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Purification of simian virus 40 tumor antigen from a line of simian virus 40-transformed human cells

(heparin-Sepharose/amino acid analysis)

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ABSTRACT **Simian virus 40 tumor antigen can be isolated in a highly purified state from the nuclei of SV80 cells, a continuous line of simian virus 40-transformed human fibroblasts. A five-step purification method was used. Its apparent molecular weight (in sodium dodecyl sulfate/polyacrylamide gels) is approximately 90,000–94,000. It contains a detectable amino-terminal residue.**

The simian virus 40 (SV40) tumor (T) antigen is a virus-encoded protein that was first recognized as a nuclear constituent in SV40-transformed cells (1, 2). T antigen is a product of the viral A gene (3–12). As such, it has been suggested to play a role in the initiation of rounds of viral DNA replication and the maintenance of virus-induced neoplastic transformation (13–17). The biochemical role of the protein in these processes is not clear. However, it is now known that T antigen is a DNA binding protein, capable of recognizing specific sequences in the SV40 chromosome (18–20). At least one of these binding sites lies close to or at the origin of viral DNA replication (19, 20). Thus, perhaps a step central to the function of the T antigen in the initiation of viral DNA replication involves the direct interaction of T or a T-cellular protein(s) complex with specific DNA sequences lying in the vicinity of or actually at the replication origin.

Thus far, *in vitro* T antigen studies have been carried out in our laboratory with partially purified protein (21, 22). Historically, purification of T antigen has been hampered by various difficulties. These have included instability of the complement-fixing activity of the antigen (23), its relatively low concentration in some SV40-infected permissive and transformed cell lines (10, 24), and low yields after multiple chromatographic procedures (10, 24). In this report, we describe the isolation of extensively purified T antigen in microgram quantities. The antigen was extracted from a cell line, SV80, shown previously to contain relatively high concentrations of the protein (24). A five-step purification procedure was used. The final fraction is represented by a single stainable band on sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis of up to 4 μg of protein. The yields of T antigen from such cells have permitted certain initial protein chemical analyses which will be described here.

MATERIALS AND METHODS

Cells. SV80 cells (an SV40-transformed derivative of a primary strain of fibroblasts from a child with anemia) and their mass cultivation have been described (24, 25). They were generously grown for us at the Massachusetts Institute of Technology Cell Culture Center, in Dulbecco's modification of Eagle's essential medium containing 10% fetal calf serum.

Complement-Fixation Tests for T Antigen. This assay was performed with 1.2 units of guinea pig complement by the published method (22).

Purification of T Antigen from SV80 Cells. Unless noted otherwise, all operations were at 4°. SV80 cells (usually 50–80 g), freshly scraped from glass roller bottles with a rubber policeman, were washed twice with ice-cold 0.01 M sodium phosphate, pH 7.4/0.14 M NaCl. The cells were allowed to swell on ice for 15 min in buffer A [0.01 M 2-(*N*-morpholino)-ethanesulfonic acid, pH 6.0/10 mM NaCl/1.5 mM $MgCl_2$/phenylmethylsulfonyl fluoride (PMSF), 100 μg/ml/tosyl-L-phenyl chloromethyl ketone (TPCK), 50 μg/ml/tosyl-L-lysyl chloromethyl ketone (TLCK), 20 μg/ml] at 5 ml per original 1 g (wet weight) of cells. They were then homogenized with five or six strokes in a glass Dounce homogenizer with a tight-fitting pestle. The nuclei were pelleted by centrifugation at 2000 rpm for 20 min in the J-20 rotor of a Beckman J21B centrifuge. The supernatant was discarded, and the nuclei were resuspended in a volume of buffer A equivalent to that used in the original homogenization. They were then vigorously pipetted up and down and subsequently centrifuged as above. The nuclear pellet was resuspended in buffer B [0.05 M Tris·HCl, pH 7.8/1 mM dithiothreitol (DTT)/10% glycerol/PMSF, 100 μg/ml/TPCK, 50 μg/ml/TLCK, 20 μg/ml] at 2 ml/g of cells and then subjected to sonication at 40–50 W (Heat Systems ultrasonic device equipped with a pointed microtip) for 15 sec in an ice bath. After a 30-sec interval, the sonication was repeated for 15 sec. This sonicate/rest cycle was performed twice more, resulting in a total sonic disruption period of 60 sec.

