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Simian Virus 40 T-Antigen: Identification of Tryptic Peptides in the C-Terminal Region and Definition of the Reading Frame

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T-antigen (the simian virus 40 A cistron protein) was purified by immunoprecipitation and electrophoresis on polyacrylamide gels from monkey kidney CV-1 cells infected with simian virus S (SV-S), *dl*1263, or *dl*1265 and digested with trypsin. The tryptic peptides, labeled with [^{35}S]methionine, [^{35}S]cysteine, or [^{3}H] proline, were fractionated either by chromatography on Chromobead-P resin or by two-dimensional electrophoresis and chromatography on cellulose thin layers. The T-antigen of SV-S was shown to give rise to a proline-rich (approximately 6 mol of proline) tryptic peptide which was absent in *dl*1265 T-antigen and hence, on the basis of DNA sequence data, must originate from the C-terminus of the SV-S protein. T-antigen from *dl*1265, but not SV-S, yielded a cysteine-rich terminal tryptic peptide. The presence of these cysteines caused the protein to be retarded during electrophoresis under the usual conditions in polyacrylamide gels. The T-antigen of *dl*1263 possessed the proline-rich terminal tryptic peptide; the data are consistent with there being only one peptide altered by the deletion. Both deletion mutants produced a T-antigen that had a higher electrophoretic mobility than SV-S T-antigen but still a larger apparent molecular weight than was predicted by the DNA sequence. The major form of T-antigen found in several lines of 3T3 cells transformed by these mutants was indistinguishable from the T-antigen found in infected cells, and in addition seemed to associate normally with the host-coded 53,000-dalton protein. Except for a minor form of T-antigen with a slightly lower mobility in gels but the same C-terminus, no other polypeptides were detected among the extracted and immunoprecipitated proteins whose electrophoretic mobility was affected by either deletion.

The proteins coded for by the early region of simian virus 40 (SV40) have been the subject of a number of recent studies. There are two well-defined polypeptides, here referred to as large-T (or T-) antigen and small-t (t-) antigen. The unmodified T-antigen protein has a theoretical molecular weight of about 85,000 and is believed to be coded for by two noncontiguous regions of the genome, 0.647-0.600 and 0.533-0.174 MU (map units). t-antigen has a molecular weight of about 20,500 and is likely to be coded for by a continuous region of the genome from 0.647 to 0.546 MU. The same reading frame is used for both proteins in the 0.647-to-0.600 MU region and, as a consequence, the first 82 of the 174 amino acids in t-antigen are identical to those found in T-antigen (barring posttranslational modifications). These conclusions are based on knowledge of the DNA sequence, detailed investigations of the mRNA molecules and splices therein, the sequence of amino acids at the N-terminus, and studies of the tryptic peptides of the proteins (3, 8, 13, 25, 29, 31, 32, 34, 37). T-antigen, the classical product of the A cistron, is required for the establishment or maintenance, or both, of the transformed state at least under certain conditions (14, 35; review in 18). The function of t-antigen is less well defined, but it also seems to be necessary, at least under some conditions, for the establishment or maintenance, or both, of the transformed state (4, 11, 28, 39). The two proteins are precipitated independently by specific anti-T sera and by sera from animals bearing SV40-induced tumors (23; L. V. Crawford, D. C. Pim, and D. P. Lane, Virology, in press).

Very little is known about either the structure of T-antigen or its function in the cell. Under conditions of minimal proteolysis, only one major band of T-antigen is usually seen in sodium dodecyl sulfate (SDS)-polyacrylamide gels of proteins from infected cells; some lines of transformed cells have additional forms. Isoelectric focusing shows the protein from infected cells to be heterogeneous (9). T-antigen is confined

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largely to the nucleus and is required for DNA replication, both viral DNA replication and induced host cell DNA replication (reviews in 18, 30). It seems to regulate its own synthesis by reducing transcription from the early region and stimulating transcription from the late region (1, 19). The antigen involved in cell-mediated rejection of SV40-transformed cells, TSTA, is related to SV40 T-antigen and is located in the cell membrane (2, 5, 10, 41). In the transformed cell, some of the T-antigen is in a complex with a cellular 53,000-dalton (53K) protein that may be important in transformation (22, 26). The ability of SV40 to facilitate adenovirus growth in monkey cells, the adeno-helper effect, is a function of T-antigen, specifically the C-terminal region (6, 12, 24).

Two viable mutants of SV40 that contain small deletions in the C-terminal region of T-antigen have been isolated after S1 nuclease cleavage of form I DNA: *dl*1263 has 33 nucleotides deleted at 0.20 MU, and *dl*1265 has 39 nucleotides deleted at 0.18 MU (7, 43). The T-antigen coded for by *dl*1263 migrates in SDS-polyacrylamide gels with an apparent size some 6,000 daltons less than wild-type T-antigen (6, 7); the expected reduction is about 1,350 daltons. The *dl*1265 T-antigen has about the same apparent molecular weight as simian virus 40-S (SV-S) T-antigen (a 600-dalton reduction is expected), but shows considerable trailing in gels (6). The adeno-helper activities of *dl*1263 and *dl*1265 are reduced by about 75 and 95%, respectively, although at high multiplicities substantial helper activity is detected. They transform efficiently (4). For both mutants, specific changes in the T-antigen protein can be predicted from the known DNA sequence given the assumption that no splicing of the mRNA occurs in the C-terminal region as a consequence of the deletions. No splices were initially detected in wild-type mRNA in this region (3), but D. Mark and P. Berg (Cold Spring Harbor Symp. Quant. Biol., in press) have recently suggested that, at late times, there is a splice at 0.20 to 0.21.

The research described in this paper was undertaken to identify the reading frame of T-antigen in the C-terminal region by comparing the tryptic peptides of the T-antigens of the deletion mutants with those of wild-type SV40 T-antigen. The DNA sequence (see Fig. 1) predicts a protein in one reading frame that has a C-terminal tryptic peptide with 6 prolines out of 11 residues, and indeed in a study of the proteins made by the adenovirus-SV40 hybrid virus Ad2$^+$ND1dp2, Fey et al. (12) detected a proline-rich tryptic peptide that appeared to be derived from the C-terminus. If so, this tryptic peptide should be altered by the *dl*1265 deletion but not the *dl*1263 deletion. However, since there is more than one region in the DNA sequence near the C-terminus of T-antigen that can code for a proline-rich tryptic peptide (nucleotides 2610 to 2640 and 2420 to 2450 of the sequence of Reddy et al. [37]), it is necessary to show unequivocally that *dl*1265 lacks the proline-rich tryptic peptide at the C-terminus of T-antigen. We show below that it does. Since there is an unexpectedly large reduction in the apparent molecular weight of *dl*1263 T-antigen, it is possible to argue that, possibly as the result of a new splice unique to this mutant, the protein has been altered to a greater extent than could be predicted simply from the DNA sequence. We show below that this is unlikely. Having identified C-terminal tryptic peptides of T-antigen, we then asked whether physically or functionally distinct forms of T-antigen possess the same C-terminus. Finally, because of the migration characteristics of the T-antigens from these mutants, it was possible to search for other proteins that might be encoded in this region of the DNA.

MATERIALS AND METHODS

Cells and virus. African green monkey kidney CV-1 cells were grown to early confluence in plastic petri dishes in E4 (Dulbecco modified Eagle medium) containing 10% calf serum. SV-S, *dl*1263, and *dl*1265 stocks were prepared as described (6). One day after replating, the cells were infected with virus at a multiplicity of about 50 and incubated at 37°C in 5% CO_2.

