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Simian Virus 40 Tumor Antigen: Isolation of the Origin-Specific DNA-Binding Domain

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To localize the origin-specific DNA-binding domain on the simian virus 40 tumor (T) antigen molecule, we used limited proteolysis with trypsin to generate fractional peptides for analysis. A 17,000-M_r peptide was found to be capable of binding not only to calf thymus DNA, but also specifically to the simian virus 40 origin of DNA replication. This ~130-amino-acid peptide was derived from the extreme N-terminus of the T antigen and represented less than one-fifth of the entire molecule. The coding sequence for this tryptic peptide was located approximately between 0.51 and 0.67 map units (excluding the intron, which maps between 0.54 and 0.59). Since the first 82 amino acids are shared between large T and small t antigens, and since the latter does not bind DNA, it can be concluded that the sequence between isoleucine 83 and approximately arginine 130 is necessary for origin-specific binding by the T antigen. We also observed that in vivo phosphorylation of the T antigen within this region completely abolished the ability of the 17,000-M_r peptide to bind DNA. This observation is consistent with the idea that DNA binding by the T antigen is regulated by posttranslational modifications.

The simian virus 40 (SV40) large tumor (T) antigen is a multifunctional protein that plays a critical role in both cell transformation and virus propagation (38). T antigen has been found (i) to bind a specific region of the SV40 genome (32, 34), (ii) to exhibit an inherent ATPase activity which catalyzes the hydrolysis of ATP (7, 37), (iii) to possess a helper activity that enhances human adenovirus reproduction in monkey cells (4, 36), (iv) to bind tightly to the cellular p53 transformation-related protein (13, 17, 18), and (v) to induce SV40 tumor-specific transplant rejection (2, 12).

The binding of T antigen to SV40 DNA involves three distinct but tandem recognition sites which span the origin of DNA replication and the early transcriptional promoter (35). Binding to the three sites occurs sequentially, and there is evidence to support the thesis that binding by T antigen is responsible for both repression of the initiation of early SV40 transcription (autoregulation) and the induction of viral DNA replication (35).

It has long been known that infection with SV40 leads to changes in cell metabolism, including an increase in cellular DNA replication (19, 20). These changes in cell metabolism have been specifically linked to the presence of T antigen. The recent isolation of monkey (22) and human (5) cellular DNA sequences that are homologous to the SV40 origin of DNA replication supports the model in which SV40-induced changes in cellular DNA replication proceed by a mechanism similar to that of stimulation of viral DNA replication in response to the binding of T antigen to the SV40 control region.

Although much is known about the control elements residing within and around the three binding sites on SV40 DNA, very little is known about the conformational requirements of T antigen in the interaction. Although the DNA sequences that comprise the binding sites provide for recognition, the T antigen provides for the specificity of the interaction. The DNA-binding domain of the T antigen has not yet been localized, and little is known about possible posttranslational modifications which may modulate its binding activities.

Earlier attempts to identify the origin-specific DNA-binding domain by analyzing mutants or truncated T antigens led to conflicting results (3, 9, 26–28, 30, 33; Y. Gluzman and C. Prives, personal communication). It has been difficult to distinguish between a region of the T antigen that may be directly involved in DNA binding and a region that may be conformationally important in inducing an active binding domain elsewhere in the same T antigen molecule. In the present study, we designed an experiment to circumvent this ambiguity by directly generating a fractional peptide of the T antigen which conserves its specificity for origin-specific DNA

binding. We provide evidence that phosphorylation of the T antigen within this domain completely obliterates its binding capacity.

