small t antigen seems to be required for the induction of a fully transformed phenotype (5, 15, 45, 48). This function may, however, be dispensable in actively growing cells (30, 43). Finally, the small t protein may provide some functions which are required for full viral oncogenicity, since F gene mutant viruses injected into neonatal hamsters induce tumors which grow more slowly and have a longer latency than those induced by wild-type SV40 (27; W. Topp, personal communication). On the basis of these observations, it is likely that small t antigen plays some role, albeit undefined, in the viral induction of both transformation and oncogenicity.

In support of the idea that the small t polypeptide may be responsible for certain morphological changes associated with the transformed phenotype, it was shown that microinjection of SV40 DNA coding for small t antigen (0.735 to 0.375 map units) induced dissolution of the cytoplasmic actin cable network in rat fibroblasts (19). Injection of DNA from an F gene deletion mutant, however, had no effect on the cytoskeletal structure. Although these results suggest that SV40 small t antigen is responsible for cytoskeletal changes, no firm conclusions can be made until similar experiments are performed with purified small t protein.

Our understanding of the biological function of SV40 small t antigen has been impeded by the difficulties involved in purifying the protein from conventional sources. To overcome this problem, we chose to construct a recombinant plasmid which would produce this eucaryotic

protein in bacterial cells. Three features of SV40 small t antigen facilitate its synthesis in *Escherichia coli*. First, unlike most eucaryotic genes, the small t structural gene is not interrupted by intervening sequences (2, 38). Second, the entire coding sequence is present on a *Hind*III restriction fragment which conveniently contains the small t initiation codon 12 base pairs (bp) from one of the ends. Finally, the only known post-translational modification of the protein is acetylation of the N-terminal methionine (36). Construction of a plasmid able to promote transcription and translation of the SV40 small t gene should therefore allow synthesis in bacterial cells of a protein which is virtually identical to authentic small t antigen.

Several bacterial plasmids have been designed to direct efficient expression of eucaryotic genes as fusion proteins (16, 29, 32). Backman and Ptashne (1) have developed a plasmid containing the *lac* regulatory sequences which allows the production of intact gene products by constructing a hybrid ribosome binding site consisting of the Shine and Dalgarno sequence from β -galactosidase (44) fused to a foreign initiation codon. This system has been used to produce phage lambda repressor and *cro* protein to levels as high as 2.5% of the total bacterial protein (1, 42) and also to overproduce human growth hormone (18). Roberts and his co-workers have used this system to achieve synthesis of SV40 small t antigen in bacteria (41). However, they detected only a low level of expression of this eucaryotic gene product. In this paper, we report the quantitation, isolation, and characterization of SV40 small t antigen overproduced by *E. coli* carrying a recombinant plasmid.

MATERIALS AND METHODS

Cells and viruses. *E. coli* strain 294 (endo I, r_k^- , m_k^+ , B1⁻) was a gift from H. Echols. *E. coli* 294 with the *lac* i^+ gene was constructed by conjugation with a strain of *E. coli* which carries this gene on an F factor, a generous gift from F. Heffron. Bacteria with drug resistance were grown in the presence of 30 μ g of ampicillin (Sigma Chemical Co.) per ml or 20 μ g of kanamycin (Sigma) per ml.

Plaque-purified SV40 strain 776 was propagated on CV-1 cells as described previously (55). CV-1 monkey cells were maintained on plastic dishes with Dulbecco-modified Eagle medium supplemented with an antibiotic-antimycotic mixture (GIBCO Laboratories) and 5% fetal calf serum (GIBCO).

Purification of plasmid DNA. Plasmid DNA was purified by a modification of the technique described by Katz et al. (25). Bacteria were grown to an optical density at 550 nm of 0.5 in M9 medium (33) supplemented with Casamino Acids, vitamin B1, and 0.05% glucose and were treated overnight with 20 μ g of chloramphenicol (Sigma) per ml to amplify the plasmid DNA. The cells were suspended in lysis buffer (50

mM Tris [pH 8.0], 25% sucrose, 1 mM EDTA) supplemented with 4 mg of solid lysozyme per ml. After 20 min at 0°C, EDTA was added to 0.15 M final concentration, and solid pronase (Calbiochem) was added to approximately 1 mg/ml. Incubation of the spheroplasts was continued at 0°C for 10 min, followed by the addition of 0.1 volume of TET buffer (50 mM Tris [pH 8.0], 60 mM EDTA, 0.3% Triton X-100) to lyse the cells. After 15 min at 0°C, the lysate was centrifuged at 20,000 rpm for 30 min to pellet the chromosomal DNA. The lysate supernatant was then phenol extracted three times, ether extracted three times, and ethanol precipitated. The plasmid supercoils were purified by banding to equilibrium in a cesium chloride-ethidium bromide gradient (8) followed by isoamyl alcohol extraction and extensive dialysis against TE buffer (10 mM Tris [pH 7.8], 1 mM EDTA).

This procedure was scaled down for small plasmid preparations ("minilysates") from 5- to 10-ml cultures. An RNase digestion (Worthington Biochemical Corp.) was included at the pronase digestion step. In place of the cesium chloride-ethidium bromide gradient, the DNA was purified by exclusion from a 2-ml Sepharose CL-2B column equilibrated in TE with 0.1 M NaCl. This DNA was concentrated by ethanol precipitation and used for restriction enzyme analysis.

Construction of plasmids. Restriction enzymes were purchased from Bethesda Research Laboratories (BRL) or New England Biolabs. All digestions were performed in the buffers recommended by BRL at 37°C for 0.5 to 10 h. Ligations were performed with T4 DNA ligase (BRL) for 12 h at 20°C in ligase buffer (66 mM Tris [pH 7.6], 10 mM MgCl₂, 1 mM spermidine, 15 mM dithiothreitol [DTT], 200 μ g of bovine serum albumin [BSA, Miles-Pentex] per ml, 1 mM ATP). Horizontal gel electrophoresis was performed by using 0.7 to 1.4% agarose (Sigma type II) in TBE buffer (45 mM Tris base, 45 mM boric acid, 1.25 mM EDTA) or Loen buffer (36 mM Tris base, 30 mM NaH₂PO₄, 1 mM EDTA). The DNA was visualized by staining with ethidium bromide and UV transillumination.