Radioactive labeling. Forty-eight hours after infection, the medium was removed. To label with L-[^{35}S]methionine (Amersham SJ.204), the monolayer was washed once with Tris-saline and then overlaid with a small volume (1.5 ml per 90-mm dish) of E4 medium lacking methionine. Radioactive methionine at a specific activity of about 1,000 Ci/mmol was added to a final concentration of 50 to 500 μCi/dish, and the cells were incubated for 2 to 4 h before harvesting. To label with [^{3}H]proline, the monolayer (in a 90-mm dish) was not washed but was drained well and overlaid with 1.5 ml of a mixture composed of 1 ml of E4 medium (lacking amino acids), 0.5 ml of [^{3}H]proline (0.5 mCi, 14 to 40 Ci/mmol; Amersham TRK.323 L-[5-^{3}H]proline), and 0.03 ml of calf serum. Cells were labeled for 3 to 4 h. To label with [^{35}S]cystine, the monolayer was rinsed once with E4 medium lacking both cysteine and methionine. L-[^{35}S]cystine (New England Nuclear NEG-020; about 500 Ci/mmol, 1 mCi/ml) was lyophilized and taken into an appropriate volume of 300-μg/ml solution of methionine. Proportions were adjusted so that each 90-mm plate contained 1.5 ml of E4 medium (lacking cysteine and methionine but with 2% calf serum) with 30 μg of methionine per ml and 0.5 to 1 mCi of [^{35}S]cystine.

Extraction and immunoprecipitation of T-antigen. All operations were done at 0°C unless specified otherwise; volumes given are the amounts used per 90-mm dish. At the end of the labeling period, the monolayers were chilled and lysed with 1.5 ml of 1% Nonidet

P-40 in 0.15 M NaCl–0.02 M Tris-hydrochloride, pH 8 (adjusted at room temperature), for 30 min. The lysate was transferred to a 15-ml centrifuge tube and centrifuged in the Sorvall HB4 rotor for 10 min at 10,000 rpm to pellet the nuclei; T-antigen is extracted from the nucleus under these conditions. The supernatant was combined with an equal volume of 0.15 M NaCl–5 mM EDTA–0.05 M Tris-hydrochloride (pH 7.4)–0.02% NaN_3–0.05% Nonidet P-40 (NET buffer) plus 0.2% bovine serum albumin. Hamster anti-T serum (100 μl) was added, and the mixture was incubated for 60 min. Washed (in NET buffer), Formalin-fixed *Staphylococcus aureus* Cowan 1 bearing protein A (SAC) (300 μl) was added, and incubation at 0°C was continued for an additional 15 min. The bacteria were removed by centrifugation at 8,000 rpm for 8 min and washed three times with NET buffer. The absorbed proteins were then eluted from the SAC by incubation at 70°C for 10 min in 200 μl of sample buffer (2% SDS, 50 mM Tris-hydrochloride, pH 6.8, 10% glycerol, and 0.001% bromophenol blue) containing 0.1 M dithiothreitol. The SAC were pelleted by centrifugation for 2 min in a Beckman Microfuge, and the supernatant was carefully withdrawn. The solution was heated at 100°C for 2 min and then stored at −70°C.

Preparative gel electrophoresis. SDS-polyacrylamide slab gels were 2 mm thick and made with 15% acrylamide and 0.075% bisacrylamide. A stacking gel was used according to the procedures described by Laemmli (21). The gels were electrophoresed at 100 V for 5 to 6 h. Wet gels were autoradiographed overnight, and the bands of T-antigen were excised from the appropriate region of the gel. For the [^{3}H]proline-labeled preparations, appropriately placed external ^{35}S-labeled T-antigen markers were used to locate the position of the T-antigen.

Generation of tryptic peptides. These procedures were adapted from those used by Smart and Ito (40). Gel slices were washed well (several hours, several changes) with destain buffer (25% methanol–7.5% acetic acid) to remove chloride ions, minced by two vigorous passages through a 5-ml syringe, and dried. The protein in the gel was oxidized for 2 h at −5°C with performic acid (17). To each dried gel mince was added 1.5 to 2 ml of a solution composed of 10 ml of freshly prepared performic acid, 4 ml of formic acid, and 1 ml of methanol. After completion of the oxidation, 15 ml of cold water was added, and the solution was frozen and lyophilized. The dried sample was combined with 5 ml of water and lyophilized a second time. Five milliliters of 0.1 M NH_4HCO_3 and 5 μl of a 10-mg/ml (in water) solution of trypsin treated with L-(tosylamido 2-phenyl) ethyl chloromethyl ketone (Worthington Biochemicals Corp., trypsin-TPCK) was added to the oxidized and lyophilized gel mince, and the mixture was incubated at 37°C with gentle agitation for 18 to 24 h. The gel pieces were removed by filtration through a 0.22-μm membrane filter (Millipore GSWP). Siliconized glass fiber GF/C filters were used in later experiments and may give a better yield of certain peptides, but the major spots were not different. The gel pieces were washed once with an additional 3 ml of 0.1 M ammonium bicarbonate, and the filtrates were combined. An additional 5 μl of a 10-mg/ml trypsin-TPCK solution was added to the filtrate, which was then incubated for 3 to 5 h at 37°C. The solution was frozen and lyophilized. Two additional lyophilizations from 5 ml of water each time were performed to remove the bicarbonate.

Chromatography on Chromobead-P resin. Tryptic peptides (in 200 to 400 μl of buffer A plus 10% formic acid) were fractionated on a column (0.4 by 24 cm) of Chromobead-P cation-exchange resin (Technicon Chemicals S.A.) at 53.5°C. The starting buffer (buffer A: 30 ml of formic acid, 3 ml of pyridine, 567 ml of water) was 1.32 M formic acid–0.62 M pyridine, pH about 2.2. The column was eluted with about 180 ml of an approximately exponential pH gradient running from 2.2 to about 5.5 and generated in a nine-chambered Buchler mixing device, using the first four chambers only. The first three chambers were each filled with 50 ml of buffer A, and the fourth chamber was filled with 50 ml of 3.1 M pyridine (buffer B: 75 ml of pyridine, 225 ml of water). One hundred and eighty fractions of 60 drops each (about 0.9 ml) were collected at a rate of about one fraction every 5 to 10 min. The flow rate and drop size decreased gradually during the run. Portions of 0.8 ml were assayed for radioactivity with 7.2 ml Aquasol (New England Nuclear Corp.) in a scintillation spectrometer with appropriate settings. The increase in quenching was about 10% at the highest pyridine concentration attained.

Two-dimensional electrophoresis and chromatography. The desired amount (2 to 20 μl) of tryptic peptides in 0.1 M acetic acid was spotted onto prewashed cellulose plates (Merck; 0.1-mm cellulose thin-layer chromatography plates). Electrophoresis in the first dimension was performed at 500 V and 21°C with an acetic acid-formic acid mixture at pH 2.05 (320 ml of glacial acetic acid, 80 ml of formic acid, 3,600 ml of water). Duration of the run varied for different separations. The chromatogram was developed by ascending chromatography at room temperature in the second dimension with a butanol-acetic acid-water-pyridine (75:15:60:60) buffer. Dried chromatograms were autoradiographed on Kodak SB 5 film. Autoradiograms are oriented so that positively charged species are migrating toward the left (the cathode).

Transformation of cells. Monolayers of BALBc 3T3 A31 mouse cells were infected with a high multiplicity of virus for several hours, trypsinized, and plated in soft agar (27). One month later colonies were picked, grown up, and tested for the presence of T-antigen by immunofluorescence and by immunoprecipitation followed by gel electrophoresis and autoradiography. Selected clones of cells transformed by SV-S, *dl*1263, and *dl*1265 were analyzed in more detail. One of the two *dl*1265 T-antigen clones (C2) analyzed in this paper contained two species of T-antigen, one with a somewhat lower mobility in gels than normal (super-T-antigen).