MATERIALS AND METHODS

Preparation of cell extracts. African green monkey kidney cells were grown in monolayer cultures (150-cm^2 bottles) in Eagle minimal essential medium supplemented with 10% fetal calf serum. Cells were infected with wild-type SV40 (strain 776) at a multiplicity of 100 PFU/cell and radiolabeled at 24 h postinfection with either [^{35}S]methionine at 100 μCi/ml (~1,000 Ci/mmol) in methionine-free medium or with $^{32}P_i$ at 500 μCi/ml in phosphate-free medium for 3 h. At the end of the labeling period, L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) was added to a final concentration of 50 μg/ml for 1 min. The cells were then rinsed with phosphate-buffered saline and lysed with 2 ml of buffer A (50 mM Tris-hydrochloride [pH 8.3], 150 mM NaCl, 0.5% Nonidet P-40). Cell lysates were clarified by centrifugation at 100,000 × *g* for 30 min and stored frozen at −70°C.

Immunoprecipitation of T antigen. Immunoprecipitations were carried out as previously described (14, 15). Briefly, 100 μl of infected-cell extract was incubated with either 1 μl of normal hamster serum, 1 μl of SV40 antitumor serum, or 100 μl of hybridoma supernatant (kindly provided by E. Harlow and L. V. Crawford, Imperial Cancer Research Fund Laboratories, London, England) for 45 min at 4°C. Formalin-fixed *Staphylococcus aureus* (Cowan I strain) (25 μl) was added for an additional 20 min, and the immunoprecipitate was washed four times with buffer A. The proteins bound to the immune complexes were released by being heated for 5 min in sodium dodecyl sulfate (SDS) buffer (62 mM Tris-hydrochloride [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 10% glycerol) and analyzed by electrophoresis in a 12.5% SDS-polyacrylamide gel (14). Peptides with molecular weights below 5,000 were not resolved but stacked up at the front under these conditions.

Preparation of tryptic peptides. Tryptic fragments were prepared by a variation of the method of Schwyzer et al. (31). Washed immunoprecipitates of the T antigen were resuspended in 200 μl of buffer A (adjusted to pH 8.0) containing the appropriate amount of TPCK-trypsin. After incubation for 30 min at 4°C, the reaction was terminated by adding 5 μl of aprotinin (10 to 20 U/ml). The suspension was centrifuged at 10,000 × *g*, and both the pellet and the supernatant were saved. Those peptides that remained bound to the immune complexes were released by heating at 95°C for 5 min in SDS buffer. The peptides released from the immune complexes during the trypsin digestion were concentrated by precipitation with 5 volumes of acetone, a procedure that we confirmed resulted in quantitative recovery of all the tryptic peptides detected.

Affinity chromatography on DNA-cellulose. The 17,000-M_r (17K) peptide released into the supernatant by trypsin digestion of the immune complexes was diluted in binding buffer (5 mM sodium phosphate [pH 6.5], 100 mM NaCl, 0.5% Nonidet P-40, 10% glycerol) and applied to a calf thymus DNA-cellulose column that was preequilibrated in the same buffer (14). The flow-through material was recycled over the column three times to assure quantitative binding. The bound material was eluted with SDS buffer.

Solution binding of SV40 DNA fragments. SV40 DNA was nick-translated with ^{32}P-labeled dATP and dGTP to a specific activity of 2×10^7 to 5×10^7 cpm/μg and then digested with endonuclease *Bst*N1. The 17K peptide was isolated from immunoprecipitates of the T antigen with BK8 serum (hamster SV40 antitumor serum), followed by treatment with trypsin at a concentration of 1 mg/ml. The resulting supernatant from the equivalent of 1 ml of cell extract was diluted into 0.5 ml of buffer A containing 1 mM EDTA–3% bovine serum albumin and adjusted to pH 6.8. The solution was then divided into two equal portions, and 50 ng of the ^{32}P-labeled DNA fragments was added to each. After 30 min at 4°C, 1 μl of either normal hamster serum or BJ5 hamster SV40 antitumor serum was added. The immune complexes were then precipitated by adding *S. aureus* after 45 min at 4°C as described above. The ^{32}P-labeled DNA fragments were dissociated from the immune complexes by heating for 5 min at 70°C in stop buffer (50 mM Tris-hydrochloride [pH 8.0], 10 mM EDTA, 0.1% SDS, 10% glycerol). The released fragments were extracted with phenol-chloroform before being analyzed by electrophoresis on a 2% agarose gel.