The sonicated suspension was centrifuged in a JA-14 rotor for 15 min at 15,000 rpm at 4°, and the supernatant was retained. It was fractionated between 30 and 60% $(NH_4)_2SO_4$ saturation, and the final pellet was redissolved in sufficient buffer C (0.05 M Tris·HCl, pH 7.8/10% glycerol/3 mM DTT/0.02% Triton X-100/PMSF 100 μg/ml/TPCK, 50 μg/ml/TLCK, 20 μg/ml) to dissolve all of the protein (usually 30–50 ml for material from a 60-g cell pack). This fraction was dialyzed for 6 hr against two changes of 40 volumes of buffer C, and any insoluble protein was removed by centrifugation. It was loaded on a column of DEAE-cellulose (DE-52, Whatman) (120 ml packed volume per 60 g of cells) equilibrated in buffer C. After washing with sufficient buffer C to bring the A_{280} of the effluent to base-line levels (approximately 2 column

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Abbreviations: SV40, simian virus 40; T antigen, simian virus 40 tumor antigen; $NaDodSO_4$, sodium dodecyl sulfate; TPCK, tosyl-L-phenyl chloromethyl ketone; TLCK, tosyl-L-lysyl chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; buffers A–I, see *Materials and Methods*; M_r, molecular weight.

volumes), a linear gradient (6–7 column volumes) was applied between 0.15 and 0.45 M NaCl in buffer C. Fractions (0.07 column volume) were collected.

After the T antigen-containing fractions were identified by complement fixation under antigen-limiting conditions (22), the peak fractions were pooled. The pool was dialyzed for 6 hr against two changes of 30 volumes of buffer D (0.05 M Tris·HCl, pH 7.8/20% glycerol/3 mM DTT/0.02% Triton X-100) and subsequently loaded on a column of QAE-Sephadex (volume = 0.20 × wet weight of cells) equilibrated in buffer D. The column was washed with 3 column volumes of buffer D and then eluted with a linear gradient (13 column volumes) between 0.15 and 0.40 M NaCl in buffer D containing the same concentrations of PMSF, TPCK, and TLCK as in buffer C; 0.1-column-volume fractions were collected.

The desired fractions containing T antigen were pooled and dialyzed for 4 hr against two changes of 100 volumes of buffer E (0.05 M Tris·HCl, pH 7.4/3 mM DTT/20% glycerol/0.02% Triton X-100). The dialyzed T antigen pool was then applied to a column of heparin–Sepharose (volume = 0.16 × weight of cells) prepared as described below. The column was washed with 3 column volumes of buffer E and eluted with a 10-column-volume linear gradient between 0.10 and 0.50 M NaCl in buffer E containing the same concentrations of PMSF, TPCK, and TLCK as in buffer C; 0.1 column volume fractions were collected. The desired T antigen-containing fractions were pooled, brought to 0.1% in Triton X-100 and 10 mM in DTT, and concentrated to less than 1 ml at 4° by ultrafiltration through a Diaflo PM-10 filter (Amicon Corp.) under nitrogen at 40 lb/in.^{2} (275 kPa). The concentrated material was then dialyzed against 100 volumes of buffer F (0.05 M Tris, pH 7.8/10 mM DTT/0.02% Triton X-100/4% glycerol) for 2 hr and then centrifuged through 5–25% glycerol gradients in buffer F containing the same concentrations of PMSF, TPCK, and TLCK as in buffer C (11.5 ml) poured atop 1–1.5 ml cushions of 40% glycerol in buffer F. Gradients were poured in cellulose nitrate tubes that had been previously boiled for 30 min in 1 mM EDTA, pH 7.8, and then exhaustively rinsed with cool distilled water. Samples were sedimented at 40,000 rpm in an SW 41 rotor at 4° for 12–15 hr. Gradients were drip collected from below, at 0°, and approximately 0.15-ml fractions were collected.