Plasmid pGL101 DNA was linearized by digestion with *Pvu*II; the enzyme was inactivated by heating at 65°C for 5 min, and the digest was stored at -20°C. SV40 DNA, prepared as described previously (23), was digested with *Hind*III and *Hpa*II, and fractionated by 1% agarose gel electrophoresis in TBE buffer. The 1,169-bp B fragment was excised from the gel, and the DNA was eluted by electrophoresis into TBE buffer and purified and concentrated by DE52-cellulose chromatography and ethanol precipitation. The DNA was resuspended in TE buffer and stored at 5°C for subsequent insertion into pGL101.

(i) **pSV250.** Gel-purified SV40 *Hind*III B fragment was treated with large fragment DNA polymerase I (BRL) at 37°C in the presence of 10 μ Ci of [α -³²P]-dTTP (Amersham), all four dNTP, and nick translation buffer (50 mM Tris [pH 7.8], 10 mM MgCl₂, 1 mM DTT, 100 μ g of BSA per ml). The reaction kinetics were followed by trichloroacetic acid precipitation. By 30 min DNA synthesis had almost plateaued, and the reaction was stopped at 60 min by phenol extraction, three ether extractions, and ethanol precipitation. A 10-fold molar excess of this blunt-ended SV40 DNA

was ligated with T4 DNA ligase to 0.2 μ g of *Pvu*II-digested pGL101 DNA in a 15- μ l reaction volume. The ligation products were then digested with an excess of *Pvu*II to linearize recircularized pGL101 DNA, and this mix was used to transform *E. coli* 294 cells by standard procedures (9). The bacteria were spread on sterile nitrocellulose filters (Schleicher and Schuell) on L agar plates containing ampicillin to select for transformants. The resultant colonies were transferred to a fresh nitrocellulose filter, and these duplicate colonies were grown, treated with chloramphenicol, lysed, and probed with nick-translated SV40 DNA (40) as described by D. Hanahan (personal communication). Clones which contained SV40 sequences were identified by autoradiography and picked, and small cultures were grown for purification of plasmid DNA as described above. Plasmids containing the SV40 *Hind*III B fragment inserted into pGL101 in the proper orientation were identified by digestion with *Hae*III, Southern blotting (47), and hybridization with nick-translated SV40 DNA. One clone containing a plasmid with the SV40 small t gene fused to the *lac* promoter was selected, and the plasmid was designated pSV250.

(ii) pSV240. Synthetic *Hind*III linkers (Collaborative Research) were labeled with [γ - 32 P]ATP (Amersham) and T4 polynucleotide kinase (New England Biolabs). Phosphorylation was then completed by the addition of an excess of cold ATP (1 mM final concentration), and the reaction was terminated by phenol extraction, three ether extractions, and ethanol precipitation. A 30-fold excess of these phosphorylated linkers was then ligated to *Pvu*II-linearized pGL101 DNA with T4 DNA ligase. Protein was removed by phenol extraction, and the pGL101 DNA was separated from unincorporated linkers by Sepharose CL-2B chromatography. The excluded fractions were pooled, ethanol precipitated, and digested with *Hind*III to remove excess linker sequences. After phenol extraction, ether extraction, and ethanol precipitation, 0.3 μ g of this modified pGL101 DNA was ligated to the gel-purified SV40 *Hind*III B fragment, and *E. coli* 294 cells were transformed with the recombinant molecules, as above. A clone containing plasmid DNA with the SV40 insert in the proper orientation, as determined by restriction mapping with *Taq*I and Southern blot hybridization, was selected, and the plasmid was designated pSV240.

(iii) HP1. pSV240 DNA was digested partially with *Hind*III to form predominantly linear molecules. BAL 31 nuclease (BRL) was then titrated to remove only a few bp from the ends of a DNA duplex by digesting the pSV240 partial digests in the presence of tracer end-labeled DNA for 30 min at 30°C in BAL 31 buffer (12 mM CaCl₂, 12 mM MgCl₂, 0.6 M NaCl, 20 mM Tris [pH 8.1]). The amount of BAL 31 nuclease which was required to remove 95% of the end label, but not reduce the size of the pSV240 DNA as detected by agarose gel electrophoresis, was selected for use on a preparative scale. BAL 31 nuclease treatment of the pSV240 partial digest was terminated by the addition of EDTA to 40 mM final concentration, and linear molecules were purified by 0.7% agarose gel electrophoresis in Loen buffer, excision of the linear DNA, electroelution, and DE52 chromatography. These molecules were recircularized with T4 DNA ligase and

used to transform *E. coli* 294. Ampicillin-resistant colonies were picked randomly, and the plasmid DNA was digested with *Hind*III and *Pst*I to identify plasmids missing the *Hind*III site between the *lac* promoter and SV40 DNA. Those clones were then tested for small t antigen expression as described below.

All manipulations were in accordance with the guidelines of the National Institutes of Health and used P2-EK1 containment.

Labeling of proteins. Bacterial clones were grown in 5 ml of M9 medium supplemented with amino acids (minus methionine), thiamine, 0.5% glycerol, and ampicillin. At an optical density at 550 nm of 0.4, 50 to 200 μ Ci of [35 S]methionine (Amersham) was added, and the culture was left at 37°C for 2 min for pulse-labeling or 1 h for steady-state labeling. The tube was then chilled to stop the pulse, and the cells were immediately pelleted by centrifugation. The bacteria were suspended in 0.5 ml of TE, 0.1 M NaCl, 0.1 mM DTT, and 50 μ g of phenylmethylsulfonyl fluoride (Sigma) per ml and sonicated, and the lysate was centrifuged to pellet the cellular debris. Induction with isopropyl- β -D-thiogalactopyranoside was accomplished by the addition of this inducer to 1 mM final concentration in the growth medium one generation before harvesting the cells.