RESULTS

The C-terminal tryptic peptide is proline rich. Figure 1 shows the nucleotide sequence of the E strand of SV40 DNA extending from near the C terminus of VP1 into the C-terminal region of T-antigen. The C-terminal tryptic peptide of

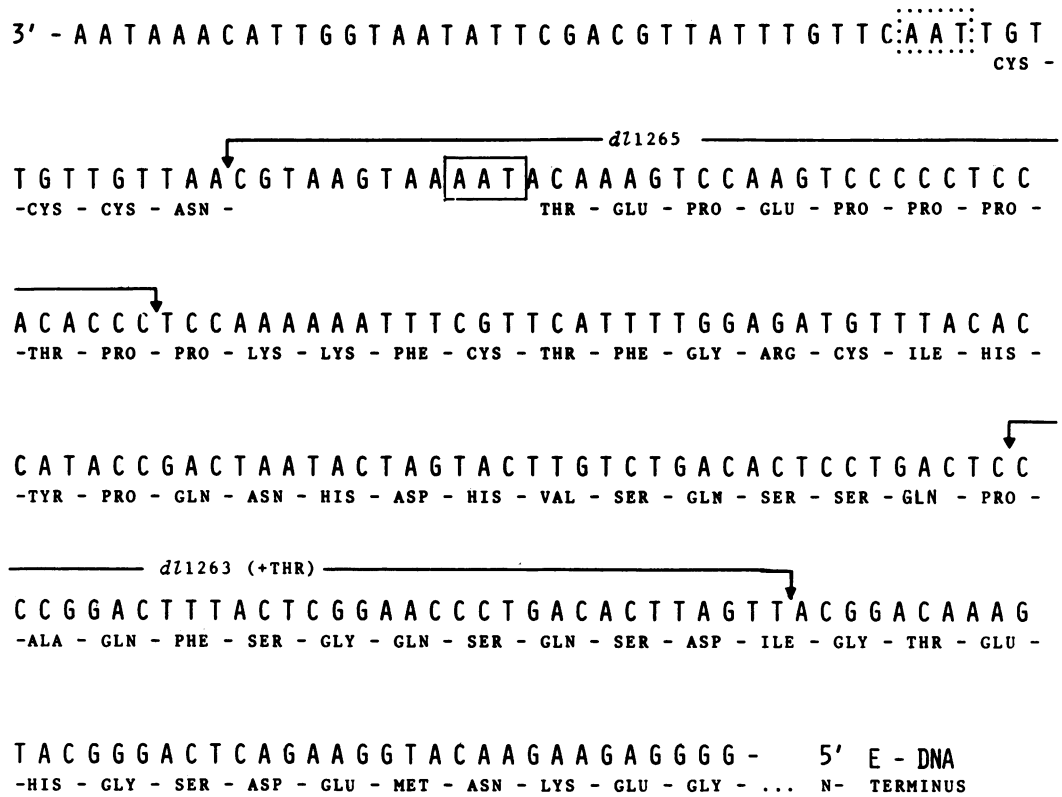

FIG. 1. *Sequence of nucleotides of the E strand of SV40 DNA (nucleotides 2551 to 2787 of the Reddy et al. [37] sequence) around the C-terminus of T-antigen. The TAA at the extreme 3' end is a potential terminator preceded by 91 in phase codons before an in-phase initiator at 2829; however, no protein corresponding to the sequence has yet been reported. Downstream (in a 5'-to-3' direction) by 46 and 53 nucleotides from this TAA sequence (and not shown in the figure) are, respectively, the 3' end of the major species of early mRNA (at 2505) and the C-terminus of VP1 (at 2497). Upstream are the TAA codons predicted to terminate translation of the dl1265 T-antigen (dotted box) and of wild-type T-antigen (solid box). The amino acids encoded upstream of these terminators are indicated below the sequence. The extents of the deletions in the mutants dl1265 and dl1263, as determined by van Heuversyn et al. (43), are indicated by arrows. The ends of the dl1263 deletion fall within codons, resulting in the formation of the codon for threonine.*

T-antigen is anticipated to contain 6 proline residues out of a total of 11 amino acids (a lysine-proline bond is relatively resistant to trypsin). The sequence indicates that *dl*1265 will lack that particular peptide. The *dl*882 deletion is a three-base deletion located at the *Hin*dII-GB junction shortly after the termination of T-antigen in the region where *dl*1265 T-antigen terminates; it should not affect the peptides derived from the wild-type T-antigen (W. Fiers, personal communication).

The complete set of proline-containing, cysteine-containing, and methionine-containing tryptic peptides predicted to be produced by complete trypsin hydrolysis of T-antigen is compiled in Table 1. We assume that the frequency of chymotryptic (or other nontrypsin) cleavages is low enough to be ignored. The peptides are predicted by the data (13, 37) on the assumption that only the 0.600-to-0.533 splice is present. There are 20 methionine-, 13 cysteine-, and 19 proline-containing peptides, which altogether include 503 of the 708 amino acids believed to compose T-antigen. We used two widely used techniques to fractionate these tryptic peptides of T-antigen: one-dimensional cation-exchange chromatography on columns of Chromobead-P resin (a sulfonated polystyrene), and two-dimensional electrophoresis and chromatography on cellulose thin layers.

Figure 2 shows a set of four chromatograms of the [^{3}H]proline-labeled tryptic peptides of T-antigen resolved on a Chromobead-P column. SV-S, *dl*882, and *dl*1263 yielded very similar patterns with seven major peaks, in addition to the flowthrough, clearly resolved; three minor peaks eluting after the last major peak were also observed reproducibly. None of these peaks cor-

TABLE 1. *Predicted tryptic peptides of SV40 T-antigen containing methionine, cysteine, or proline*

Tryptic peptide[a]	No. of amino acids	Residue			Tryptic peptide	No. of amino acids	Residue		
		Methionine	Cysteine	Proline			Methionine	Cysteine	Proline
1–3	3	1	0	0	305–307	3	0	1	0
8–21	14	1	0	0	309–315	7	0	0	1
22–31	10	1	0	1	335–347	13	0	1	0
38–39	2	0	1	0	358–371	14	1	0	0
40–45	6	0	0	1	372–400	29	3	1	1
52–53	2	1	0	0	401–410	10	1	0	0
55–60	6	1	0	0	411–418	8	1	1	1
62–67	6	1	0	0	426–432	7	0	0	1
68–127	60	1	1	5	433–446	14	0	1	0
132–136	5	0	0	1	447–456	10	0	0	1
137–154	18	0	0	1	484–498	15	0	0	1
155–165	11	0	1	0	518–535	18	1	0	3
175–178	4	1	0	0	544–550	7	0	0	1
187–202	16	0	0	1	555–559	5	0	1	0
215–224	10	0	2	0	568–595	28	1	0	1
229–238	10	1	0	0	615–616	2	1	0	0
239–253	15	0	0	2	617–630	14	1	0	0
254–266	13	0	0	1	653–691	39	1	1	2
272–281	10	1	0	0	692–697	6	0	1	0
282–304	23	2	2	0	698–708	11	0	0	6

[a] Each peptide is identified by the position of its N- and C-terminal amino acids, starting with the N-terminal amino acid in the protein as specified by the sequence data of Reddy et al. (37) and Fiers et al. (13). Peptides 653 through 691 and 698 through 708 are the ones prediced to be altered by the *dl*1263 and *dl*1265 deletions, respectively.

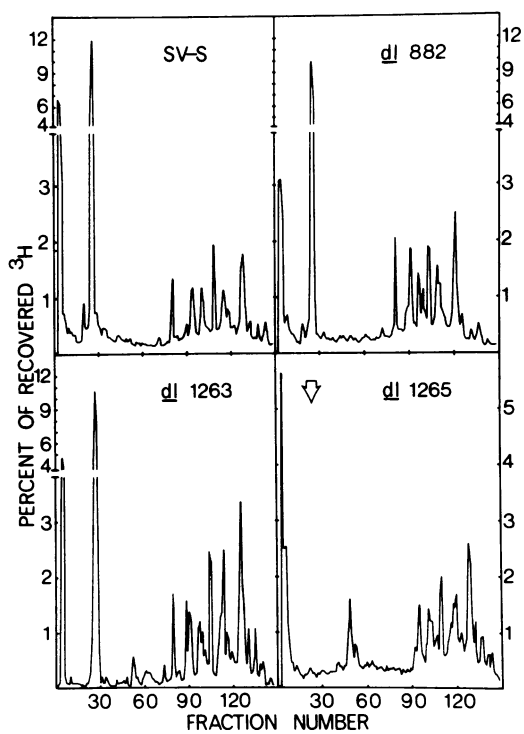

FIG. 2. *Chromobead-P elution profiles of [^{3}H]proline-labeled tryptic peptides of T-antigen coded for by SV-S, dl882, dl1263, and dl1265. Peptides were prepared, the columns were loaded and eluted, and the radioactivity was determined as described in Materials and Methods. The profiles have been normalized, and the percentage of the total recovered radioactivity in each fraction is shown. Recovered radioactivity, about 50% of the input, from each column was: SV-S, 1 × 10^4 cpm; dl882, 2.9 × 10^4 cpm; dl1263, 1.1 × 10^4 cpm; dl1265, 3.2 × 10^4 cpm. The flowthrough is in fractions 3 through 5. Note the change in scale of the SV-S, dl882, and dl1263 profiles.*

responded to free proline, which eluted between fractions 37 and 41. The amount of radioactivity in each of the seven peaks is given in Table 2, and it can be seen that there was five to seven times more radioactivity in the first peak than in the others. This suggests that it is the expected C-terminal peptide. It is not possible to be more quantitative because (i) the terminal peptide may be recovered in especially high yield since only one cleavage is required; (ii) some peptides may be underrepresented due to poor solubility; and (iii) one or more peaks may represent a mixture of peptides.