The complement-fixing activity of the antigen can be unstable to heat and protein dilution (23). We have found it to be partially stabilized by maintaining a DTT concentration of ≥1 mM in all buffers, by avoiding excessive dilution of T antigen during the various steps, and by working as rapidly as possible.

Preparation of Heparin–Sepharose. Heparin (Wilson Pharmaceuticals, stage 14) was generously provided by Robert and Judith Rosenberg. It was dissolved in 0.10 M $NaHCO_3$ at a concentration of 0.33 mg/ml. Freshly washed Sepharose 4B was CNBr-activated and then added at a concentration of 0.50 g/mg of heparin (26, 27). This suspension was stirred for 16 hr at 4°. The coupled Sepharose was washed exhaustively with cold H_2O and then with 50 volumes of cold buffer G (0.05 M Tris·HCl, pH 7.4/20% glycerol/1 mM DTT/1 M NaCl) and poured into a column in this buffer. Columns of this material could be reutilized after washing with several volumes of buffer H (0.05 M Tris·HCl, pH 7.4/2 M NaCl) and re-equilibration in buffer E.

NH_2-Terminal Analysis. This was performed by a standard micro dansylation technique (28). Samples were dialyzed against three changes of 200 volumes of 0.02% Triton X-100 or 0.02% $NaDodSO_4$ for 24 hr before dansylation (identical results were obtained in either instance). Dansyl amino acids were identified by two-dimensional thin-layer chromatography on polyamide layers (28) and identified under short-wave UV light. Photographs of such plates were taken with a Polaroid Land camera containing type 105 positive-negative film. The camera lens was shielded with a red filter. NH_2-terminal analysis was also performed by the micro Edman technique using [^{14}C]phenylisothiocyanate (New England Nuclear Corp.) (29).

Amino Acid Analysis. This was performed by the standard procedure of Spackman *et al.* (30). Samples (15–60 μg) were brought to 0.02% in $NaDodSO_4$ and dialyzed against 0.02% $NaDodSO_4$ for 24 hr. They were then hydrolyzed for 20 hr at 110° in 5.7 M HCl in evacuated, acid-washed, glass ampoules.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. This was performed in tube (0.5 × 8.5 cm) and slab (13 × 8 × 0.1 cm) gels by the method of Laemmli (31). After electrophoresis was completed, the gels were fixed in 10% trichloroacetic acid for 1 hr. Where indicated, tube gels were frozen in a dry ice bath and cut into 1-mm slices. Gels were stained with 0.25% Coomassie brilliant blue in methanol/acetic acid/H_2O, 5:1:5 (vol/vol), and then destained in 7.5% acetic acid/5% methanol.

Iodination. Samples of T antigen (5–15 μg) were dialyzed for 4 hr against two changes of 0.05 M $NaPO_4$, pH 7.5/0.01% Triton X-100 prior to iodination with ^{125}I (11–17 mCi/μg of I, Amersham/Searle) by the chloramine T method (32).

Immunoprecipitation. For ^{125}I-labeled T antigen, a minor modification of the method of Ahmad-Zadeh *et al.* (33) was used. Hamster anti-T serum (10 μl) (4 × 0303 from R. Wilsnack) was added to a fixed aliquot (30 μl) of solution containing radioactive T antigen. To another 30-μl sample of antigen, 10 μl of nonimmune hamster serum was added. After incubation for 1 hr at 4°, 20 μl of goat anti-hamster-IgG was added to each sample. After a 16 hr incubation at 4°, the precipitates were pelleted by centrifugation, washed three times in 3 ml of 2.5 mM Tris, pH 7.4/1 mM DTT/0.5 M LiCl/1 mM EDTA at 4° and dissolved by boiling in $NaDodSO_4$/gel electrophoresis sample buffer (31) for further analysis.

For ^{35}S-labeled T antigen, the method of Tegtmeyer *et al.* (8) was followed.

Protein Analysis. A modification (34) of the Lowry technique was used.