RESULTS

Specificity of the antisera. We previously suggested, from analysis of the truncated T antigens encoded by adenovirus 2 (Ad2)-SV40 hybrids, that the amino acid sequences specified by SV40 DNA between 0.50 and 0.63 map units are necessary for origin-specific DNA binding (26). To obtain direct evidence that this region of the T antigen molecule is not only conformationally important but is, in fact, sufficient to act as the binding site with specificity for the origin of replication, we attempted to isolate a fractional peptide derived from the appropriate region of the T antigen by limited proteolysis with trypsin.

It has been observed that when T antigen in the form of an immune complex was subjected to limited proteolysis with trypsin, several discrete fractional peptides were obtained (16, 29, 31). The bond most susceptible to cleavage is between arginine 130 and lysine 131, yielding a 17K peptide derived from the N-terminal 130 amino acids (Fig. 1). This 17K peptide was estimated to control all of the sequences that we suspect are required for origin-specific DNA binding (26) and represented less than one-fifth of the T antigen molecule.

To test the ability of the 17K peptide to bind DNA, it was necessary to select antibodies which would react specifically with T antigen but not with other viral gene products present in an infected-cell extract, such as small t antigen or capsid protein VP1, to avoid ambiguity in assigning the origin of the fractional peptides. Furthermore, these antibodies should selectively release the 17K peptide and preferably no

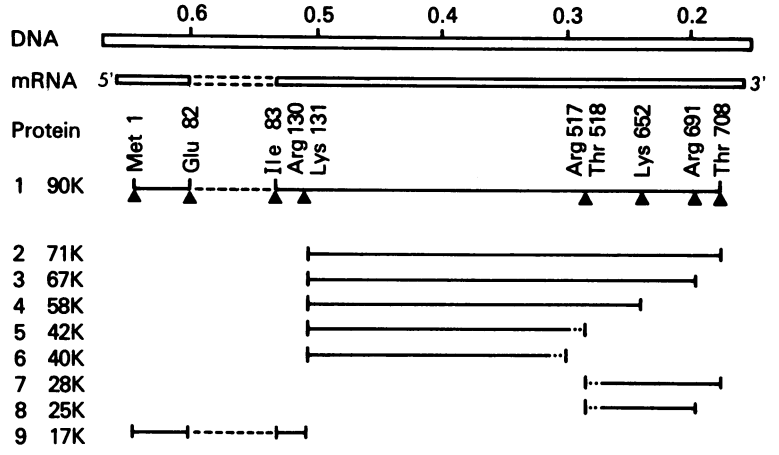

FIG. 1. Tryptic cleavage map of the SV40 T antigen. Aligned in the diagram are the entire early region of the SV40 genome (DNA), an early RNA transcript, with the intron depicted by a broken line (mRNA), and the 90K T antigen encoded by this spliced mRNA (protein). Also included are the locations of the known fractional peptides generated by limited proteolysis of the T antigen by trypsin. Dots at the end of a molecule indicate that the exact position has not been confirmed.

other T antigen-derived peptides from the immune complex after digestion with trypsin. These prerequisites would appear to preclude the use of monoclonal antibodies, which, although they may be specific for T antigen, would probably release most of the fractional peptides except the one which carries the antigenic determinant it recognizes. For this reason, we chose to only use sera from hamsters bearing SV40-induced tumors (antitumor sera). We rejected sera (such as BJ5) with any reactivity against viral products other than the T antigen and concentrated on sera (e.g., BI6 and BK8) which were not only specific for T antigen but also had a high titer against the C-terminus of the molecule, as determined by reactivity to the T antigen-related peptides encoded by the $Ad2^{+}ND1$ and $Ad2^{+}ND2$ viruses (Fig. 2). The specificity of this latter class of antitumor sera (including BI6 and BK8) was confirmed by their inability to precipitate any protein other than T antigen (as is true with monoclonal antibodies [11] [Fig. 2, lanes e and f], which react with either the N-terminus [PAb416] or the C-terminus [PAb405] alone).