CV-1 monkey cells were infected with 10 PFU of SV40 per cell and left at 37°C until approximately 10% of the cells showed cytopathic effects. The cells were then washed twice with phosphate-buffered saline and incubated with DME medium minus methionine for 1.5 to 2 h at 37°C. From 0.1 to 1 mCi of [35 S]methionine was then added, and the plates were left for 2 h for pulse-labeling or 12 h for steady-state labeling. The cells were then washed twice with phosphate-buffered saline and lysed by the addition of 1 ml of buffer B (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40), and the cellular debris was pelleted by centrifugation. Extracts were stored at -80°C if not used immediately.

Immunoprecipitation and gel electrophoresis. Cell extract was diluted into buffer B, and an appropriate amount of antibody was added, usually 5 to 10 μ l. After 2 to 4 h at 0°C, a 10% suspension of Formalin-fixed *Staphylococcus aureus* Cowan I (ATCC 12598) (26) in buffer B was added, using two volumes of 10% *S. aureus* per volume of antibody. After 1 to 2 h at 0°C, the *S. aureus* cells were pelleted by centrifugation, washed three times with buffer B, and suspended in sample buffer (0.5 M Tris [pH 6.8], 5% β -mercaptoethanol, 3% sodium dodecyl sulfate [SDS], 10% glycerol, 0.05% bromophenol blue). The resuspended *S. aureus* cells were boiled for 2 min and pelleted by centrifugation, and the supernatant sample buffer was loaded onto an SDS-polyacrylamide gel with a 7 to 18% gradient of acrylamide (51).

Purification of radioactive small t antigen. A 10-ml culture of HP1 cells was pulsed for 5 min with 500 μ Ci of [35 S]methionine as described above. This crude extract was then subjected to immunoprecipitation with 200 μ l of hamster anti-T immunoglobulin G, and the precipitated proteins were fractionated by preparative SDS-polyacrylamide gel electrophoresis as described above. The band of small t antigen, identified by autoradiography, was excised, and the protein was eluted by electrophoresis directly into a

dialysis bag containing 0.2 mg of BSA. The SDS was removed by dialysis against increasing concentrations of urea, up to 8 M, followed by decreasing concentrations of urea in the presence of 1% BSA. After final dialysis against 1% BSA, precipitated material was pelleted by centrifugation, and the purified small t antigen (ca. 10^7 cpm) was used for tryptic fingerprint analysis or radioimmunoassay.

Radioimmunoassay. The sample to be assayed (up to 20 μ l) was added to 100 μ l of radioimmunoassay buffer (phosphate-buffered saline, 0.1% Nonidet P-40, 0.1% BSA) containing 0.03 to 0.05 μ l of hamster anti-T antibody. After 20 min at 0°C, 1,000 cpm (2 to 10 ng) of purified radioactive small t antigen was added, and the immune complex formation was continued for another 20 min. The complex was collected with 2 μ l of a 10% suspension of *S. aureus* Cowan I cells by incubation at 0°C for 10 min. The cells were pelleted by centrifugation and resuspended in 10 μ l of water, and radioactivity was quantitated by scintillation counting. The amount of antibody used in the assay was determined by titration as the amount required to precipitate 50 to 70% of the pure radioactive small t antigen.

To assay extract from SV40-infected monkey cells, large T antigen was first removed by precipitation with 5 μ l of anti-D2 serum. The supernatant from that immunoprecipitation was then subjected to the standard radioimmunoassay described above.

Column chromatography of small t antigen.

(i) **HP1.** For preparative purposes, HP1 cells were grown to late log phase in a rich medium (0.7% Casamino Acids, 0.7% tryptone [Difco Laboratories], 0.7% yeast extract, 0.7% brain heart infusion, 20 mM K_2HPO_4 [pH 7.2], 0.5% glycerol, 20 μ g of ampicillin per ml). The cells were lysed by sonication in 10 mM Tris (pH 7.8)–1 mM EDTA–0.2 M NaCl–0.1 mM DTT–50 μ g of phenylmethylsulfonyl fluoride per ml, and the lysate was centrifuged at 100,000 $\times g$ for 1 h. Nucleic acids were removed from the S-100 by the addition of fresh 1% protamine sulfate (Sigma) to 0.2% final concentration and removal of the precipitate by centrifugation. The supernatant was then brought to 60% saturation with ammonium sulfate (Schwarz-Mann), and the precipitate was collected by centrifugation, resuspended in the appropriate buffer, and loaded onto a gel filtration column. The columns were equilibrated in either 20 mM Tris (pH 7.8)–1 M LiCl–0.1 mM DTT or 20 mM Tris (pH 7.4)–50 mM NaCl–0.1 mM EDTA–0.1 mM DTT. For a large-scale preparation (15 g of HP1 cells), 20 ml of the resuspended ammonium sulfate pellet was loaded onto a Biogel A1.5m column (80 by 3.5 cm), and 5-ml fractions were collected, to generate a profile similar to that seen in Fig. 7.

(ii) **SV40.** One hundred 100-cm plates of CV-1 monkey cells were infected with 5 PFU of SV40 per cell, and the cells were harvested after approximately 3 days at 37°C. After swelling in Dounce buffer (0.01 M sodium phosphate [pH 7.0], 0.01 M NaCl, 4 mM $MgCl_2$, 0.1 mM DTT, 50 μ g of phenylmethylsulfonyl fluoride per ml), the cells were disrupted by 20 strokes in a type B ground glass homogenizer and centrifuged at 5,000 rpm for 5 min to pellet the nuclei. The supernatant cytoplasmic fraction was brought to 60%

saturation with ammonium sulfate, and the precipitate was pelleted by centrifugation and resuspended in 2 ml of 20 mM Tris (pH 7.4)–50 mM NaCl–0.1 mM DTT. This was fractionated on a Biogel A1.5m column (60 by 1.5 cm) equilibrated with the same buffer, and 1.2-ml fractions were collected. In all cases, the nucleic acids and protein were monitored by absorbance at 260 nm and 280 nm, respectively. The small t antigen was detected by radioimmunoassay.