The *dl*1265 T-antigen did not yield the peptide in peak 1, providing confirmation that this proline-rich peptide is indeed the C-terminal peptide (698 to 708 in Table 1). This is evidence that the reading frame in this region is that given in Fig. 1 and predicted by van Heuverswyn et al. (43). There was a new peak present around fraction 50, and the sharp peak around fraction 80 was missing. These differences were repro-

TABLE 2. *Radioactivity in Chromobead-P peaks of [^{3}H]proline-labeled tryptic peptides*[a]

Peak	Radioactivity (cpm)			
	SV-S	*dl*882	*dl*1263	*dl*1265
1	2,100	5,500	3,100	—
2	260	900	280	—
3	300	1,100	550	1,300
4	350	1,300	470	1,700
5	390	1,200	540	1,200
6	470	1,700	870	2,500
7	630	2,100	1,000	2,700

[a] In the four experiments, the seven peaks were within fractions 24 through 28, 78 through 81, 88 through 96, 96 through 104, 103 through 110, 108 through 121, and 119 through 131. For each experiment, the fractions around the peaks were summed and background was subtracted. Recoveries of some peptides are likely to be less than 1 M; the relatively broad peak 6 is likely to be composed of two overlapping peptides. There are in theory five tryptic peptides (see Table 1) that have more than one proline residue, but the three largest are likely to be excluded from, or trapped on, the column. Peak 7 may be peptide 239 through 253 (Table 1) because of its size and sharpness; we did not perform a double label (with [^{35}S]methionine or [^{35}S]cysteine) to verify the absence of these amino acids.

duced in a second run of this digest and in a second independent preparation.

New cysteine-containing peptides in *dl*1263 and *dl*1265. The nucleotide sequence data allow specific predictions to be made about the changes in tryptic peptide patterns consequent upon the deletions. There should be only one change, a shift in the position of one peptide, in the *dl*1263 pattern. Since the peptide contains both cysteine and methionine, this change should be seen with either ^{35}S-labeled amino acid. Figure 3 shows two pairs of chromatograms of *dl*1263 T-antigen tryptic peptides, one set labeled with [^{35}S]methionine (a, b) and one set labeled with [^{35}S]cysteine (c, d). The only reproducible difference we could detect in the *dl*1263 preparations is the presence of one new peptide (arrows in Fig. 3b and 3d). This peptide was present in small amounts, perhaps because of low solubility. That both amino acid labels produced a spot in the same position is evidence for the validity of the assignment (only four other peptides contained both amino acids, and one of these was probably too large to be detected). The solubility of the corresponding larger peptide in SV-S may make it difficult to detect, but a possible candidate is indicated by the arrow in Fig. 3c.

The sequence obtained by van Heuverswyn et al. (43) and given in Fig. 1 predicts that there should be a prominent new cysteine-containing peptide from the C-terminus of *dl*1265 T-antigen. The other tryptic peptides should be the same as those from SV-S T-antigen. The C-terminal tryptic peptide of SV-S T-antigen contained neither cysteine nor methionine. The autoradiograms shown in Fig. 4 reveal two strong new spots, one electrophoresing with a negative charge at pH 2.1 and both chromatographing only a short distance in the second dimension. These two spots probably represent the same peptide with all, or all but one, of the cysteine residues oxidized (see Discussion); multiple spots could also arise because of the two adjacent lysines at the N-terminus of the C-terminal tryptic fragment.

The Chromobead-P elution profiles of the [^{35}S]cysteine-labeled tryptic peptides of SV-S and *dl*1265 T-antigens were similar to each other (Fig. 5). According to Table 1, there are nine cysteine-containing tryptic peptides possessing fewer than 20 amino acids; eight peaks are clearly evident in the chromatogram. The cysteine-containing peptides characteristic of *dl*1265, and four other regularly observed peptides, did not stick to the negatively charged resin and were present in the flowthrough (see Fig. 6d). Thus, the new proline-containing peptide of *dl*1265 T-antigen seen at fraction 50 in Fig. 2 is not the new C-terminal cysteine-rich peptide (which should contain one proline residue).

There were a number of negatively charged cysteine-containing peptides at pH 2.1 (Fig. 6a), presumably the result of the negatively charged cysteic acid residues. Surprisingly, the one prominent methionine-containing peptide that migrated with a negative charge at this pH did not correspond to any of the cysteine-containing peptides (Fig. 6b, c), nor did it contain phosphate (data not shown). Why it possessed a negative charge at this pH is not evident. Several positively charged peptides appeared in similar positions in the autoradiograms of the cysteine- and methionine-labeled peptides.

Electrophoretic behavior of *dl*1263 and *dl*1265 T-antigens. From the DNA sequence, one can predict that the T-antigens of *dl*1263 and *dl*1265 should be reduced in size by about 1,350 and 600 daltons, respectively, in comparison with SV-S T-antigen (predicted size of 85,000 daltons); indeed, in SDS-polyacrylamide gel electrophoresis both proteins were found to migrate more rapidly than SV-S T-antigen. This is illustrated in Fig. 7, which shows autoradiograms of 10.5 and 9.0% gels (Fig. 7a and 7b; respectively). The proteins were alkylated with *N*-ethylmaleimide to prevent sulfhydryl interactions. Depending upon the conditions and the gel concentration, the *dl*1263 T-antigen (lane 4)