Release of the 17K peptide by trypsin. Antitumor sera identified as recognizing only T antigen were tested for their ability to release the 17K peptide into the supernatant after treatment of the immune complex with increasing concentrations of trypsin (Fig. 3A and B). At a low trypsin concentration (lanes b and f), both the BI6 and BK8 sera gave an overall cleavage pattern quite similar to that reported previously (31). In addition, most if not all of the cleavage products remained bound to the antibodies in the form of immune complexes. The origin of most of these fractional peptides has already been determined (31), and each is referred to by a number in the present study (Fig. 1 and 3). Peptide 5, for example, had a molecular weight of 42K and extended from lysine 131 possibly to arginine 517. Peptide 9, which migrated with a molecular weight of ~15K in our gel system, appeared to be identical to the 17K peptide derived from the N-terminal 130 residues.

Although the overall patterns of the peptides that remained bound by the various antitumor sera were quite similar (Fig. 3A and B), there were distinguishable differences. Peptides 8 and 9 were reproducibly more abundant with the BI6 serum than with the BK8 serum. This could be the result of either the shielding of a potential trypsin-sensitive site by the immunoglobulin molecule or the release from the immune complex of a particular tryptic peptide not associated with an antibody molecule.

Increasing concentrations of trypsin resulted in the further digestion of some of the larger peptides, and at a trypsin concentration of 1 mg/ml no peptide was left associated with the immune complex. Analysis of the supernatant after trypsin digestion showed that, with the exception of peptide 9, virtually none of the major peptides was released from the immune complex. In fact, peptide 9 was released only by the BK8 serum (Fig. 3B) and not by the BI6 serum (Fig. 3A). To our express advantage, peptide 9 was not only selectively released by one of the antitumor sera, it was also resistant to further digestion at a high trypsin concentration (Fig. 3B). At 1 mg of trypsin per ml, peptide 9 was the

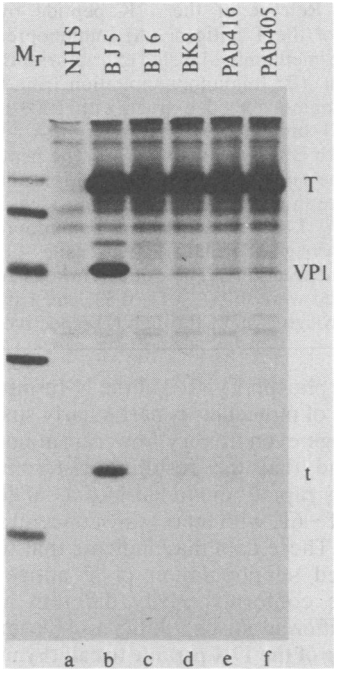

FIG. 2. Specificity of antibodies directed against T antigen. Immunoprecipitates from a [^{35}S]methionine-labeled extract of monkey cells infectedwith wild-type SV40 for 24 h were analyzed by electrophoresis on a 12.5% polyacrylamide-SDS gel. The antibodies used included (lane): normal hamster serum (NHS) (a), broadly reactive hamster SV40 antitumor serum (BJ5) (b), two hamster SV40 antitumor sera (BI6 and BK8) with limited reactivity (c and d), and two monoclonal antibodies (PAb416 and PAb405) with specificity against either the N-terminus (e) or the C-terminus (f) of the T antigen. The molecular weight markers (M_r) used were phosphorylase b (92.5K), bovine serum albumin (68K), ovalbumin (45K), carbonic anhydrase (30K), and cytochrome *c* (12.3K).

only methionine-containing peptide detectable in the supernatant from the complexes formed with the BK8 serum.

