JOURNAL OF VIROLOGY, Feb. 1979, p. 587-596
0022-538X/79/02-0587/10$02.00/0

Vol. 29, No. 2

Effect of Alkylation on the Physical Properties of Simian Virus 40 T-Antigen Species

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Received for publication 25 July 1978

We analyzed large and small species of T-antigen by immunoprecipitation and two-dimensional gel electrophoresis. The T-antigen species were subjected to electrophoresis either directly or after reduction and alkylation with *N*-ethylmaleimide. Treatment with *N*-ethylmaleimide improved the resolution of large-T by two-dimensional gel electrophoresis and was a requirement for the resolution of small-t antigen on two-dimensional gels. Large-T did not form a discrete protein spot, but rather formed a streak from approximately pH 6.5 to 6.9 on isoelectric focusing gels. Small-t formed a sharp protein spot at approximately pH 7.2 when subjected to electrophoresis under non-equilibrium conditions which extended the pH gradient to include proteins with basic isoelectric points. Treatment with *N*-ethylmaleimide decreased the mobility of the T-antigen species during sodium dodecyl sulfate gel electrophoresis. We suggest that the apparent increase in molecular weight was due to the association of *N*-ethylmaleimide with cysteine-rich regions of these proteins. Viable deletion mutants of simian virus 40 which do not induce the synthesis of small-t but produce small-t-related polypeptides were used to localize the cysteine-rich region of small-t to between 0.54 and 0.59 on the genetic map of simian virus 40.

Cells transformed by simian virus 40 (SV40) synthesize several virus-specific polypeptides. The same is true after infection of permissive cells, i.e. before the onset of viral DNA synthesis, although their synthesis continues throughout infection. Antisera from hamsters bearing tumors induced by virus-free transformed cells can be used to immunoprecipitate these virus-specific polypeptides from cell extracts. In both transformed and infected cells a major polypeptide of about 94,000 daltons, large-T, can be detected. It corresponds to the product of the A cistron (31, 37). Related polypeptides of about 85,000 daltons and less are also seen, particularly in extracts of infected rather than transformed cells (1, 6). Their appearance depends on the extraction conditions used, and they may result from proteolytic degradation of the 94,000-dalton species (39). Temperature-sensitive mutations in the A cistron affect the properties of large-T (2, 17, 40, 41). Cells transformed with such mutants lose some of their transformed properties when grown at high, nonpermissive temperatures (4, 16, 20, 24, 38). These results implicate large-T in the maintenance of the transformed state. A discrete small polypeptide, small-t, with an apparent molecular weight of 17,000 to 20,000 is also observed both in transformed and lytically infected cells. Similar low-molecular-weight polypeptides were observed in cell-free protein-synthesizing systems and shown to share tryptic peptides with large-T (10, 25, 27).

Small-t is now clearly implicated in transformation, because deletion mutants which do not produce wild-type small-t (8, 34) also have a much reduced ability to transform rat cells to anchorage-independent growth (3a). These mutants produce large-T which is apparently normal in size and amount, indicating that large-T alone may not be sufficient for initiation and/or maintenance of the transformed state. The situation with polyoma virus hr-t mutants (3) is very similar in that these have deletions in the equivalent region of the genome (9) and the transforming activity of the virus is greatly reduced (3, 35).

SV40 T-antigens are clearly involved in the initiation of viral DNA replication and control of viral mRNA synthesis as well as in the maintenance of the transformed state, making them of central importance in the life cycle of the virus. Estimates of the molecular weights of large-T antigens of polyoma virus and SV40 obtained by gel electrophoresis and guanidine-agarose chromatography have varied widely (11,

12, 31, 32). This seemed to indicate some unusual structure for the large-T polypeptides. Also, it was difficult to reconcile the higher estimates of molecular weight with the defined coding capacity of the SV40 early region. For these several reasons we decided to analyze the isoelectric focusing properties of the T-antigen species to determine whether this would help us understand their unusual properties.

Using the standard method of equilibrium isoelectric focusing (21), we found initially that very little of either polypeptide entered the gel. A method is described for circumventing this problem to some extent by reduction and alkylation of proteins before electrophoresis, and this may be of more general use for proteins which behave anomalously in two-dimensional gel systems. It allowed us to make estimates of the isoelectric points of the large-T and small-t species. In addition, alkylation affected the apparent molecular weights of all species of T-antigen. Small-t-related polypeptides, produced by SV40 mutants with deletions in the 0.54 to 0.59 region, were used to localize the region affected by alkylation to the C-terminal half of small-t.

MATERIALS AND METHODS

SV40 and deletion mutants. Strain SV-S (36) was used as the wild-type virus strain in SV40. The deletion mutants of SV40, derived from SV-S, have already been described by Shenk et al., (33) (*dl* 883, *dl* 884, *dl* 885, *dl* 886, *dl* 888, *dl* 889, *dl* 890 and *dl* 891) and by Cole et al. (7) (*dl* 1264). A stock of in(*ts*)1501 (30) was kindly supplied by H. Rothschild.