RESULTS

Purification of T Antigen Protein. The first step in the purification sequence was the preparation of nuclei from freshly harvested cells by Dounce homogenization (21). In the procedure described here, a significant modification was made. Cells were ruptured by homogenization in hypotonic medium at pH 6 instead of at pH 7.8. Tegtmeyer *et al.* (8) have previously shown that T antigen efficiently leaks out of nuclei at pH 8 but not at pH 6. We confirmed this observation and found at least a 2-fold increase in the yield of immunoreactive material from sonicated nuclei prepared by homogenization at pH 6 compared to pH 7.8. Three known inhibitors (TPCK, TLCK, and PMSF) of certain proteolytic enzymes were added in an effort to inhibit or decrease ambient proteolytic activity that might degrade the protein during extraction and purification (6, 35). Nuclei were then sonicated at pH 7.8 and the extract was fractionated with $(NH_4)_2SO_4$ between 30% and 60% saturation.

The $(NH_4)_2SO_4$ fraction was chromatographed on

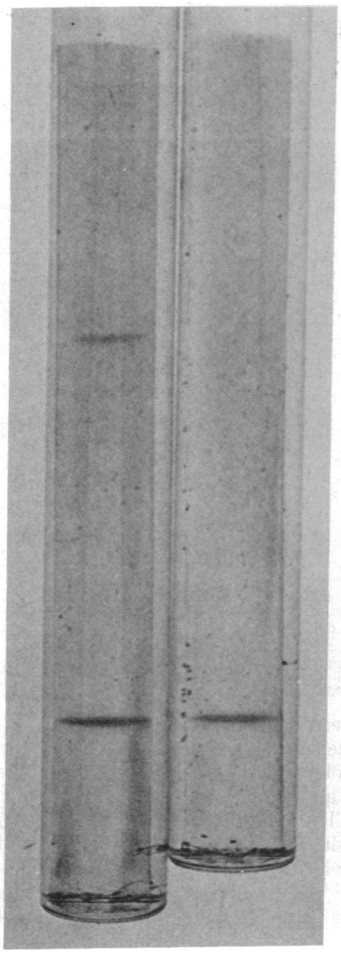

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of glycerol gradient-purified T antigen. Approximately 4 μg of the glycerol gradient material was applied to a 7.5% tube gel with a 3% stacking gel prepared and run according to the method of Laemmli (31). Staining was with Coomassie blue. (*Left*) T antigen fraction plus dye marker. (*Right*) Dye marker alone. The lower band in each gel represents the dye front. In this gel system, the protein migrated with an apparent molecular weight of 92,000 compared to phosphorylase *a* (94,000), bovine serum albumin (69,000), and ovalbumin (45,000) standards.

DEAE-cellulose, as reported earlier (21), but with the addition of Triton X-100 to the buffer in an effort to minimize nonspecific adherence of antigen to cellulose, tubing, and glassware. The peak antigen-containing fractions (eluting at approximately 0.2 M NaCl) were identified by complement fixation, pooled, and then dialyzed and sequentially chromatographed on columns of QAE-Sephadex and heparin-Sepharose. The QAE material eluted at approximately 0.3 M NaCl. With the heparin-Sepharose column the peak was at approximately 0.25 M NaCl. The desired heparin-Sepharose fractions were pooled and concentrated by ultrafiltration and then sedimented through a 7–25% glycerol density gradient in which the antigen migrated in a unimodal distribution.

The yield of antigen in the final step was routinely ≥3% (occasionally as high as 10%) in repeated preparations (Table 1) and averaged 75–100 μg/60–80 g of cells. The specific activity routinely increased at least 200-fold over that of the crude extract. The purity and yield of the final material were significantly affected by how pools of antigen were selected from the prior purification steps. Two factors facilitated the selection of fractions for a given pool—carrying out complement-fixation assays under conditions of limiting antigen concentration, and analyzing the degree of purity of individual fractions in and around T antigen peaks by $NaDodSO_4$/gel electrophoresis.