RESULTS

Construction of plasmids designed to express SV40 small t antigen. We have used the plasmid pGL101 to achieve expression of the SV40 small t gene in bacteria. This plasmid consists primarily of pBR322 DNA sequences between the *Eco*RI site and the *Pvu*II site which contain the gene coding for ampicillin resistance. Spanning the *Eco*RI–*Pvu*II junction is a 95-bp restriction fragment containing the *E. coli* UV-5 *lac* promoter, operator, and β -galactosidase ribosome binding site (24). This fragment is sufficient to promote efficient transcription and translation of any gene which has its initiation codon fused to the ribosome binding site in the proper orientation.

Digestion of SV40 DNA with the restriction enzyme *Hind*III generates a 1,169-bp fragment, designated the B fragment, which contains the entire small t gene with the initiation codon located 12 bp from one of the ends. This fragment was purified by preparative agarose gel electrophoresis and then inserted into the *Pvu*II site of pGL101 by using two different procedures (Fig. 1).

The first recombinant plasmid was constructed by modifying the ends of the SV40 *Hind*III B fragment. *E. coli* large fragment DNA polymerase I was used to synthesize a DNA complement to the single-stranded ends of the *Hind*III fragment to generate blunt ends. Plasmid pGL101 DNA was then digested with *Pvu*II, and the modified SV40 restriction fragment was inserted with T4 DNA ligase. Because hybrid molecules do not contain any *Pvu*II restriction sites, the ligation products were digested extensively with *Pvu*II before transformation to reduce the background of recircularized pGL101 DNA. The circular recombinants were then used to transform *E. coli* 294, and transformants were selected by growth in the presence of ampicillin. Bacterial colonies containing SV40 sequences were identified by a modification of the Grunstein and Hogness (21) screening procedure. Several such clones were selected, and the plasmid DNAs were analyzed by restriction enzyme mapping and Southern blot hybridization to confirm the presence and determine the orientation of the inserted SV40 *Hind*III B fragment. A

FIG. 1. Schematic representation of plasmid construction designed to express SV40 small t antigen. Preparation of the DNA and construction of the hybrid plasmids is described in the text.

clone carrying the small t gene fused to the *lac* promoter in the proper orientation was selected, and the plasmid was designated pSV250.

Roberts et al. (42) have demonstrated that overproduction of proteins utilizing the plasmid *lac* promoter system can be achieved by randomly varying the distance between the bacterial ribosome binding site, postulated by Shine and Dalgarno (44), and the initiation codon of the inserted gene. A change of as few as 3 bp can affect the level of expression by as much as 20-fold (42). With this in mind, we constructed a plasmid which contained a restriction site between the Shine and Dalgarno sequence and the small t AUG codon. This recombinant allowed direct manipulation of the base sequence within this crucial region.

PvuII was used to linearize pGL101 DNA, and *HindIII* linkers were ligated to the plasmid by using T4 DNA ligase. The SV40 *HindIII* B fragment was then inserted into this *HindIII* site, and the hybrid molecules were used to transform *E. coli* 294. The transformants were screened for colonies containing SV40 sequences, as before. One clone with the SV40

insert in the proper orientation was selected, and the plasmid was designated pSV240.

This plasmid has two *HindIII* sites, one of which lies between the bacterial Shine and Dalgarno sequence and the small t initiation codon. Plasmid pSV240 DNA was subjected to partial digestion with *HindIII* to generate a population of molecules, some of which were cut only at the hybrid ribosome binding site. These molecules were then treated with nuclease BAL 31 to decrease randomly the distance between the two sequences. Linear molecules were then purified by agarose gel electrophoresis, recircularized with T4 DNA ligase, and used to transform *E. coli* 294. Forty transformants containing the modified pSV240 molecules were selected and screened by restriction enzyme mapping of plasmid DNA for loss of the *HindIII* site near the *lac* promoter. Fourteen such clones were identified and tested by immunoprecipitation for their ability to produce small t antigen. As expected, a variety of different levels of expression was detected. Three clones, carrying plasmids designated HP1, HP2, and HP3, appeared to produce substantially more small t protein than

did pSV240. Since these plasmids exhibited approximately the same high level of production, HP1 was arbitrarily selected for quantitation and characterization of the viral protein.

Synthesis of SV40 small t antigen in bacteria. We have two antibodies available to detect the early gene products of SV40. One of these antibodies was isolated from the serum of hamsters bearing SV40-induced tumors and is specific for the virally coded large T and small t antigens (3, 13). The other antibody was made by injecting rabbits with purified D2 protein isolated from HeLa cells infected with the adenovirus-SV40 hybrid virus Ad2⁺D2 (54). The D2 protein lacks the amino terminal region of large T antigen which is shared with the small t polypeptide (22). Antibodies directed against D2 will therefore react only with large T antigen-specific determinants and not cross-react with small t antigen (54). Since the SV40 *Hind*III B fragment contains one-half of the large T structural gene as well as the entire small t coding sequence, it could potentially promote the synthesis of truncated large T-related proteins. The use of the anti-T and anti-D2 sera allows us to distinguish these proteins from SV40 small t antigen.