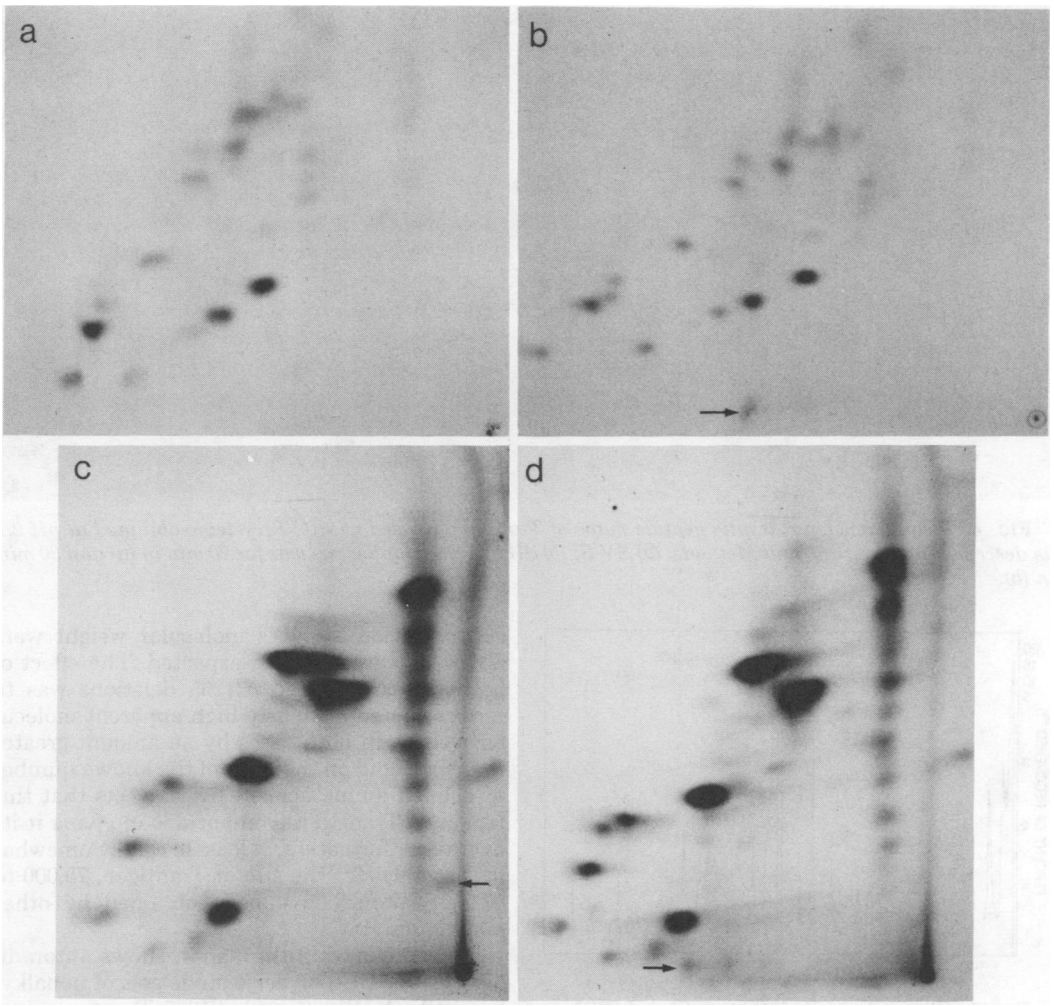

FIG. 3. *Two-dimensional tryptic peptide maps of T-antigen obtained at pH 2.1 as described in Materials and Methods. (a) SV-S [^{35}S]methionine labeled; (b) dl1263 [^{35}S]methionine labeled; (c) SV-S [^{35}S]cysteine labeled; (d) dl1263 [^{35}S]cysteine labeled. The arrows in b and d indicate the new spot observed in dl1263 fingerprints. This peptide was observed in low amounts in every preparation of methionine- and cysteine-labeled peptides (two each). The arrow in c indicates a candidate for the peptide 653-659 in SV-S. This spot was not seen in the methionine-labeled preparations or the earlier cysteine-labeled preparations, perhaps because they had been passed through nitrocellulose filters; siliconized glass fiber filters were used in this experiment. Electrophoresis was for 55 min. The anode is on the right.*

showed a mobility increase relative to SV-S (lane 3) that corresponded to an apparent size reduction of 2,000 to 6,000 daltons; the decrease in the mobility of the *dl*1265 T-antigen (lane 2) was just detectable. There was a distinct band at a higher molecular weight position than T-antigen in both the SV-S and *dl*1263 patterns (longer exposures revealed a similar band in *dl*1265). In these gels we did not detect any other polypeptides whose size was affected by these deletions. The track of proteins from uninfected cells (lane 1) indicates the degree of radiochemical purity of the T-antigen used in these studies.

We used an approach for determining apparent molecular weights that is based on a calculation of the molecular weight from the retardation coefficient, K_R, in the Ferguson equation log (RF) = log (Y_0) − $K_R T$. Y_0 and K_R are measures of molecular free mobility and molecular size, respectively; T is the percentage acrylamide in the gel (16). Provided that the conformations of the proteins are similar, the molecular weight can be estimated from protein stand-

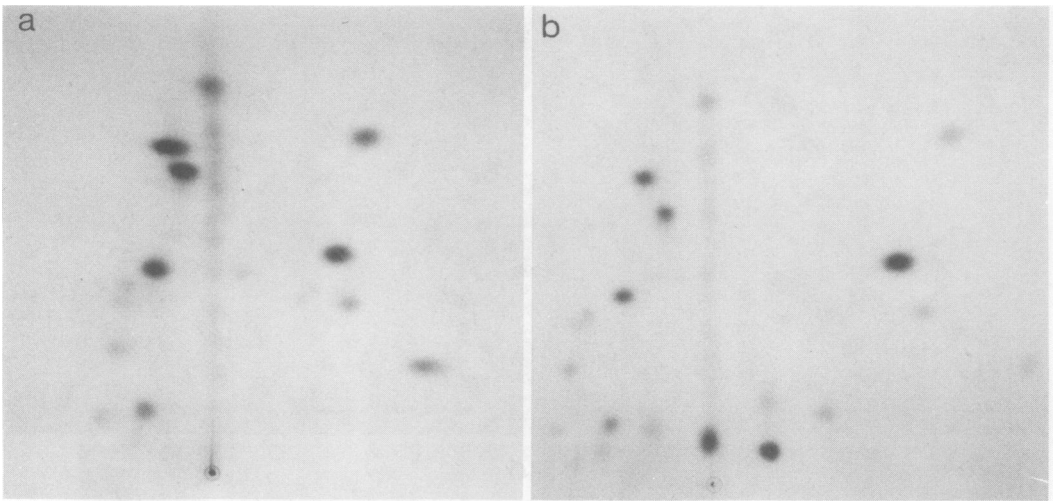

FIG. 4. *Two-dimensional tryptic peptide maps of T-antigen labeled with [^{35}S]cysteine obtained at pH 2.1 as described in Materials and Methods. (a) SV-S; (b) dl1265. Electrophoresis was for 20 min in (a) and 30 min in (b).*

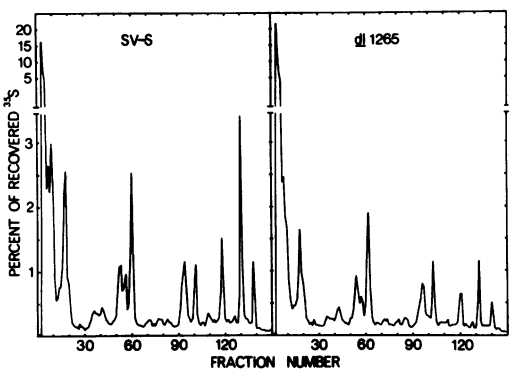

FIG. 5. *Chromobead-P elution profiles of [^{35}S]cysteine-labeled peptides of T-antigen coded for by SV-S and dl1265. Peptides were prepared, the chromatography was performed, and the radioactivity was determined as described in Materials and Methods. The profiles are normalized, and the percentage of the total recovered radioactivity in each fraction was determined. Recovered radioactivity, about 50% of the input for each column, was: SV-S, 1.1 × 10^5 cpm; dl1265, 4.8 × 10^4 cpm.*

ards with the assumption that it is linearly related to K_R.

The results of an analysis of T-antigen using this approach are given in Table 3. The anomalous behavior of T-antigen is striking, indicating a higher apparent molecular weight than that of the more slowly migrating phosphorylase a, and confirms that its conformation in SDS is different from that of the standard protein markers. Others have commented on this (e.g., Rundell et al. [38]). It is retarded by increasing acrylamide concentration as if its molecular weight were about 35% greater than expected. The effect of both the *dl*1263 and *dl*1265 deletions was to reduce the anomalously high apparent molecular weight, in both cases by an amount greater than expected on the basis of the known number of deleted amino acids. This suggests that this region of T-antigen is important in giving it its overall conformation, at least in SDS. Somewhat lower values for the size of T-antigen, 75,000 to 80,000 daltons, have been obtained by other techniques (15).

Figure 8, lanes 2 through 4, shows autoradiograms of SDS-polyacrylamide gels of nonalkylated SV-S, *dl*1263, and *dl*1265 T-antigens, respectively, immunoprecipitated from infected cells. The proteins were labeled with [^{35}S]methionine; they were portions of the same preparations that were alkylated and electrophoresed in the gels shown in Fig. 7. The T-antigen of *dl*1265 characteristically migrated as a diffuse band at a higher molecular weight position in the gel than was usual for T-antigen; alkylation abolished this trait. We presume that transient interactions of the cysteines at the C-terminus of one protein molecule with cysteines elsewhere in the same or another molecular result in an aggregation or trapping of the protein that retards its passage through the gel.