Identification of the 17K peptide. In our gel system, in which the SV40 small t antigen migrated with a molecular weight of 17K (Fig. 4, lane a), peptide 9 appeared as a 15K component (Fig. 4, lane b). To verify that peptide 9 was identical to the N-terminal 17K peptide detected by others (16, 29, 31), we asked whether this peptide (i) could be recognized specifically by a monoclonal antibody directed against the N-terminus of the T antigen and (ii) was also phosphorylated during metabolic labeling.

Portions of [^{35}S]methionine-labeled peptide 9, released by trypsin digestion from the immune complexes obtained with the BK8 serum, were analyzed either directly on an SDS-polyacrylamide gel or after immunoprecipitation with either a control serum, a broad-spectrum antitumor serum (BJ5), a monoclonal antibody (PAb416) directed against the N-terminus of the T antigen, or a monoclonal antibody (PAb405) directed against the C-terminus of the T antigen. Peptide 9 was quantitatively precipitated only with the BJ5 antitumor serum and with the monoclonal antibody directed against the N-terminus. In addition to assigning the origin of peptide 9 (the 17K peptide), this latter observation also confirmed the homogeneity of peptide 9 as observed on the gel. It is interesting to note that immunoprecipitation of peptide 9 resulted in the formation of a ~29K component (Fig. 4, lanes d and e) that was not present in the input material and that was apparently resistant to both SDS and reducing agents. Its size indicated that it is probably a covalent dimer of peptide 9.

Previous studies indicated that the 17K peptide is phosphorylated (16, 29, 31). To confirm this observation, we treated ^{32}P-labeled T antigen immunoprecipitated with the BK8 serum with increasing concentrations of trypsin (Fig. 3C). At a trypsin concentration of 0.037 mg/ml, a cleavage pattern identical to that previously reported was found to be associated with the immune complex. All of the [^{35}S]methionine-containing peptides generated at this trypsin concentration (except peptides 5 and 6) were reproducibly found to be phosphorylated. In contrast to the [^{35}S]methionine experiment, in which peptide 9 was detected in the supernatant only at high trypsin concentrations (Fig. 3B), large amounts of ^{32}P-labeled peptide 9 were released even at a trypsin concentration of 0.037 mg/ml (Fig. 3C). This released component, however, was highly unstable to increasing concentrations of trypsin. We do not believe that this instability was due to contaminating phosphatase activity, as two other trypsin fragments remained visible. The finding of the unstable ^{32}P-labeled peptide was in marked contrast to the [^{35}S]methionine-containing peptide, which, apart from being released only at high trypsin concentrations, was also highly resistant to further cleavage.

The ^{32}P-labeled peptide 9 was also derived from the N-terminus of the T antigen, as indicated by the finding that it could be specifically immunoprecipitated only with the N-terminus-specific monoclonal antibody PAb416, but not with the control antibody. Together, these observations suggest that a majority of the T antigen molecules are not phosphorylated at the N-terminus, that this population is susceptible to trypsin cleavage only at high concentrations, and that the resulting N-terminal 17K peptide cannot be further degraded into smaller components under our experimental conditions. Our data also suggest that a small subpopulation of T antigen molecules (probably less than 10% of the