To detect SV40-related proteins expressed in bacteria and compare them with authentic SV40 small t antigen, both SV40-infected monkey cells and HP1 bacterial cells were pulse-labeled with [³⁵S]methionine, and the extracts were subjected to immunoprecipitation with anti-D2 and anti-T sera. A control preimmune serum was also used to identify proteins which were nonspecifically precipitated (Fig. 2A, lanes 1 and 2). The immune complexes were collected on protein A-bearing *S. aureus* cells and then fractionated by SDS-polyacrylamide gel electrophoresis. As expected, the rabbit anti-D2 serum precipitated only the SV40 large T antigen from the infected monkey cell extract, whereas the hamster serum precipitated both the large T and small t antigens (Fig. 2A, lanes 3 and 5). No proteins in the HP1 extract were specifically precipitated with the rabbit anti-D2 serum, but the hamster anti-T antibody selectively precipitated a 12-kilodalton (kd) protein and a 15-kd protein which comigrated with the SV40 small t antigen (Fig. 2A, lanes 4 and 6). These results demonstrate that the HP1-encoded small t protein is the same size and has the same antigenic determinants as authentic SV40 small t antigen. Furthermore, with the exception of the 12-kd protein, there appear to be no additional SV40-related proteins synthesized in HP1.

Since our ability to detect small t antigen is dependent on the use of anti-T antibody, we were interested in determining the efficiency of

our immunoprecipitations. SDS-polyacrylamide gel electrophoresis of a crude extract prepared from pulse-labeled HP1 cells allowed us to visualize directly a 15-kd protein which comigrated with SV40 small t antigen (Fig. 2C, lane 4). After anti-T immunoprecipitation and gel electrophoresis of the supernatant fraction, however, none of this protein could be detected (Fig. 2C, lane 3). Furthermore, the pattern displayed by the supernatant was almost identical to that of an extract prepared from *E. coli* cells carrying a control nonproducer plasmid (Fig. 2C, lane 5). These results indicate that our immunoprecipitation technique is capable of selectively and quantitatively removing small t antigen from a crude cell extract.

Quantitation of small t antigen produced by recombinant plasmids. Plasmids pSV240, pSV250, and HP1 were constructed to provide different levels of expression of small t antigen. To compare the relative amounts of small t protein being produced by these clones, bacteria carrying pSV240, pSV250, and HP1 were pulse-labeled with [³⁵S]methionine, and the crude extracts were subjected to anti-T immunoprecipitation with an excess of antibody, followed by SDS-polyacrylamide gel electrophoresis (Fig. 2B). Plasmid pSV250 was found to produce two-fold more small t antigen than did pSV240, and HP1 produced approximately 25-fold more of this protein than did pSV240. As expected, the relative levels of small t expression varied considerably with changes in the structure of the hybrid ribosome binding site.

Having demonstrated that HP1 is a high producer of SV40 small t antigen, we were interested in quantitating the amount of small t protein present in bacteria carrying this plasmid. Since pulse-labeling reflects the rate of synthesis rather than absolute amount, HP1 cells were labeled under steady-state conditions. A crude extract containing 10⁶ cpm of labeled protein was then subjected to immunoprecipitation with an excess of anti-T antibody (Fig. 3A, lane 3). By excising the band of antigen from the gel, 12,000 to 25,000 cpm could be recovered. Since the small t polypeptide contains approximately threefold more methionine residues than does an average protein (14), we calculated that small t antigen represents 0.4 to 0.8% of the total labeled protein in the extract.

To confirm this quantitation, we immunoprecipitated small t antigen from a crude extract of HP1 cells and directly visualized the proteins by Coomassie blue staining (Fig. 3B). Approximately 2 to 3 μ g of small t antigen can be immunoprecipitated from 300 μ g of protein. This is equivalent to 0.6 to 1% of the total cellular protein, or 40,000 to 80,000 molecules of small t

FIG. 2. Characterization of small *t* antigens by immunoprecipitation and gel electrophoresis. SV40-infected monkey cells and clones pSV240, pSV250, and HP1 were pulse-labeled with [35 S]methionine, and the crude extracts were subjected to immunoprecipitation with the antibodies described below. The immune complexes were collected on protein-A bearing *S. aureus* cells (26), fractionated on a 7 to 18% gradient of polyacrylamide containing SDS (51), and visualized by autoradiography as described in the text. (A) Labeled extracts from SV40-infected monkey cells (lanes 1, 3, and 5) and HP1 cells (lanes 2, 4, and 6) were subjected to immunoprecipitation with control rabbit anti-hamster serum, rabbit anti-D2 serum, and hamster anti-T antibody, respectively. (B) Samples containing 2×10^6 cpm of extract from SV40-infected monkey cells and 1.5×10^6 cpm of extracts from pSV240, pSV250, and HP1 cells were subjected to immunoprecipitation with an excess of anti-T antibody. (C) Lane 1 depicts an anti-T immunoprecipitate from SV40-infected monkey cells. A sample containing 4×10^5 cpm of extract from HP1 cells was subjected to anti-T immunoprecipitation, and the precipitated proteins are depicted in lane 2; 1×10^5 cpm of the supernatant from this immunoprecipitation was run in lane 3. A sample containing 10^5 cpm of HP1 cell extract was run in lane 4, and an equal amount of radioactive protein from a pSV240-like clone, carrying a plasmid with the SV40 insert in the wrong orientation, was run in lane 5. (D) HP1 DNA was used to transform a derivative of *E. coli* 294 which carries the *lac i'* gene and thus overproduces *lac* repressor (34). Anti-T immunoprecipitation and gel electrophoresis were performed on cells either with or without induction with isopropyl- β -D-thiogalactopyranoside.

antigen per HP1 bacterial cell.

We then compared the level of expression of HP1 with that of cells productively infected with SV40. Infected monkey cells were labeled with [35 S]methionine under steady-state conditions. A crude extract was prepared, and 10^6 cpm of labeled protein was treated with an excess of anti-T antibody (Fig. 3A, lane 2). By excising the band of small *t* antigen from the gel, approximately 0.03 to 0.06% of the total labeled protein could be recovered.