Preparation of cell extracts. Confluent monolayers of CV-1 African green monkey kidney cells in 35-mm plastic plates (approximately 10^6 cells) were infected with 10 to 20 PFU of the appropriate virus stock per cell. After a 70-h incubation at 37°C, the medium was removed and replaced with medium lacking methionine plus 100 to 300 μCi of [^{35}S]methionine (1,000 Ci/mmol; Radiochemical Centre, Amersham, England) in a volume of 0.25 ml per plate. At the end of the labeling period, usually 3 h at 37°C, the monolayers were washed in Tris-saline and lysed in 300 μl of buffer containing 0.15 M NaCl, 0.02 M Tris, pH 8.0, and 1% Nonidet P-40 (NP40). After 30 min at 0°C the lysate was collected and centrifuged for 1 min (Beckman Microfuge B), and the supernatant was collected.

Immunoprecipitation and alkylation. Volumes of 100 to 300 μl of extract were mixed with an equal volume of a buffer containing 0.15 M NaCl, 5 mM EDTA, 0.05 M Tris, pH 7.4, 0.02% sodium azide, and 0.05% NP40 (NET buffer) plus 0.2% bovine serum albumin and 20 μl of normal hamster serum. After incubation overnight at 4°C, the normal immunoglobulin G was removed with 100 μl of a 10% suspension of Formalin-fixed *Staphylococcus aureus* Cowan 1 bearing protein A (SAC) (15). Anti-T hamster serum (20 μl) was then added, and the samples were incubated for an additional 1 h at 4°C before another treatment with 20 μl of 10% SAC suspension. The bacteria were washed three times with NET buffer and then eluted with 20 μl of 1% sodium dodecyl sulfate (SDS) containing 0.02 M dithiothreitol. After 1 h at 25°C the SAC were removed as completely as possible by centrifugation (incomplete removal interferes with alkylation), and the sample was heated at 100°C for 3 min, rapidly cooled, and exposed to *N*-ethylmaleimide (NEM) for 1 h at 0°C. The NEM was prepared fresh at 0.2 M, and 10 μl was added to each sample. For one-dimensional gels 10 μl of 50% glycerol containing 0.5 M mercaptoethanol was added to stop the reaction. For two-dimensional gels 5 μl of 1 M mercaptoethanol was added, followed by 20 μl of lysis buffer A (21), 10 μl of NP40, and urea to 9.5 M.

One-dimensional electrophoresis. Samples prepared as above with or without alkylation were analyzed on gradient polyacrylamide slab gels (14 by 14 by 0.1 cm) by using the SDS-Tris-glycine system of Laemmli (18). The ratio of acrylamide to bisacrylamide was 29.2:0.8, and the gradient was from 7 to 20% acrylamide. After electrophoresis for about 5 h at 100 V, the gels were stained with 0.1% Coomassie brilliant blue in 50% trichloroacetic acid, destained in 7% acetic acid, and dried before exposing to X-ray film.

Two-dimensional electrophoresis. Samples were prepared as described above and subjected to either equilibrium isoelectric focusing or non-equilibrium pH gradient electrophoresis in the first dimension.

Isoelectric focusing was performed as described previously (21, 22); proteins were subjected to isoelectric focusing for a total of 6,800 V-h through gels containing 1.6% Ampholine, pH 5 to 7, and 0.4% Ampholine, pH 3.5 to 10. Non-equilibrium pH gradient electrophroesis was performed as described by O'Farrell et al. (23). Electrophoresis was for a total of 1,600 V-h, and the gels contained 2.0% Ampholine, pH 3.5 to 10.

Estimates of isoelectric points were made by referring protein mobility to measurements of the pH gradient formed under parallel conditions (21, 23). In all cases the second-dimension SDS gel contained a uniform concentration of 12.5% acrylamide (from a stock acrylamide solution containing 29.2% acrylamide and 0.8% bisacrylamide). The gels were stained, destained, and dried for autoradiography as described above for one-dimensional SDS gels.

RESULTS

Preliminary isoelectric focusing studies. For the isolation of T-antigen species from [^{35}S]methionine-labeled extracts of SV40-infected or transformed cells, we relied on their reaction with specific antisera and adsorption to SAC (15). The immune complexes were removed from the adsorbent either with lysis buffer A (21) containing urea and NP40 or with 1% SDS. Analysis of the extracts of SDS-polyacrylamide gels showed two major bands at 90,000 to 100,000 daltons and 15,000 to 20,000 daltons. However, analysis of extracts dissolved in lysis buffer for the standard two-dimensional system showed that both polypeptides remained mostly at the origin of the isoelectric focusing gel and only

moved in the second dimension after exposure to SDS. A small and variable amount of large-T did enter the gel, but as a streak rather than forming a discrete protein spot or spots. This was not due to extreme basicity or acidity of the polypeptide because it was the case whether the sample was loaded at the acidic or at the basic end of the gel. This streaking prevented a meaningful estimation of the isoelectric point of large-T. Because background proteins contained in immunoprecipitates entered the gel, it was unlikely that immunoprecipitation itself was responsible; other antigens enter isoelectric focusing gels normally after an identical procedure (13, 14). Because large-T antigen binds strongly to DNA (5), this might have prevented the polypeptide from entering the gel; however, exhaustive treatment of the immunoprecipitate with micrococcal nuclease did not alter the properties of the antigens. The most likely explanation was that the large-T and small-t polypeptides were in aggregates, either separately or with each other, and that treatment with lysis buffer A was not sufficient to disrupt these complexes.