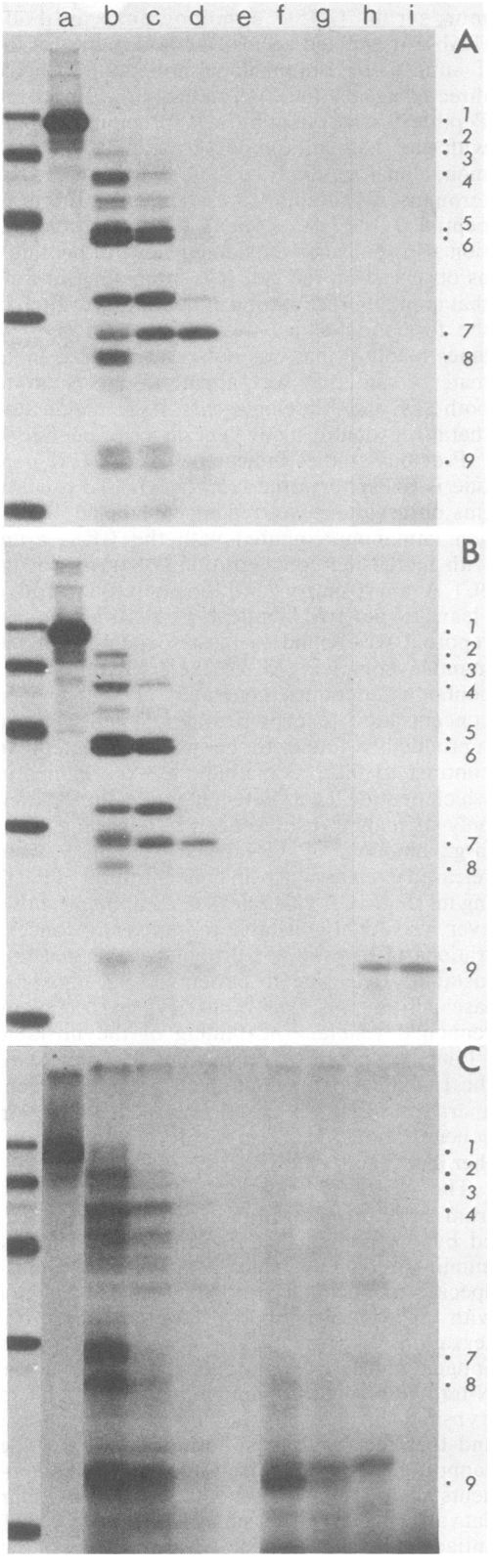

FIG. 3. Release of the 17K peptide by trypsin digestion of the T antigen. (A) Immunoprecipitation from a [^{35}S]methionine-labeled extract with BI6 antitumor serum. (B) Immunoprecipitation from the same [^{35}S]methionine-labeled extract with BK8 antitumor serum. (C) Immunoprecipitation from a ^{32}P-labeled extract with BK8 antitumor serum. The peptide numbers are the same as those in Fig. 1. Molecular weight markers are shown in the left-hand lane (see the legend to Fig. 2). Lane a, Untreated immunoprecipitate. Lanes b through e, Pellet fractions; lanes f through i, supernatant fractions. The concentrations of TPCK-trypsin used were 0.037, 0.11, 0.33, and 1.0 mg/ml in lanes b through e and f through i, respectively.

total) are phosphorylated at the N-terminus, that this class of molecules is particularly susceptible to cleavage even at very low trypsin concentrations, and that the resulting N-terminal 17K peptide is rapidly degraded into a smaller component of ~6K with increasing concentrations of enzyme. These data may indicate that the phosphorylated subpopulation of T antigen molecules are conformationally different and thus have a different susceptibility to trypsin.

Binding of the 17K peptide to calf thymus DNA. Since both the [^{35}S]methionine-labeled 17K peptide released at 1 mg of trypsin per ml and the ^{32}P-labeled 17K peptide released at 0.037 mg of trypsin per ml were present in the supernatant and not complexed to antibodies, they could be tested for their ability to bind calf thymus DNA immobilized on cellulose.

The appropriate supernatant fractions were loaded on DNA-cellulose columns at a salt concentration of 0.1 M and at pH 6.5 (Fig. 5). The material that did not bind to the column was examined. The fraction that did bind was subsequently eluted with SDS. Gel analysis of the unbound and bound fractions indicated that more than 90% of the [^{35}S]methionine-labeled 17K peptide, released by treatment with 1 mg of trypsin per ml, bound calf thymus DNA at 0.1 M NaCl (Fig. 5, lanes a and b). On the other hand, virtually none of the ^{32}P-labeled 17K peptide or its 6K breakdown product, released by treatment with 0.037 mg of trypsin per ml, was retained on the DNA column at the same salt concentration (Fig. 5, lanes c and d).

These data suggest that the N-terminal 17K peptide can bind efficiently to DNA and that phosphorylation within this region of the T antigen molecule can completely obliterate its binding capacity.