Table 1. Purification of T antigen from SV80 cells

Fraction	Total CF units*	Recovery, %	Specific activity, CF units/mg
Nuclear sonicate	2×10^6	(100)	4×10^3
Ammonium sulfate pool	1.5×10^6	75	5×10^3
DEAE-cellulose pool	7.6×10^5	38	1.3×10^4
QAE-Sephadex pool	2.3×10^5	11.5	5×10^4
Heparin-Sepharose pool	2.5×10^5	12.5	2.5×10^5
Glycerol gradient pool	1.5×10^5	5	2×10^6

This T antigen preparation was from 76 g of SV80 cells. The glycerol gradient pool contained 75 μg of protein at a protein concentration of 0.03 mg/ml.

* One CF unit is defined as a deflection of 50% in the titration of T antigen versus erythrocyte hemolysis (ΔA_{413}), as described in *Materials and Methods*.

Purity of the Resolved Material. The glycerol gradient pool yielded material characterized by a single visible band (Coomassie brilliant blue staining) when approximately 4 μg of protein was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1). Its apparent molecular weight (M_r) was 94,000. It migrated in parallel with a phosphorylase *a* standard (Fig. 2) and identically to ^{35}S-labeled T antigen prepared by specific immunoprecipitation of the protein from a crude extract of freshly labeled SV80 cells. Unlike the other observable radioactive bands, the M_r 94,000 ^{35}S-labeled band was not precipitated with nonimmune control serum (data not shown).

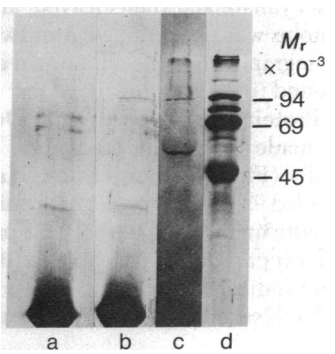

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of purified T antigen and of immunoprecipitated ^{35}S-labeled T antigen from a crude SV80 extract. The glycerol gradient-purified T antigen (300 μl) shown in Fig. 1 was precipitated in acetone at −23°, after addition of 100 μg of ribonuclease (Sigma fraction XI). The precipitated material was centrifuged at 1500 × *g* for 30 min and the dried pellet was dissolved in $NaDodSO_4$ sample buffer and applied to a 10% slab gel with a 3% stacking gel as described in *Materials and Methods*. [^{35}S]Methionine-labeled T antigen was immunoprecipitated from crude SV80 nuclear extracts, and the precipitate was dissolved in sample buffer and applied to the same gel as described in *Materials and Methods*. After electrophoresis the gel was stained with Coomassie brilliant blue, dried under reduced pressure, and autoradiographed. Protein standards are phosphorylase *a* (94,000), bovine serum albumin (69,000), and ovalbumin (45,000). Lanes: a, ribonuclease alone; b, acetone-precipitated glycerol gradient material plus ribonuclease; c, [^{35}S]methionine-labeled T antigen immunoprecipitate; d, standards. A parallel nonimmune hamster serum immunoprecipitate of the same ^{35}S-labeled extract contained all of the bands noted in lane c except for the 94,000 band (data not shown).

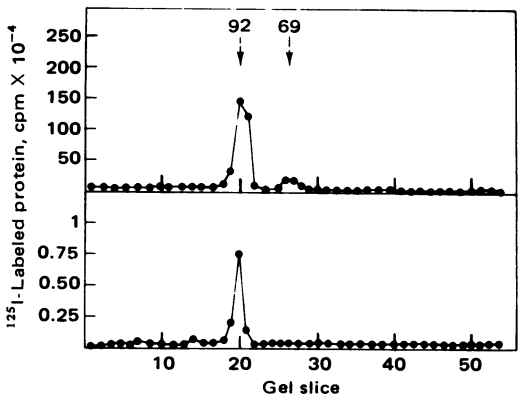

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of ^{125}I-labeled glycerol gradient-purified T antigen. Glycerol gradient-purified T antigen, lactoperoxidase (Sigma, M_r 92,000), and bovine serum albumin (M_r 69,000) were iodinated as described in *Materials and Methods* and electrophoresed in a 10% tube gel (0.5 × 6 cm). Then the gels were sliced with a Mickle gel slicer and the radioactivity of each slice was measured in a gamma counter. (*Upper*) Lactoperoxidase and bovine serum albumin. (*Lower*) T antigen.