Heating in SDS containing dithiothreitol or mercaptoethanol appeared to disrupt the aggregates, as shown by the migration of the polypeptides on SDS gels. Unfortunately, SDS dissociates from the polypeptides during electrophoresis, and this treatment alone did not facilitate entry into the isoelectric focusing gel. The procedure finally adopted involved heating in SDS plus dithiothreitol to disrupt aggregates and reduce disulfide bridges and alkylation to block the sulfhydryl groups irreversibly, followed by addition to excess NP40 to reverse the effect of SDS and, finally, addition of urea to 9.5 M. The proteins were alkylated with NEM because this reagent is specific for cysteine residues under the conditions used here and should not have altered the charge of the modified polypeptide (29).

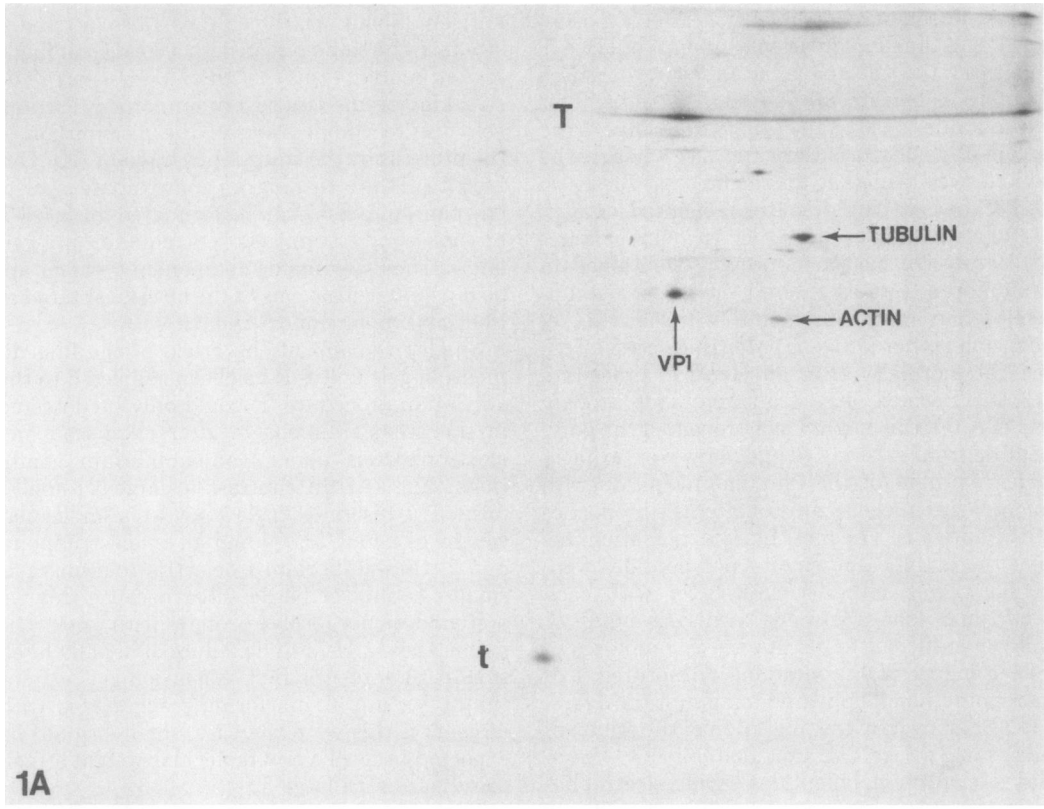
Two-dimensional analyses of alkylated polypeptides. The amount of large-T entering the isoelectric focusing gel was greatly increased by alkylation, but the properties of the polypeptide entering the gel were not otherwise markedly different. It formed a streak when analyzed by either equilibrium isoelectric focusing (21) or non-equilibrium pH gradient electrophoresis (23) (Fig. 1 A and B). Note that background proteins actin, SV40 capsid protein VP1, and tubulin, contained in immunoprecipitates, all formed discrete spots under these conditions. The streaking of large-T indicates that, even after alkylation, it retains some of its unusual properties with respect to aggregation or solubility. A lower-molecular-weight species of T-antigen (about 55,000 daltons) also formed a streak in the same region with a correspondingly higher mobility in the SDS dimension. The streaking seems less severe on non-equilibrium gels due to compression of the distribution of the proteins by the steeper pH gradient (Fig.1A). In spite of the streaking, an approximate isoelectric point of pH 6.7 can be estimated for large-T on non-equilibrium gels. On equilibrium gels large-T has two major components, which appear as intensifications of the streak centered at isoelectric points of 6.5 and 6.9.

Small-t remains at the origin of the first dimension gels unless it has been subjected to the alkylation procedure. It could only be detected in the second dimension after alkylation and electrophoresis under non-equilibrium conditions (Fig. 1A). In contrast to large-T, small-t formed a discrete single spot at a molecular weight of about 20,000 and an isoelectric point of 7.2. On equilibrium isoelectric focusing gels small-t could not be detected because this gel system does not resolve proteins with isoelectric points above pH 7.0. The failure of nonalkylated small-t to enter the first dimension gels cannot simply be due to its forming aggregates with large-T; if this were the case, small-t would be expected to form a low-molecular-weight streak coordinate with large-T.

The effect of alkylation on the mobilities of T-antigen species. Our primary reason for studying the effect of alkylation was to improve the isoelectric behavior of the T-antigen species. However, alkylation also affected the apparent molecular weight of the species.