Characteristics of T-antigen in transformed cells. It is important to know whether the primary structure of T-antigen in the transformed cell is the same as in permissive infection, and to this end we asked whether the characteristic C-terminal peptides identified above are present in the T-antigen present in transformed

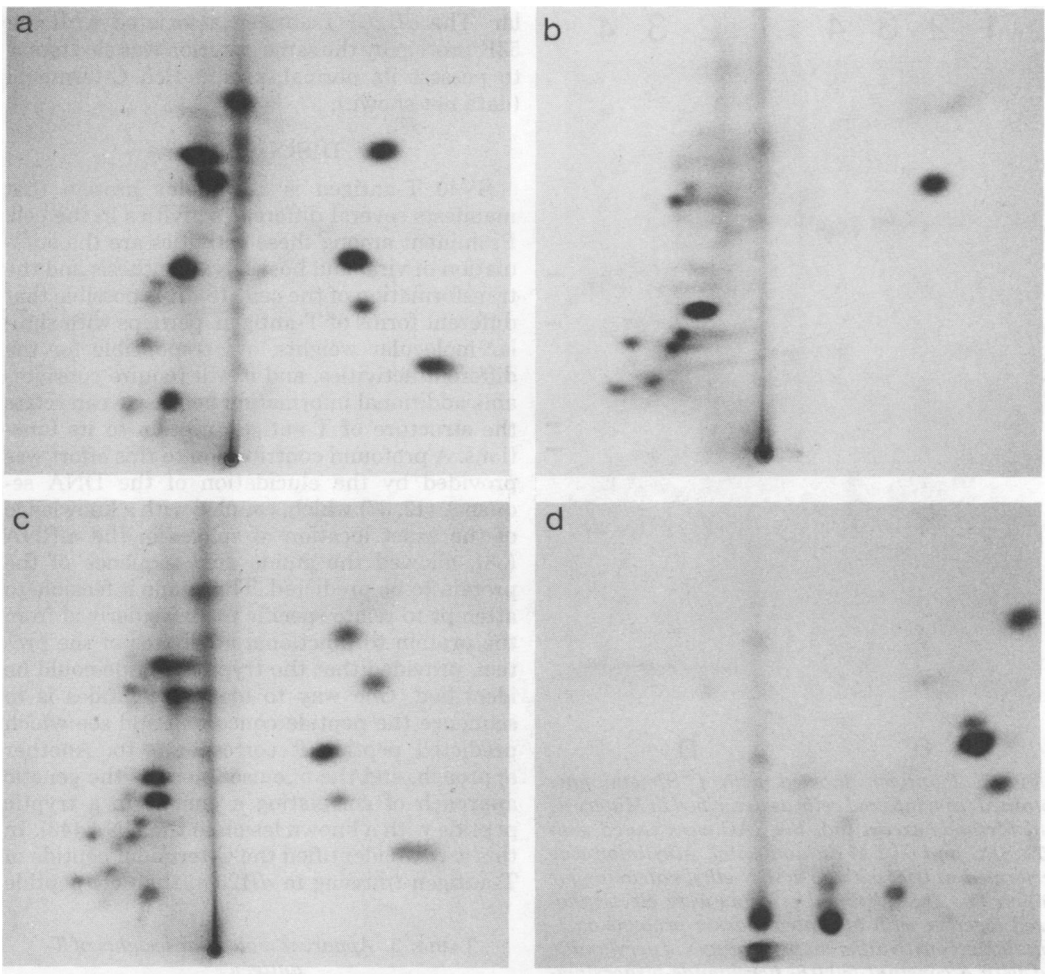

FIG. 6. *Two-dimensional tryptic peptide maps of [^{35}S]cysteine- and [^{35}S]methionine-labeled SV-S T-antigen from infected CV-1 cells. Electrophoresis was for 20 min in (a), (b), and (c). (a) [^{35}S]cysteine-labeled peptides; (b) [^{35}S]methionine-labeled peptides; (c) a mixture of (a) and (b). (d) Peptides present in the flowthrough of a Chromobead-P column of [^{35}S]cysteine-labeled tryptic peptides derived from T-antigen purified from CV-1 cells infected with dl1265. Electrophoresis was for 30 min.*

cells. The presence of the proline-rich C-terminal tryptic peptide of T-antigen present in a clone of 3T3 A31 cells transformed by *dl*1263 is shown in Fig. 9 (fractions 29 and 30). There was a general similarity between the other peaks and the peaks generated from T-antigen produced in infected cells (see Fig. 2), but the finer details were obscured by the lower yield and generally higher background obtained in preparations of T-antigen from transformed cells. We conclude that the major form of T-antigen in these cells has the normal *dl*1263 C-terminus.

We looked in more detail at the proteins present in two clones of *dl*1265 transformants and immunoprecipitated with anti-53K protein serum (F5), two different anti-T sera, and control antisera. Although the F5 antiserum reacts specifically with the 53K protein and not directly with purified T-antigen (F. McCormick, F. Chaudry, R. Harvey, R. Smith, P. W. Rigby, E. Paucha, and A. E. Smith, Cold Spring Harbor Symp. Quant. Biol., in press), it nevertheless will precipitate T-antigen because T-antigen and the 53K protein occur as a complex in transformed cells (22). The proteins were electrophoresed on SDS-polyacrylamide slab gels (Fig. 10) both with and without alkylation to determine whether there were any species other than the major 95K protein that could be identified by virtue of their lacking the cysteine-rich C-terminus. These gels lead us to the conclusion that T-antigen associated with the 53K protein and

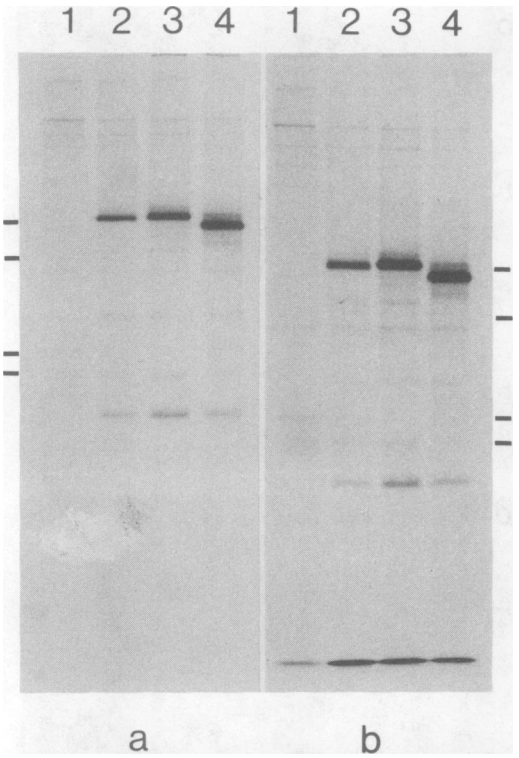

FIG. 7. *T-antigen labeled with [^{35}S]methionine obtained from infected cells as described in Materials and Methods except that the SAC were eluted with 1.5% SDS and 0.02 M dithiothreitol. Alkylation was performed at 0°C with 66 mM N-ethylmaleimide for 60 min (9). The alkylated proteins were electrophoresed together with alkylated marker proteins on 1-mm slab gels with different percentages of acrylamide but with a constant (30:0.8) acrylamide-bisacrylamide ratio. (a) 10.5% acrylamide; (b) 9.0% acrylamide. Channels 1 through 4 represent extracts from mock-infected cells and from cells infected with dl1265, SV-S, and dl1263, respectively. Lines at the sides indicate, from top to bottom, the positions of phosphorylase a (97.4K), transferrin (80K), catalase (60K), and glutamate dehydrogenase (53K).*

precipitated by a specific anti-53K serum (F5, lanes 1 and 6) retains the normal cysteine-rich *dl*1265 C-terminus because there was no band in the position of T-antigen in the unalkylated gels. Proteins in lanes 2, 5, 7, and 10 were brought down with normal sera; proteins in lanes 3, 4, 8, and 9 were precipitated with anti-T sera.

We also investigated the [^{35}S]cysteine-labeled tryptic peptides present in the *dl*1265 T-antigen both associated with and not associated with the host-coded 53K protein on the basis of its sedimentation behavior (McCormick et al., in press). Two-dimensional fingerprints of these peptides (Fig. 11) clearly show that they were very similar. The *dl*1263 T-antigen associated with the 53K protein by the same criterion was also found to possess its normal, proline-rich C-terminus (data not shown).