In other experiments with the standard slab gel system, the purified protein and immunoprecipitated ^{35}S-labeled T antigen band migrated in parallel with apparent M_r of 90,000–94,000. When the glycerol gradient fraction was iodinated with ^{125}I by the chloramine T technique (32) and then analyzed by NaDodSO$_4$/gel electrophoresis, a single radioactive peak was identified (Fig. 3). Its apparent M_r, based on its migration relative to lactoperoxidase (92,000) and bovine serum albumin (69,000) standards was approximately 92,000.

The glycerol gradient fraction was treated with dansyl chloride and then acid hydrolyzed and analyzed for dansylated amino acids (28). A single α-dansyl amino acid was identified, dansylproline (Fig. 4). The protein was also treated with [^{14}C]phenylisothiocyanate and then cleaved, and radioactive phenylthiohydantoins were determined after two-dimensional thin-layer chromatography. A single amino acid derivative, that of proline, was found (data not shown).

The Purified Protein Carries T Antigen Determinant(s). An attempt was made to immunoprecipitate ^{125}I-labeled T antigen specifically (Fig. 5). Hamster anti-T antigen serum bound the ^{125}I-labeled 92,000 M_r species but nonimmune serum did not. The absolute quantity of ^{125}I-labeled protein precipitated varied from preparation to preparation. It was routinely >10% of the input radioactivity.

Amino Acid Analysis. The glycerol gradient-purified material was acid hydrolyzed and analyzed for amino acids by a standard method (30). The protein was relatively rich in certain residues: glutamate and/or glutamine, aspartate and/or asparagine, leucine, and glycine (Table 2). Because wet thumbprints are known to be rich in glycine and serine (36) and micro quantities of protein were being studied here, sham-purified, protein-free samples prepared in parallel with T antigen were also analyzed. The estimated amount of possible adventitious

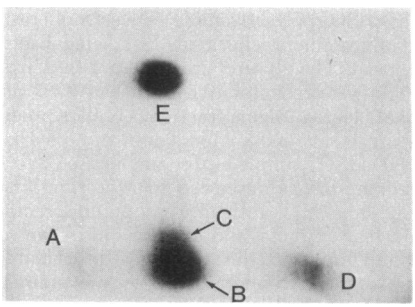

FIG. 4. NH_2-terminal analysis. Approximately 20 μg of glycerol gradient-purified protein was dialyzed overnight against three changes of 0.01% Triton X-100 in H_2O. The protein was then dried under reduced pressure, dansylated, and acid hydrolyzed and the resulting dansylated amino acids were analyzed. Spots: A, origin; B, dansylic acid; C, internal tyrosine–*o*-dansyltyrosine; D, internal lysine–ϵ-dansyllysine; E, dansylproline.