Infected cells were extracted by various procedures as detailed in the legend to Fig. 2, and duplicate samples were analyzed with and without alkylation. The different procedures produced variation in the yield of T-antigen and in the relative amounts of the 94,000- and 85,000-dalton species (25). The change in the mobility of large-T on alkylation was small, but it was seen consistently and corresponded to an increase in apparent molecular weight of 2,000 to 3,000. There was little or no change in the mobility of the 85,000-dalton species (Fig. 2f and g). The data suggest that a proteolytic cleavage near the end of large-T may remove a region which is sensitive to alkylation.

The decreased mobility, as a function of alkylation, is shown more dramatically for small-t (Fig. 2). Alkylated small-t showed an increase in apparent molecular weight of approximately 2,000. NEM binds covalently to cysteine residues; the increased molecular weight of NEM-treated antigens could be explained by suggesting that these proteins are rich in cysteine resi-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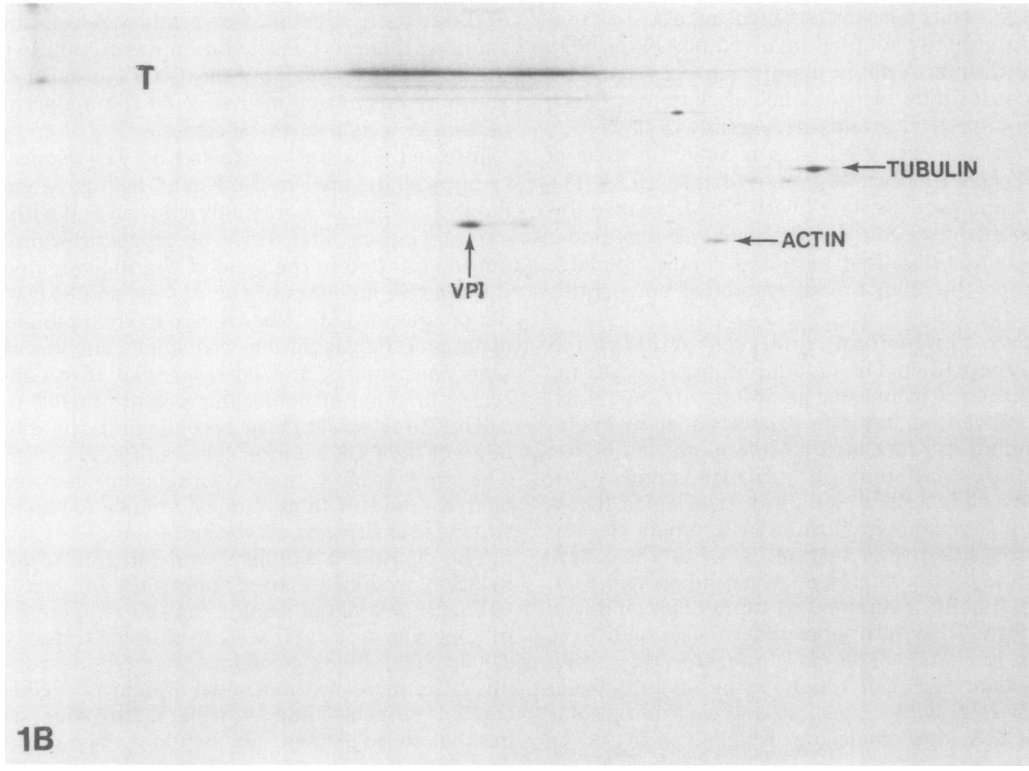
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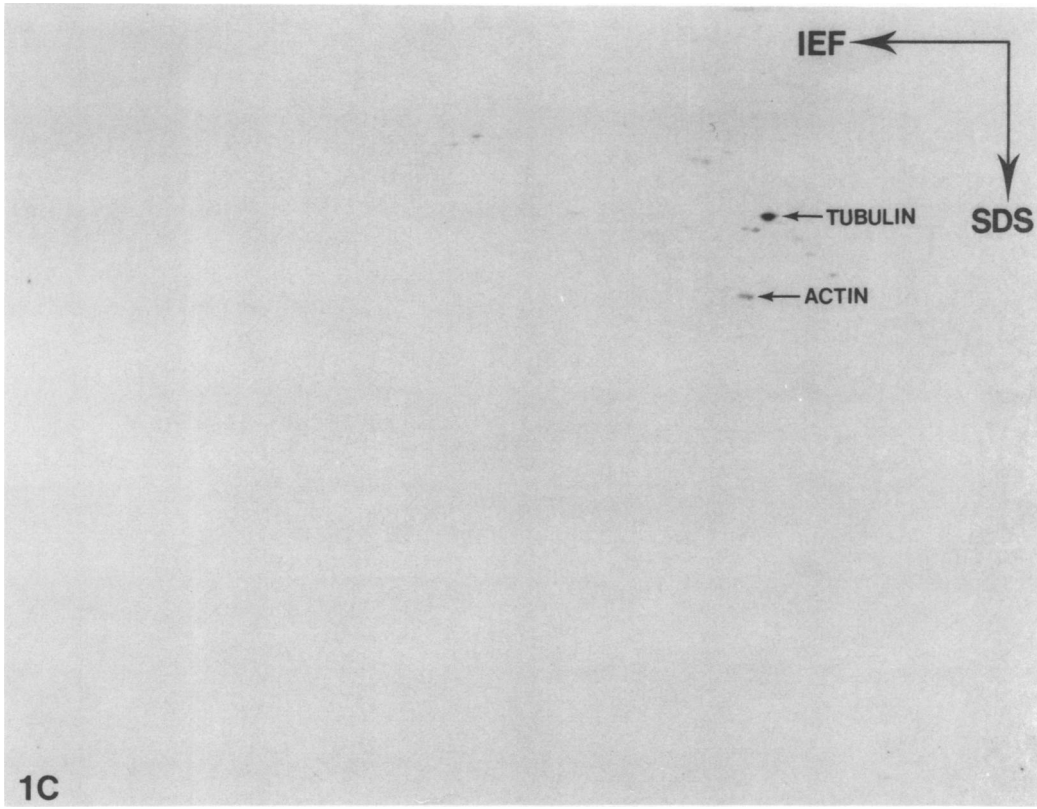