Sequence determination of the hybrid ribosome binding sites. The nucleotide se-

quence of 50 bp encompassing the hybrid ribosome binding site of pSV240, pSV250, HP1, HP2, and HP3 was determined by the Maxam and Gilbert (31) sequencing method (Fig. 4). As predicted, pSV240 contained one *Hind*III linker between the *lac* promoter and the SV40 insert, and pSV250 was missing 3 bp of the linker. HP3 is a 12-bp deletion mutant of pSV240, whereas HP1 and HP2 are missing an identical 11-bp sequence. The distance from the Shine and Dalgarno sequence to the small AUG codon is thus reduced from 20 bp in pSV240 to 9 bp in HP1 and HP2 and 8 bp in HP3. This indicates that

FIG. 3. Quantitation of small *t* antigen produced by HP1. (A) Samples containing 10^6 cpm of extract from SV40-infected monkey cells and HP1 cells labeled under steady-state conditions were treated with anti-T antibody, and the precipitates were subjected to gel electrophoresis. The end lanes contain 6.5×10^4 cpm of crude extract from these cells. The immunoprecipitated small *t* antigens, shown in the center lanes, were excised and quantitated as described in the text. (B) An unlabeled crude extract of HP1 cells containing 300 μ g of protein was subjected to immunoprecipitation with 10 μ l of rabbit anti-D2 serum or hamster anti-T IgG. The precipitated proteins were fractionated by slab gel electrophoresis and directly visualized by staining with Coomassie brilliant blue (Bio-Rad Laboratories). These samples were not heated before gel electrophoresis.

there are sequences in pSV240 that reduce the efficiency with which the mRNA can be bound by the 30S ribosomal subunit or, possibly, which attenuate transcription.

Characterization of the small *t* antigen produced by HP1. Our motive for constructing a bacterial plasmid which overproduces SV40 small *t* antigen was to facilitate purification of the protein for functional analysis. We were therefore interested in characterizing the HP1-encoded protein and comparing it with authentic SV40 small *t* antigen. Having determined that the size and antigenicity of the HP1-encoded and authentic small *t* antigens were identical, we

next used the more sensitive technique of isoelectric focusing to characterize the physical properties of these two proteins. Crude extracts prepared from both [35 S]methionine-labeled HP1 cells and *E. coli* cells carrying a control nonproducer plasmid were subjected to isoelectric focusing in the first dimension and then SDS-polyacrylamide gel electrophoresis in the second dimension (Fig. 5A and B). Under these conditions it was possible to separate both small *t* antigen and the 12-kd protein from most other bacterial proteins. The HP1-encoded antigen was found to migrate in the isoelectric focusing dimension with an isoelectric point of 7.2 to 7.4, identical to the reported isoelectric point of authentic SV40 small *t* protein (Fig. 5C) (12). In addition, a mixture of small *t* antigen isolated by anti-T immunoprecipitation from both SV40-infected monkey cells and HP1 cells comigrated during two-dimensional gel electrophoresis (Fig. 5D). Therefore, the size and the charge of the HP1-encoded small *t* antigen were identical to that of the authentic virally coded protein.

Upon isoelectric focusing, we observed charge heterogeneity in small *t* antigen isolated from both SV40-infected monkey cells and HP1 cells. In most cases three different species were identified, although more forms could be resolved with a longer exposure.

We performed tryptic fingerprint analysis of the small *t* antigen expressed in HP1 and compared its pattern with that of the authentic SV40 small *t* protein. Small *t* antigen was purified from pulse-labeled SV40-infected monkey cells and HP1 cells by anti-T immunoprecipitation and SDS-polyacrylamide gel electrophoresis. These purified radioactive antigens were then oxidized with performic acid and subjected to digestion with tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin. The peptides were fractionated by electrophoresis at pH 2.1 in one dimension and ascending chromatography in the second dimension. All except one of the tryptic peptides comigrated in both dimensions (Fig. 6). The unique peptide corresponded to the N-terminal fragment of small *t* antigen in the fingerprint pattern described by Linke et al. (28). The altered mobility of the HP1-encoded small *t* peptide could, therefore, be due to the absence of N-terminal acetylation known to occur in eucaryotic cells (36), although other post-translational modifications of the HP1-encoded protein cannot be ruled out. As observed by other investigators (46), some of the peptides seem to be over-represented in the digest of small *t* antigen isolated from one source relative to the other. However, even tryptic digests of identical proteins exhibit such quantitative dif-

FIG. 4. Nucleotide sequence of the hybrid ribosome binding sites. Plasmid DNA was digested with *Hinf*I, end-labeled by a polynucleotide kinase exchange reaction with [γ - 32 P]ATP (7), and then cleaved with *Pst*I. The 890-bp fragment containing the hybrid ribosome binding site for small t antigen was purified by gel electrophoresis and sequenced according to the technique of Maxam and Gilbert (31). The ribosome binding sequence, hypothesized by Shine and Dalgarno (44), is abbreviated to SD, and the SV40 sequence is drawn in bold letters. The direction of transcription is from left to right.

ferences, suggesting that this variability is inherent in the technique.

The structure of the 12-kd protein synthesized by HP1 was similarly analyzed by tryptic fingerprinting. Three, and possibly four, small t peptides were absent in the 12-kd protein pattern (Fig. 6, peptides a, b, c, and d). Peptides a, b, and c have all been mapped to the amino terminus of small t antigen (28). The 12-kd protein may therefore be considered to be an amino-terminal deletion of small t antigen. Further analysis will be necessary to ascertain the exact structure of this 12-kd protein.