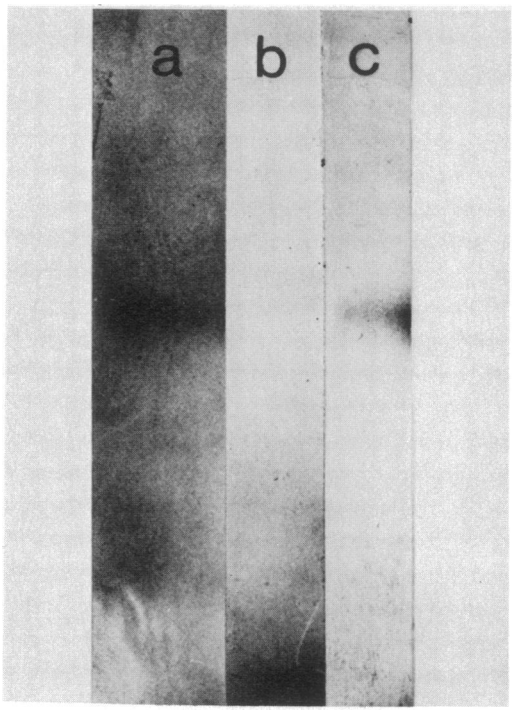

FIG. 5. Specific immunoprecipitation of ^{125}I-labeled glycerol gradient-purified material. Aliquots of ^{125}I-labeled glycerol gradient-purified protein (M_r, 92,000–94,000) were incubated with hamster anti-T antigen and nonimmune sera and then precipitated with second antibody. The solubilized precipitates were electrophoresed in a 10% polyacrylamide slab gel. The gel was fixed, dried, and autoradiographed. Lanes: a, ^{125}I-labeled protein + anti-T antigen serum precipitate; b, ^{125}I-labeled protein + nonimmune serum precipitate; c, ^{125}I-labeled glycerol gradient-purified protein alone.

Table 2. Amino acid analysis of T antigen from SV80 cells

Residue	Mol/1000 mol
Asx	112 ± 2
Thr	51 ± 3
Ser	82 ± 7
Glx	147 ± 13
Pro	44 ± 5
Gly	97 ± 7
Ala	81 ± 8
Val	51 ± 6
Ile	42 ± 2
Leu	98 ± 4
His	26 ± 3
Lys	70 ± 2
Arg	48 ± 4
Tyr	27 ± 4
Phe	37 ± 3
Met	20 ± 1

Amino acid analyses were performed on glycerol gradient-purified material (15–40 μg) that had been dialyzed for 24 hr against three changes of 0.02% NaDodSO$_4$. The data shown are means ± maximal variations in three analyses of independent pools.

amino acid contamination that could have occurred here was ≤10% for glycine, serine, alanine, and lysine. No other residues were detected in the sham preparation.

DISCUSSION

Highly purified T antigen has been isolated from a line of SV40-transformed human cells by a five-step procedure. It can be obtained from SV80 cells in a yield ≥3% by this method. This value, while not ideal, is acceptable for an unstable protein present in relatively low concentration in the cell and is sufficient to permit certain protein chemical and DNA binding experiments.

The resolved protein was highly purified by several criteria: (*i*) the presence of a single stained band when 4 μg of protein was applied to a gel; (*ii*) the presence of a single ^{125}I-labeled protein band when the glycerol gradient-purified fraction was exogenously labeled; and (*iii*) a single dansylated NH_2-terminal residue. These results also suggest that the protein isolated by this method consists of a unique polypeptide. One cannot, however, assume from such results that the antigen naturally exists in this state in the cell. Moreover, since T antigen is known to be subject to limited proteolysis during its extraction (35), we recognize the additional possibility that, in the cell, the presently observed amino-terminal proline might be internal to the natural end group.

The isolated protein is T antigen as defined by its ability to be bound selectively by specific hamster anti-T antigen IgG. It resembles *in vivo* labeled protein immunoprecipitated directly from crude extracts of freshly harvested cells in terms of its migration in $NaDodSO_4$/polyacrylamide gels. These results do not rule out the possibility of some differences when more detailed chemical studies of the protein and of its coding gene are undertaken.

The observed $NaDodSO_4$/gel electrophoretic behavior of purified and crude immunoprecipitated T antigen indicates an approximate M_r of 90,000–94,000. It is problematic to accept that this is the correct size of the protein in view of the fact that recent results from other laboratories suggest that the 5′ border of the T antigen cistron lies to the right of 0.54 map unit and the 3′ border close to 0.17 map unit (9, 37–39). Viable deletion mutants lacking significant quantities of DNA between 0.54 and 0.59 map units encode a "normal" size T antigen (9, 39). Moreover, nucleotide sequence analysis reveals termination codons in all three reading frames at approximately 0.54 map unit (37). Assuming these loci to be boundaries of the largest possible T cistron, its maximal coding capacity would be approximately 65,000–70,000 daltons. Comparison of the NH_2-terminal sequence of the protein with the appropriate segment of the SV40 DNA sequence should reveal the location of the translational start of the T antigen cistron. This information coupled with direct M_r determinations by methods other than $NaDodSO_4$/gel electrophoresis should permit insights into the basis for the apparent discrepancy between the observed molecular weight on $NaDodSO_4$ gels and the estimated gene size.

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