FIG. 1. *Two-dimensional electrophoresis of T-antigen species. Extracts of uninfected or SV40-infected cells labeled with [^{35}S]methionine were treated with anti-T serum, and the immunoprecipitates were alkylated with NEM before dissolving in lysis buffer A for electrophoresis as described in the text. The two-dimensional separation of extracts prepared from infected cells after electrophoresis by non-equilibrium pH gradient electrophoresis (A) or by equilibrium isoelectric focusing (B) are displayed. Uninfected cell extract subjected to non-equilibrium pH gradient electrophoresis is shown in (C). (A) and (B) are from in(ts)1501-infected cells because these gave higher overall incorporation of [^{35}S]methionine into the T-antigen species than did SV-S-infected cells. The results obtained with SV-S and in(ts)1501 were otherwise indistinguishable. Large-T and small-t antigens are labeled T and t, respectively. Other proteins (actin, tubulin, and the SV40 capsid protein VP1) are also indicated.*

dues which bind NEM. As discussed below, recent results on the DNA sequence of the region of SV40 encoding small-t antigen also indicate that this protein is cysteine rich. The addition of NEM to cysteine residues may increase the molecular weight of small-t directly and/or change the mobility of small-t by causing it to take up a more extended configuration in the absence of disulfide bridges.

It should be noted that NEM treatment does not cause a generalized shift in molecular weight of proteins but rather appears to have a selective effect on a few proteins contained in immunoprecipitates. In addition to large-T and small-t there are two protein bands with apparent molecular weights of 60,000 (61,000 after alkylation) and 28,000 (30,000 after alkylation) which are affected by NEM treatment. These proteins, which may be related forms of T-antigen, were seen only when detergent extraction was used (Fig. 2b), rather than freezing and thawing (Fig. 2d). Lane and Robbins (19) have also observed a 60,000-dalton polypeptide in immunoprecipitates formed by using rabbit anti-T serum.

Initially we were concerned to establish that deletions in the 0.54 to 0.59 region were in the structural gene for small-t antigen and not some control element. In a previous paper it was reported that a smaller polypeptide, assumed to be a fragment of small-t, was detected in extracts of cells infected with *dl* 1264. Using different labeling conditions, Sleigh et al.(34) were able to detect fragments in several other deletion mutants from the 0.54 to 0.59 regions. Figure 3 shows that by using more efficient immunoprecipitation conditions with high serum input we were also able to detect putative small-t fragments in extracts of cells infected by most of the