Chromatographic properties of the HP1-encoded small t antigen. Having achieved a high level of production of intact small t antigen, we began working on the purification of this protein from *E. coli* carrying the HP1 plasmid. Since no biochemical activity for the small t protein has yet been identified, we depended on anti-T antibodies as a specific probe for the presence of the antigen. Initially, a complement fixation assay was attempted, but small t antigen was found to be inefficient in fixing complement, even when it was present in large amounts. We therefore developed a sensitive and rapid radioimmunoassay. One unit of small t antigen (approximately 5 to 10 ng) was defined as the amount of protein required to give 50% inhibition of complex formation between pure radioactive small t antigen and a limiting amount of anti-T antibody.

Our initial attempt at the purification of HP1-encoded small t antigen involved subjecting a crude preparation of HP1 extract to gel filtration chromatography. Surprisingly, about 50% of the

antigen was found to elute with an apparent molecular weight of greater than 10^6 (Fig. 7).

SDS-polyacrylamide gel electrophoresis and direct visualization of the antigen by Coomassie blue staining were used to confirm the presence of the small t protein in the excluded and included fractions (Fig. 8A). Approximately three-fold more 15-kd protein was visible in the excluded fraction than in the included fraction. This result could be accounted for if two-thirds of the cross-reacting antigen in the included fraction were the 12-kd protein.

We have tried 4 M NaCl, 0.2 M β -mercaptoethanol, 6 M urea, a variety of detergents, and extremes in pH in an attempt to dissociate the small t protein into its monomer form. None of these agents, either alone or in combination, was sufficient to disrupt the small t complex. However, with extreme denaturation conditions such as those employed during SDS-polyacrylamide gel electrophoresis, we found the antigen migrating as a monomeric species. The small t molecules are therefore not covalently bound to one another, except perhaps by labile disulfide-type bonds. We are currently pursuing alternate conditions to achieve dissolution of the high-molecular-weight complex.

Having established that small t antigen was present in HP1 cells in a high-molecular-weight form, we were interested in characterizing the gel filtration properties of authentic SV40 small t antigen. A crude cytoplasmic extract of SV40-infected monkey cells was fractionated by agarose A1.5m chromatography, and the small t protein was found to elute in a broad peak extending from approximately 500,000 daltons

FIG. 5. Two-dimensional gel electrophoresis of small *t* antigen from SV40 and HP1. The following samples from [35 S]methionine pulse-labeled cells were subjected to isoelectric focusing in one dimension and SDS-polyacrylamide gel electrophoresis (PAGE) in the second dimension (35). Panels: (A) 7×10^5 cpm of an extract from cells carrying a pSV240-like plasmid, with the SV40 insert in the wrong orientation; (B) 7×10^5 cpm of HP1 extract; (C) 2×10^3 cpm of immunoprecipitated small *t* antigen from SV40-infected monkey cells; (D) A mixture of 2×10^3 cpm each of authentic SV40 and HP1-encoded small *t* antigen purified by anti-T immunoprecipitation.

down to monomeric size (Fig. 7). Once again, gel electrophoresis of anti-T immunoprecipitates from several column fractions confirmed the presence of small *t* antigen in a high-molecular-weight form (Fig. 8B). P. Tegtmeyer, T. Spillman, and F. R. Schuetz (Cold Spring Harbor Symp. Quant. Biol., in press) have also reported size heterogeneity of authentic SV40 small *t* antigen isolated from lytically infected monkey

cells. The behavior of the HP1-encoded small *t* protein as a high-molecular-weight complex is therefore not unique to the bacterial system. The larger small *t* complex and high ratio of oligomers to monomers in the bacterial extract may be due to the increased intracellular concentration found in HP1 cells.

It is also possible that some component of the bacterial cell is responsible for the shift of small

t antigen to a very high molecular weight. To test for this, an extract from *E. coli* 294 cells was mixed with a cytoplasmic extract from SV40-infected monkey cells and subjected to gel filtration chromatography. The small t elution pattern looked identical to that from infected monkey cells alone, indicating that, under these conditions, no bacterial components are contributing to the formation of the high-molecular-weight complex.

We have used phenyl-Sepharose chromatography as well as a variety of ion exchange resins such as DEAE-cellulose, Bio-Rex 70, phosphocellulose, and hydroxyapatite to fractionate further the small t polypeptide. In most cases, the antigen was found to behave heterogeneously. To date we have been able to achieve a 100-fold purification of this protein, and we are currently

pursuing alternative means of purifying the HP1-encoded small t antigen to homogeneity.

DISCUSSION

We have maximized the expression of SV40 small t antigen by constructing a bacterial plasmid which fuses the SV40 F gene to the *E. coli lac* promoter-operator sequence. Recently, other investigators have successfully used similar techniques to achieve expression of various prokaryotic and eucaryotic viral genes (1, 41, 42). We have modified their procedure by first inserting the foreign gene into a vector carrying the *lac* promoter and then reconstructing the hybrid ribosome binding site by treatment with BAL 31 nuclease. This enzyme has a single-stranded endonuclease activity and also acts exonucleolyti-

FIG. 6. Tryptic fingerprint analysis of small t antigen from SV40 and HP1. Proteins labeled with [35 S]-methionine were purified by anti-T immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and electroelution as described in the text. Samples were subjected to performic acid oxidation and digested with tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington) as described previously (46). Samples of 1 to 5 μ l (ca. 50,000 to 100,000 cpm) were spotted on Brinkman glass-backed cellulose thin-layer plates, subjected to electrophoresis at pH 2.1 (8% acetic acid, 2% formic acid) for 1,000 V-h, and then chromatographed in the second dimension for 4 to 5 h in butanol-pyridine-acetic acid-water (97:75:15:60). The plates were dried and autoradiographed. The arrows indicate peptides discussed in the text.