DISCUSSION

SV40 T-antigen is a complex protein that manifests several different activities in the cell. Prominent among these activities are the stimulation of viral and host DNA synthesis and the transformation of the cell (18). It is possible that different forms of T-antigen, perhaps with similar molecular weights, are responsible for the different activities, and it will require considerable additional information before we can relate the structure of T-antigen protein to its functions. A profound contribution to this effort was provided by the elucidation of the DNA sequence (13, 37) which, coupled with a knowledge of the exact location of splices in the mRNA (36), allowed the amino acid sequence of the protein to be predicted. This made it feasible to attempt to relate specific peptides derived from the protein to functional attributes of the protein, provided that the tryptic peptide could be identified. One way to identify peptides is to sequence the peptide concerned and see which predicted peptide it corresponds to. Another approach, and the one used here, is the genetic approach of correlating a change in a tryptic peptide with a known lesion in the DNA (43). In this way we identified the C-terminal peptide of T-antigen (missing in *dl*1265), the new peptide

TABLE 3. *Apparent molecular weights of T-antigen*[a]

Protein	Mol wt	Y_0	K_R
Glutamate dehydrogenase	53,000	3.673	0.084
Catalase	60,000	3.640	0.087
Transferrin	80,000	2.851	0.091
Phosphorylase a	97,400[b]	2.704	0.098
*dl*1265 T-antigen	109,000	2.944	0.101
*dl*1263 T-antigen	112,000	3.119	0.102
SV-S T-antigen	115,000	3.013	0.103

[a] Values of Y_0 and K_R were calculated by a least-squares fit of the Ferguson equation (see text), using the RF values determined at four different gel concentrations (T = 7.5, 9.0, 10.5, and 12%). Portions of two of the slab gels are shown in Fig. 7. Each preparation was electrophoresed in two channels, and the markers (glutamate dehydrogenase, catalase, transferrin, phosphorylase a) were electrophoresed in three channels; RF values were averaged. The molecular weights of T-antigen were then calculated from a least-squares fit to the equation molecular weight = $a + b(K_R)$, using the four standards to determine the values of a and b.

[b] From reference 42.

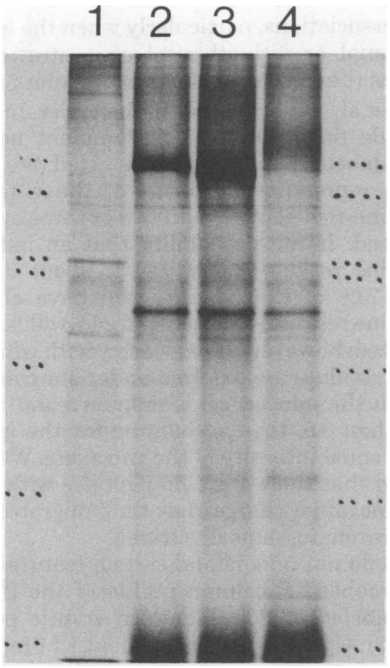

FIG. 8. *Unalkylated [^{35}S]methionine-labeled immunoprecipitated proteins electrophoresed on SDS-polyacrylamide gels, stained, and autoradiographed. The preparations were portions of the same samples that were alkylated and electrophoresed as shown in Fig. 7; gels contained 12% acrylamide. (a) Mock infected; (2) SV-S infected; (3) dl1263 infected; (4) dl1265 infected. The dotted lines indicate, from top to bottom, the positions of phosphorylase a (97.4K), transferrin (80K), catalase (60K), glutamate dehydrogenase (53K), alcohol dehydrogenase (41K), carbonic anhydrase (29K), and soybean trypsin inhibitor (21.5K).*

resulting from the *dl*1263 deletion, and the new C-terminal peptide of *dl*1265.

One conclusion from this work is that the reading frame used in the C-terminal region of T-antigen is that hypothesized by Fiers et al. (13) and Reddy et al. (37). This is consistent with the fact that the early mRNA terminates before the second of the two regions that could code for a proline-rich peptide is reached (36). Since the deletion in *dl*1265 is a multiple of three nucleotides, it will not cause a shift in the reading frame; had the proline-rich peptide been derived from the 2420-to-2450 region, the *dl*1265 T-antigen would have been expected to retain it.

Because the T-antigen from *dl*1263 retains the C-terminal proline-rich peptide, it appears that the large reduction in its apparent molecular weight is not the result either of an acquired splice that deletes all or part of the usual C-terminus of T-antigen or of proteolytic degradation of the C-terminus of the mutant protein. The presence of an internal splice unique to *dl*1263 still remains a possibility. However, the facts (i) that only one new peptide was detected, (ii) that a number of two-dimensional fingerprints of [^{35}S]cysteine- and [^{35}S]methionine-labeled tryptic peptides generated in different ways revealed no other changes (data not shown), and (iii) that the Chromobead-P pattern in Fig. 2 showed no differences between *dl*1263 and SV-S [^{3}H]proline-labeled peptides argue against this possibility. The suggestion (6) that the faint band seen in preparations of the *dl*1263 T-antigen at about the position of SV-S T-antigen corresponded to the "true" (unspliced) *dl*1263 T-antigen and that the major species of T-antigen resulted from the introduction of a new splice seems less likely in view of the presence of a similar faint band at a correspondingly higher molecular weight position in gels of alkylated SV-S T-antigen (Fig. 7). The simplest interpretation is that the effect of the *dl*1263 deletion, and *dl*1265 also, is to remove some of the conformational "anomaly" of the major species of T-antigen so that it migrates closer to its "correct" position in gels.

The behavior of the *dl*1265 T-antigen in gels is diagnostic. When not alkylated, some of the protein tended to trail behind the main band of

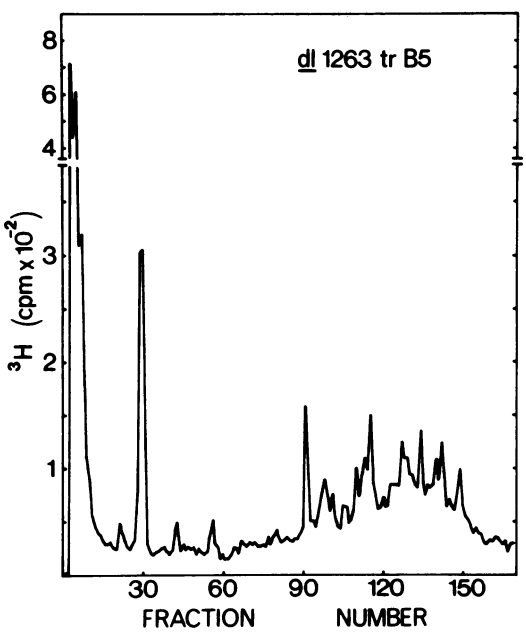

FIG. 9. *Chromobead-P chromatography of the [^{3}H]proline-labeled tryptic peptides of T-antigen purified from 3T3 cells transformed by dl1263 (clone trB5). Conditions were slightly different from those used in Fig. 2, and the peak at fraction 90 corresponds to the peak seen at a slightly earlier position in the chromatograms of Fig. 2.*