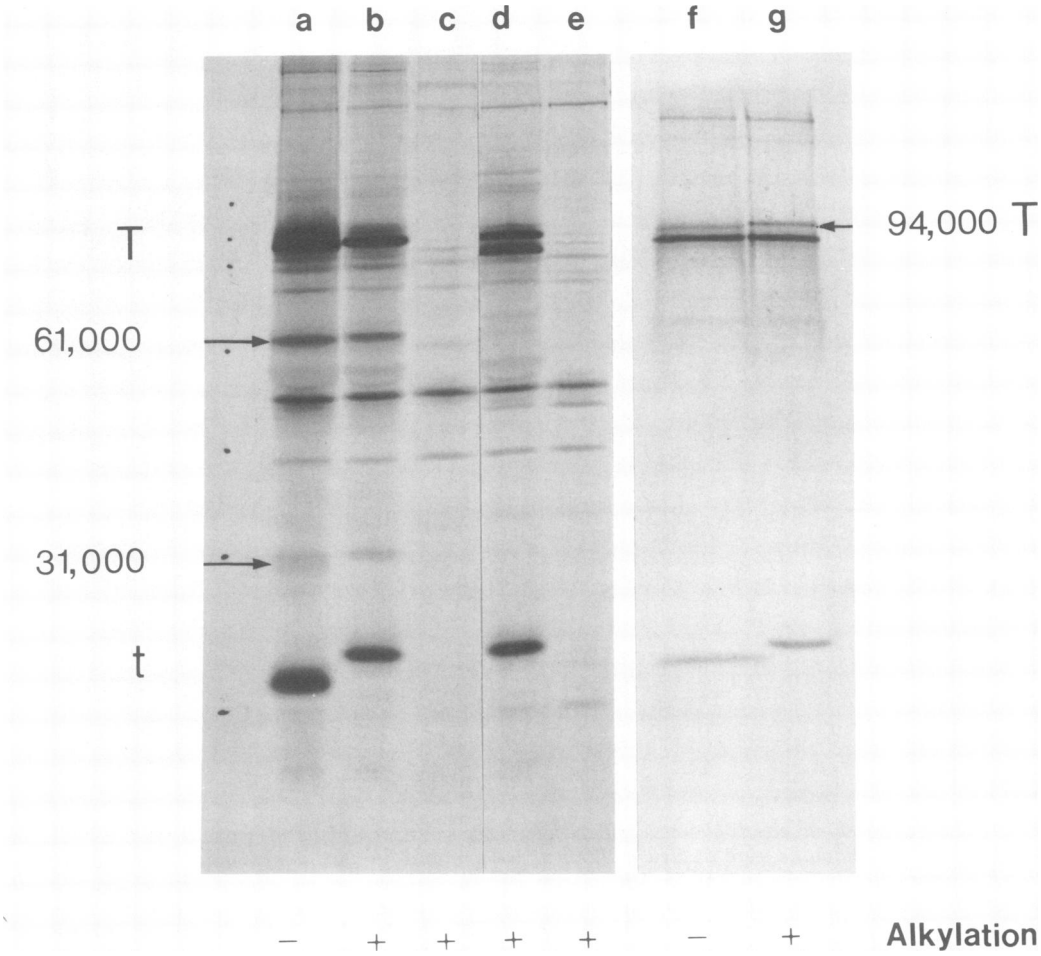
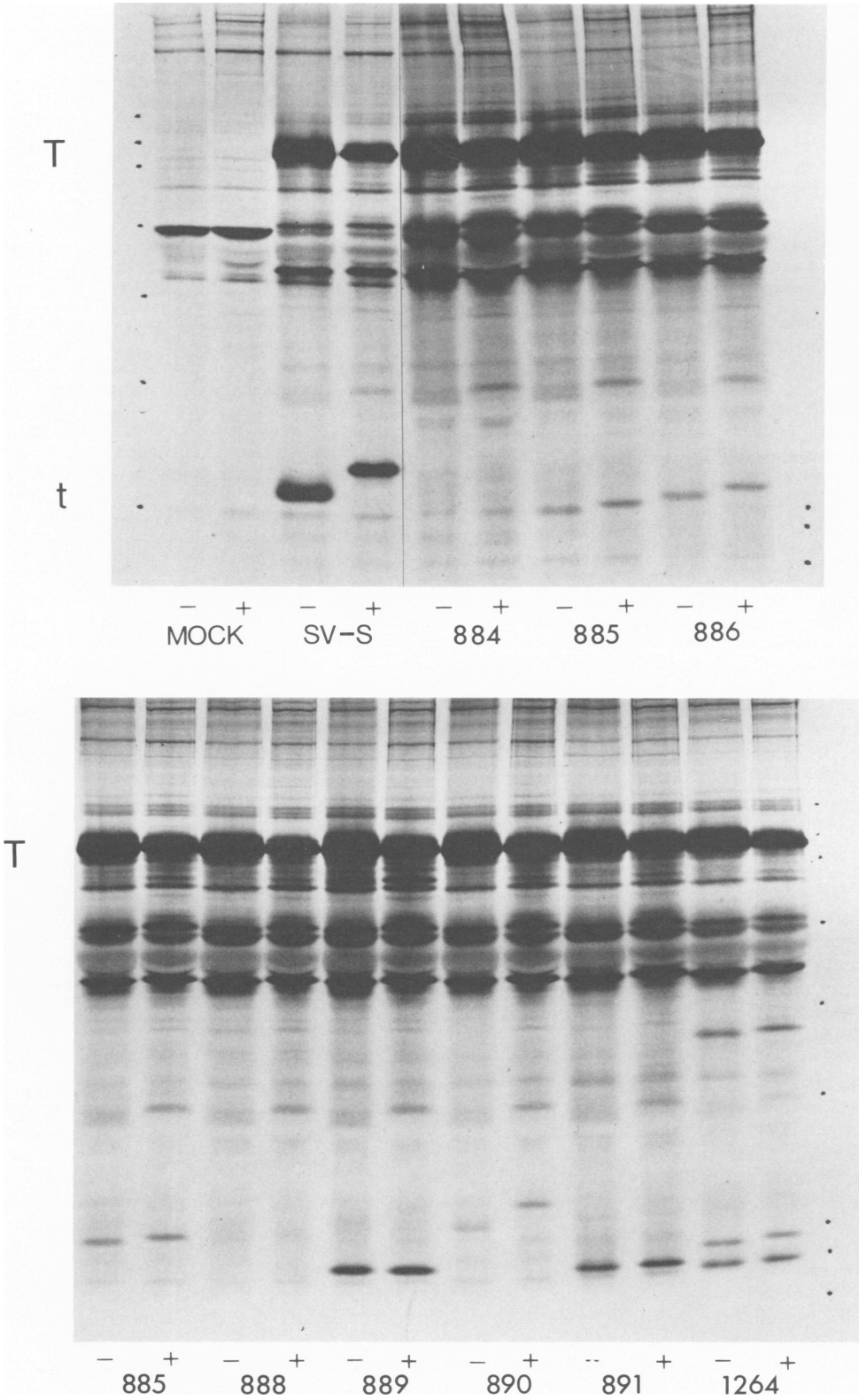