FIG. 7. Agarose A1.5m column chromatography of extract from HP1 cells and from SV40-infected monkey cells. Crude extracts from 15 g of HP1 cells (1,300 mg of protein) and one hundred 100-cm plates of SV40-infected monkey cells (12 mg of protein) were prepared and subjected to Bio-Gel A1.5m column chromatography as described in the text. Small t antigen was detected by radioimmunoassay by using anti-D2 serum to preabsorb large T antigen in the infected monkey cell fractions, as described in the text. Nucleic acids and protein were determined spectrophotometrically by absorbance at 260 nm and 280 nm, respectively. The peak of small t antigen in fraction 25 of the infected monkey cell profile could not be detected by gel electrophoresis and Coomassie staining and thus may represent residual large T antigen not preabsorbed by the anti-D2 serum.

cally to digest processively from the termini of a DNA duplex (20), leaving blunt ends which can be rejoined by T4 DNA ligase. These properties of BAL 31 nuclease make it ideal for the construction of "expression plasmids" and for in vitro deletion mutagenesis in general.

By transforming HP1 into a derivative of *E. coli* 294 which overproduces *lac* repressor, we have confirmed that expression of small t antigen is under the control of the *lac* regulatory sequences. The expression of small t antigen in this host was effectively abolished. However, upon induction with isopropyl- β -D-thiogalactoside, maximum levels of expression could be achieved (Fig. 2D). In contrast, when this gratuitous inducer was added to a culture of HP1 in its normal *E. coli* 294 host, only a slight increase in expression could be detected. This is to be expected as there are approximately 10 molecules of *lac* repressor present in a wild-type *E. coli* cell (17), and these few molecules can have little effect on the 30 to 40 HP1 plasmids which are present in a transformed bacterium (6).

Under our growth conditions, HP1 produced 40,000 to 80,000 molecules of small t antigen per cell, or approximately 1,000 to 3,000 molecules of small t antigen per plasmid. Thus, HP1 was functioning at 20 to 60% of its maximum theo-

retical efficiency in comparison to a fully induced β -galactosidase gene (1).

Figure 3A demonstrates that the concentration of small t antigen in an extract of HP1 cells was 10-fold higher than that in an equivalent amount of SV40-infected monkey cell extract. The quantitation of small t antigen in this eucaryotic system is imprecise, because detection of the protein can be achieved only by immunoprecipitation (13). However, assuming that 0.05% of the total protein in the SV40-infected monkey cells is small t antigen, then 1 liter of HP1 cells at late log phase contains as much of the antigen as can be recovered from 1.2×10^5 plates of SV40-infected monkey cells. This level of production should facilitate the purification of this protein to homogeneity.

The small t antigens produced by HP1 and SV40 were found to be virtually indistinguishable on the basis of electrophoretic mobility, antigenic determinants, isoelectric point, and tryptic fingerprint analysis. The only detectable difference between these proteins was the slower migration of the HP1-encoded N-terminal tryptic peptide during ascending chromatography. This difference is most likely due to the absence of amino-terminal acetylation in the bacterial protein. Like many eucaryotic proteins (4), small t antigen isolated from SV40-infected monkey

FIG. 8. SDS-gel electrophoresis of high-molecular-weight and monomeric small *t* antigen. Samples from gel filtration column fractions containing high-molecular-weight and monomeric small *t* antigen were fractionated by SDS-polyacrylamide gel electrophoresis either before or after immunoprecipitation. The proteins were visualized by staining with Coomassie brilliant blue. (A) Samples containing 150 U of small *t* antigen from HP1 cells as either a high-molecular-weight complex (lanes 1 to 3) or in monomeric form (lanes 4 to 6) are depicted. A sample of the total protein (lanes 1 and 4), the supernatant of an anti-*T* immunoprecipitation (lanes 2 and 5), and the immunoprecipitated proteins (lanes 3 and 6) were electrophoresed in neighboring lanes. (B) Samples containing 200 U of small *t* antigen from SV40-infected monkey cells which elute from gel filtration as a high-molecular-weight complex (lanes 1 and 2) or as monomeric antigen (lanes 3 and 4) were subjected to immunoprecipitation with anti-D2 serum to preabsorb the large *T* antigen (lanes 1 and 3). The supernatant from that immunoprecipitation was then treated with anti-*T* antibody to precipitate small *t* antigen (lanes 2 and 4). Large *T* antigen has been proteolyzed to its 89-kd and 84-kd forms due to the extraction procedure used (46).

cells is acetylated at its amino terminus (36). Functional analysis of the purified HP1-encoded protein will reveal whether this modification is required for biological activity.

We found that pSV240, pSV250, and HP1 all produced a 12-kd SV40-coded protein in addition to the 15-kd small *t* antigen. Tryptic fingerprint analysis of the 12-kd protein revealed a structure identical to that of small *t* antigen, with the exception of several amino-terminal tryptic peptides. Since the expression of the 12-

kg protein could be regulated with *lac* repressor, its transcript must also derive from the *lac* promoter. The expression of the 12-kd protein may be due to protein synthesis initiation at an in-phase AUG codon located within the small *t* gene. In fact, there is a potential AAGGAGG Shine and Dalgarno sequence located upstream from the fourth and fifth in-phase AUG codons of the small *t* coding sequence (38). The stable duplex that this sequence could form with the 3' end of *E. coli* 16S rRNA may favor internal initiation of translation (44, 50).

The 12-kd protein appears to represent a fortuitous amino-terminal deletion of SV40 small *t* antigen. It should be possible to construct other deletion mutations in the small *t* coding sequence of HP1 by using site-directed in vitro mutagenesis techniques. These mutant small *t* proteins may be useful for studying the function(s) of this viral tumor antigen.

In spite of the high level of production afforded by HP1, the purification of small *t* antigen from this clone is not straightforward, due to the aggregation properties of the protein. We are currently employing unconventional techniques which should allow us to purify this protein to homogeneity. We have begun to raise multivalent and monoclonal antibodies directed against small *t* antigen to determine its intracellular location in SV40-infected and transformed cells. In addition, we intend to purify the high-molecular-weight complex of small *t* antigen, monomeric antigen, and 12-kd protein and test their biological activities by microinjection into mammalian cells. Finally, we hope to test these different species for potential enzymatic activities.

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