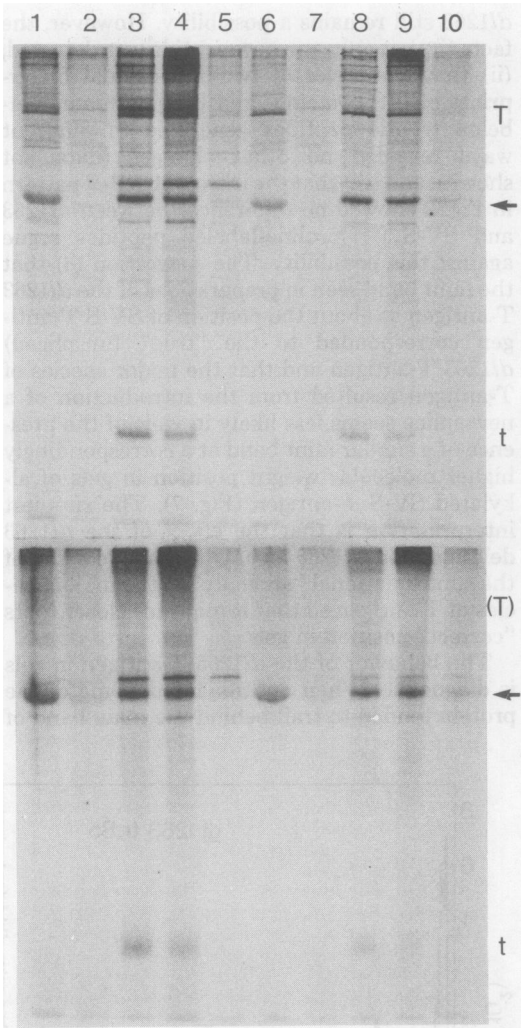

FIG. 10. *Autoradiograms of SDS-polyacrylamide gels of [^{35}S]cysteine-labeled proteins immunoprecipitated from two clones (C2 and C8 in tracks 1 through 5 and 6 through 10, respectively) of 3T3 cells transformed by dl1265. Samples were run with (upper panel) and without (lower panel) alkylation by N-ethylmaleimide. The various tracks contain proteins immunoprecipitated with F5 anti-53K serum raised in rabbits against denatured, gel-purified 53K protein (1, 6); normal rabbit serum (2, 7); no. 17 anti-T hamster serum (3, 8); no. 23 anti-T hamster serum (4, 9); and normal hamster serum (5, 10). T and t indicate positions of T-antigen and t-antigen, respectively. Arrows indicate the position of the host-coded 53K protein.*

T-antigen. This was often quite severe (Fig. 8). When alkaylated, however, the protein behaved normally; also, in the less cross-linked preparative gels there was less trailing. We presume that the C-terminus is exposed and the cysteines are available to form transient intra- or intermolecular associations, particularly when the mercaptoethanol or dithiothreitol concentrations are low, as they are after the protein enters the gel. Cole et al. (6) also noted this behavior. In tryptic peptide fingerprints, two prominent new cysteine-labeled peptides were detected. We believe these represent two forms of the C-terminal cysteine-rich peptide with two or three cysteines oxidized. If the probability that an individual cysteine residue is oxidized is about 0.9 (17), then 73% of the peptides will have all three cysteine residues oxidized and 24% will have two oxidized; however, the efficiency with which cysteine residues are oxidized under our conditions within the minced gel is unknown and may be less than 0.9, thus accounting for the approximate equal intensity of the two spots. We determined that there were no peptides with such a high negative charge that they migrated off of the chromatograms illustrated.

We do not understand certain features of the Chromobead-P column profile of the [^{3}H]proline-labeled *dl*1265 T-antigen tryptic peptides (Fig. 2). One is a new peak, about 1 M in proline residues, that was not evident at that position in the [^{35}S]cysteine-labeled profiles (the terminal peptide contained one proline and three cysteines [actually cysteic acid after oxidation] and was in the flowthrough). Also, the peak at fraction 80 was missing. The most economical hypothesis is that the new peak represented a peptide that was a different form of the peptide which was missing. Further studies with these and other deletions should help to clarify the situation.

There were several peptides from SV-S T-antigen that migrated with a negative charge at pH 2.1. The methionine-containing peptide possessed neither cysteine nor phosphate, and we do not know what caused its negative charge. There were four cysteine-containing peptides, some of which may be related to each other. Cysteic acid is presumably responsible for some of their negative charge; none of them corresponded to free cysteic acid (data not shown).

The major species of T-antigen found in transformed cells, either associated or not associated with the host-coded 53K protein, had the same C-terminal tryptic peptide as found in the T-antigen synthesized in a permissive cell. Additionally, the [^{35}S]cysteine-labeled tryptic peptides of the 53K-associated and the free T-antigen yielded fingerprints similar to each other and to the fingerprint obtained for T-antigen in a lytic infection. In these transformed cells, about half of the T-antigen appeared to be associated with the 53K protein and about half appeared to be free. Association of T-antigen

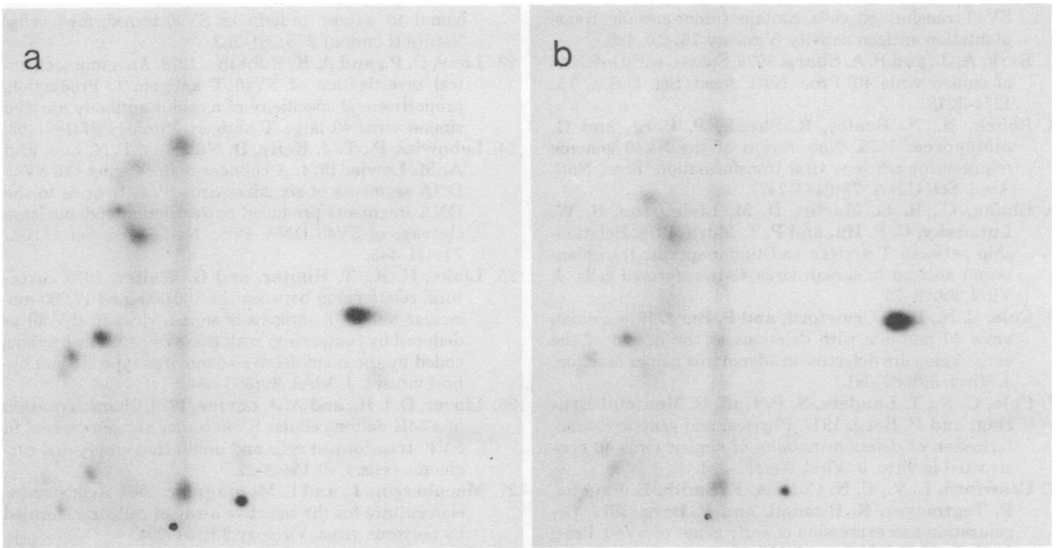

FIG. 11. *Fingerprints of the [^{35}S]cysteine-labeled tryptic peptides derived from rapidly sedimenting (a) and slowly sedimenting (b) T-antigen. [^{35}S]cysteine-labeled proteins were extracted from 3T3 cells transformed by dl1265 (clone C8) and centrifuged on a sucrose gradient as described by McCormick et al. (in press). T-antigen was purified by immunoprecipitation and gel electrophoresis before trypsin hydrolysis. The electrophoresis was for 25 min at pH 2.1.*

with the 53K protein has been shown by Lane and Crawford (22) and by McCormick et al. (in press); we infer an association in our experiments on the basis of their cosedimentation under the conditions used by McCormick et al. (in press). It is possible that these two forms of T-antigen correspond to the rapidly and slowly sedimenting forms of T-antigen observed by Kuchino and Yamaguchi (20) and to the DNA-binding and nonbinding forms of T-antigen observed by Prives and Beck (33).

Since the T-antigens in transformed and permissive cells are in many cases not perceptibly different in size in polyacrylamide gels, it is possible that they are essentially the same protein. Posttranslational modification (phosphorylation or glycosylation, for example) has not been excluded, nor is it precluded that some transformed cell lines have a T-antigen of altered size. The question of interest is whether there is obligatorily more than one form of T-antigen. In our experiments, less than 10% of the T-antigen precipitated from infected or transformed cells with the serum used here (hamster anti-T serum prepared against tumor cells) differed from the major species of T-antigen in size or C-terminal sequence. We draw this conclusion not from the tryptic peptide data but from our inability (i) to detect any proteins other than T-antigen in immunoprecipitates of *dl*1263 T-antigen whose size was affected by the *dl*1263 deletion and (ii) to detect a band at the position of normal T-antigen in gels of nonalkylated *dl*1265 T-antigen where the major species was displaced to a higher molecular weight position. If immunoprecipitates of T-antigen contained significant amounts of a minor species of T-antigen whose C-terminal region was read in a different reading frame than the major species, then we should have detected it in one or the other of these two cases. There was a faint band that migrated just behind the main band of T-antigen in gels of alkylated proteins from infected cells. This form could be significant (a glycosylated form of T-antigen on the cell surface?) and merits further study, particularly in membrane fractions from transformed cells.

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