FIG. 2. *Effect of extraction conditions and alkylation on T-antigen species. Cultures of SV-S-infected CV-1 cells were labeled with [^{35}S]methionine from 69 to 72 h after infection. Extracts were made either with NP40 as described in the text (a, b, c) or by freezing and thawing (the method of Carroll and Smith [6] (d, e, f, g). The extracts were pretreated with normal hamster serum and SAC and immunoprecipitated in the following ways: lane a, immunoprecipitated with anti-T serum; lane b, as for lane a, then alkylated; lane c, as for lane b, but with normal hamster serum; lane d, extracted by freezing and thawing, immunoprecipitated with anti-T serum, and then alkylated; lane e, as for lane d, but with normal hamster serum. Lanes f and g are from a separate experiment in which additional mercaptoethanol (1 M) was added to the samples. The change in mobility of the 94,000-dalton species of large-T is clearly seen here. Lane f, as for lane d, but without alkylation; lane g, as for lane d. Alkylated samples are labeled +, and nonalkylated samples are labeled − below the appropriate slot. The positions of marker proteins are indicated on the left. These were, in order of increasing mobility: β-galactosidase, 130,000 daltons; phosphorylase a, 94,000 daltons; lactoperoxidase, 80,000 daltons; catalase, 60,000 daltons; alcohol dehydrogenase, 41,000 daltons; carbonic anhydrase, 29,000 daltons; and myoglobin, 17,200 daltons.*

FIG. 3. *Effect of alkylation on small-t and related polypeptides. CV-1 cells, mock infected or infected with SV-S or the indicated deletion mutant, were labeled with [^{35}S]methionine at from 70 to 72 h after infection. Extracts were made and immunoprecipitated as described in the text, except that 50 μl of hamster anti-T serum was used instead of 20 μl. Half of each sample was alkylated with NEM (+) and half was not alkylated (−). Equivalent amounts of cell extract (except for SV-S) were subjected to electrophoresis through 7 to 20% gradient polyacrylamide gels; the loading of the SV-S samples was half that of the other samples. The positions of large-T (T) and small-t (t) are indicated, and the marker proteins are the same as those described in the legend to Fig. 2, with the addition of alkylated myoglobin (17,200 daltons) and alkylated α-chymotrypsin (16,700 and 10,100 daltons; upper right).*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885 888 889 890 891 1264

TABLE 1. *Relation to size and position of deletion to fragment size*[a]

Virus	Deletion		Expected size of fragment		Apparent molecular weight		
	Extent	Position	Terminated immediately	Only deleted codons absent	Untreated	Alkylated	Difference
SV-S			(20,000)	(20,000)	18,000	20,000	2,000
dl 883	−23	0.54	20,000	20,000	None detected		
dl 884	−184	0.57 to 0.54	16,000	16,000	ND[b]	15,000	
dl 885	−38	0.56	18,000	18,000	16,500	17,000	500
dl 886	−15	0.56	18,000	19,500	17,500	18,500	1,000
dl 889	−46	0.57	16,000	18,000	14,500	14,500	0
dl 890	−53	0.58	14,000	18,000	17,200	18,700	1,500
dl 891	−41	0.59	12,000	18,000	14,500	14,500	0
dl 1264	−120	0.58 to 0.55	15,000	15,000	16,500	16,700	200

[a] The sizes and positions of the deletions in these mutants are taken from Shenk et al. (33) and Cole et al. (7). The positions and extents of the deletion may not be accurate to better than ±0.01, so that the expected sizes of the mutant polypeptides must be considered to be approximate (33). More recent estimates of deletion size, based on DNA sequencing, are in most cases much lower (G. Volckaert et al. and T. E. Shenk et al., submitted for publication). The predictions of the sizes of the fragments are based on either termination occurring at the point where the deletion starts (terminated immediately) or translation continuing after the deletion to the normal termination site (only deleted codons absent). Continued translation might be in the correct reading frame or in an alternative reading frame which by chance has a termination codon close to the normal position of termination. These are the extreme possibilities, and it is likely that in most cases translation continues after the deletion for some distance but terminates before the normal termination site. In principle it is also possible that translation in an alternative reading frame could continue past the normal termination site to give an oversize polypeptide, although in view of the DNA sequence data this seems unlikely here. The apparent molecular weights of the polypeptides in this table are taken from their mobilities relative to marker polypeptides and are an indication only of the relative mobilities of the fragments. In *dl* 1264, where two fragments were observed, the data refer to the larger fragment only. The smaller fragment may be a breakdown product of the larger polypeptide. Extracts of cells infected with polypeptide *dl*883 or *dl*884 showed little or no material in the region of small-t.

[b] ND, Not detected.

mutants previously studied (Table 1). The amount of incorporation into these small-t fragments varied and was always less than that into wild-type small-t. This may be due to reduced synthesis of the fragment, lower methionine content, rapid breakdown of these abnormal polypeptides, or some combination of these alternatives. Table 1 summarizes the predicted and observed molecular weights of the deletion mutants analyzed. In general, the size of the fragments correlates with their map position. Presumably some fragments are produced due to translation beyond the mutant site in an incorrect reading frame leading to subsequent premature chain termination, e.g. *dl* 1264. Sleigh et al. (34) also noted the production of a large fragment, only slightly smaller than wild type, in *dl* 890, and these results are consistent with read-through translation. Other fragments may be the result of proteolytic cleavage of a larger more unstable polypeptide (for example, *dl* 885 or *dl* 889).

The results presented in Fig. 3 and summarized in Table 1 suggest that the degree of sensitivity to alkylation by NEM varies with the size of the mutant polypeptide. For example, the large fragments produced by *dl* 890 and *dl* 886 showed the greatest difference in mobility between alkylated and unalkylated forms, and the smallest fragments produced by *dl* 889 and *dl* 891 showed virtually no shift on alkylation. Because the effect of alkylation was minimal in mutants lacking much of the region between 0.59 and 0.54, such as *dl* 1264, this effectively localizes the region responsible for the alkylation-induced change in mobility to within the C-terminal half of the small-t polypeptide. The varying effect of alkylation on the small-t-related polypeptides clearly make it difficult to correlate the sizes of polypeptide produced by these deletion mutants with the locations of the alterations in their DNA.

DISCUSSION

The results presented provide some insight into the physical properties of large-T and small-t antigens. We have estimated the isoelectric point of large-T to be pH 6.7 and that of small-t to be pH 7.2. The two forms of T-antigens share some of their amino acid sequences, as shown by their common tryptic peptides (10, 25, 27), but otherwise their properties are rather distinct. Large-T binds strongly to DNA (5), whereas small-t shows no sign of DNA binding (26) and is found mostly in the cytoplasm of infected cells after Dounce homogenization (P.

Tegtmeyer, personal communication). Because small-t, which does not bind to DNA, is more basic than large-T, the DNA binding of large-T cannot be due to a simple charge interaction.

There is strong evidence that the region of SV40 which codes for small-t antigen is from coordinates 0.655 to 0.55. The N-terminal amino acid sequence of small-t corresponds with the nucleotide sequence at 0.655 (25a). A polypeptide with tryptic peptides in common with small-t can be synthesized in vitro by using cRNA synthesized from DNA in this region (25). Deletion mutants between 0.59 and 0.55 on the map of SV40 fail to synthesize wild-type small-t (8, 34). Interestingly, deletions within the region coding for small-t result in the production of smaller polypeptides. These are most probably related to small-t because they are specifically immunoprecipitated by anti-T serum but not by normal serum, and the size of the fragments varies in different mutants. In addition, wild-type small-t shares antigenic determinants with large-T (19), and small-t fragments produced by deletion mutants *dl* 890 and *dl* 1264 are also specifically immunoprecipitated by antiserum to the 85,000-dalton species of large-T (L. Crawford and D. Lane, unpublished data).

We have shown that the relative mobility of small-t on SDS gels is strikingly sensitive to alkylation; after alkylation the apparent molecular weight of small-t is increased by 2,000. Furthermore, using deletion mutants of small-t, we have shown that the region of small-t which is sensitive to alkylation is localized to 0.59 to 0.54 on the genetic map. We suggest that this region of the small-t polypeptide is rich in cysteine residues which bind NEM and account for the increased molecular weight. This notion is supported by the recent determinations of the nucleotide sequence of SV40 DNA (9a, 28, 42). It is now possible to predict the complete amino acid sequence of SV40-coded polypeptides. One reading frame with a start a 0.655 and extending to 0.55 gives an uninterrupted run of 174 codons. This sequence contains 11 cysteine residues, all but one in the C-terminal half of the polypeptide, i.e., the region corresponding to 0.59 to 0.54 where the relevant deletion mutants map. The reaction of all 100 cysteine residues with NEM would increase the molecular weight of the polypeptide by 1,375. It is also possible that part of the increase in apparent molecular weight could be due to removal of the opportunity for intramolecular interaction of sulfhydryl groups; thus, after alkylation a polypeptide could assume a more extended configuration, resulting in decreased electrophoretic mobility. Such a change in configuration could also account for the effect of alkylation on the isoelectric focusing properties of small-t.

Large-T antigen is also sensitive to alkylation, suggesting that it may be rich in cysteine residues. The behavior of large-T during isoelectrophoresis or non-equilibrium pH gradient electrophoresis suggests intermolecular interaction of large-T molecules. Because reaction with NEM sharpens the band of large-T, at least part of the interaction between molecules of large-T may be affected by the presence of sulfhydryl groups. Clearly, more properties of large-T antigen remain to be found which may explain its propensity for aggregation.

It is hoped that the combination of immunoprecipitation, alkylation, and non-equilibrium pH gradient electrophoresis described here will be useful to investigators hoping to study the synthesis of T-antigen species by two-dimensional electrophoresis.

ACKNOWLEDGMENTS

Some of the work reported in this paper was undertaken during the tenure of an American Cancer Society Eleanor Roosevelt International Cancer Fellowship awarded by the International Union against Cancer to L.V.C. and American Cancer Society California Division junior fellowship J-311 to P.Z.O.

We are indebted to G. Stark for advice on alkylation and the hospitality of his laboratory (L.V.C.), to H. Rothschild for the stock of in(*ts*)1501, to P. Berg and C. Cole for the SV40 deletion mutants, and to J. Smart for molecular weight markers. The support and encouragement of H. Goodman to P.Z.O. during this work is indeed appreciated.

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