Specific binding at the SV40 origin of replication. To determine whether the amino acid sequences within the 17K peptide were sufficient to induce SV40 origin-specific DNA binding, the 17K peptide released by treatment with 1 mg of trypsin per ml was incubated with a ^{32}P-labeled *Bst*N1 digest of SV40 DNA. Detection of bind-

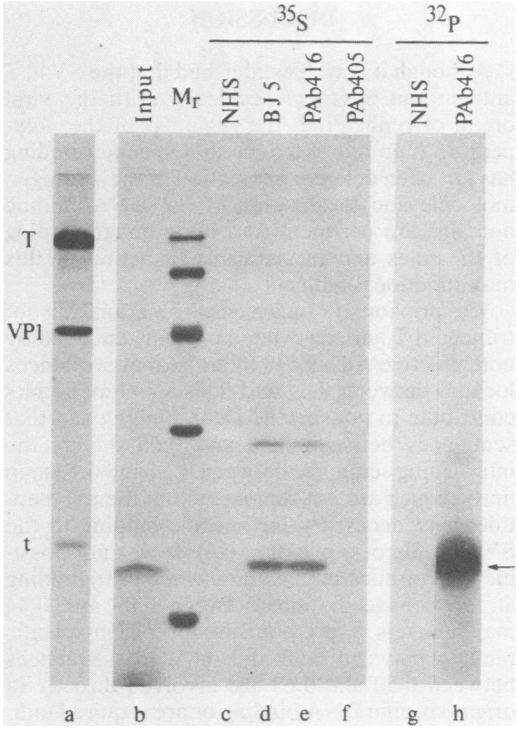

FIG. 4. Characterization of the 17K peptide. The 17K peptide released into the supernatant after trypsin digestion (1 mg/ml) of immune complexes containing [^{35}S]methionine-labeled T antigen (lane b) was compared in terms of apparent molecular weight with the small t antigen (lane a) and tested for immunoreactivity to a panel of antibodies, including normal hamster serum (NHS) (lane c), hamster SV40 antitumor serum (lane d), and monoclonal antibodies directed against either the N-terminus (lane e) or the C-terminus (lane f) of the T antigen. In a similar experiment, the 17K peptide released from ^{32}P-labeled T antigen by trypsin (0.037 mg/ml) was tested against control antibody (lane g) and monoclonal antibody against the N-terminus of the T antigen (lane h). The molecular weight markers (M_r) are described in the legend to Fig. 2. The arrow indicates the position of the 17K peptide.

ing was facilitated by immunoprecipitation with either control serum or antitumor serum as described previously (23). Although none of the SV40 DNA fragments was precipitated by the control serum, the 311-base-pair G fragment which spans the viral origin was specifically brought down by the antitumor serum (Fig. 6). As a negative control, a parallel fraction generated by immunoprecipitation with the BI6 instead of the BK8 serum was tested for specific binding of the G fragment. This parallel fraction did not contain the 17K peptide (see Fig. 3A), and no binding to SV40 DNA was observed (data not shown). As a further control, a [^{35}S]methionine-labeled fraction was used to effect G fragment-specific binding, and the resulting G fragment containing immunoprecipitate was found to contain the 17K peptide and its apparent dimer as the only [^{35}S]methionine-containing component (data not shown). This finding showed that the 17K peptide is sufficient to recognize the SV40 origin of DNA replication and to induce specific binding. Although we do not believe that a contaminating T peptide distinct from the 17K fragment is responsible for the origin-specific binding, the possibility does exist that such a peptide is present in undetectable amounts.

A similar experiment with the phosphorylated form of the 17K fragment was not done because the supernatant fraction containing this peptide included several other partial peptides derived from the T antigen (Fig. 3C). It is interesting to recall that the phosphorylated 17K peptide did not bind calf thymus DNA (Fig. 5c).

In a parallel analysis, the trypsin-released 17K peptide was found to be significantly more efficient than T antigen in effecting G fragment binding. This observation clearly argues against

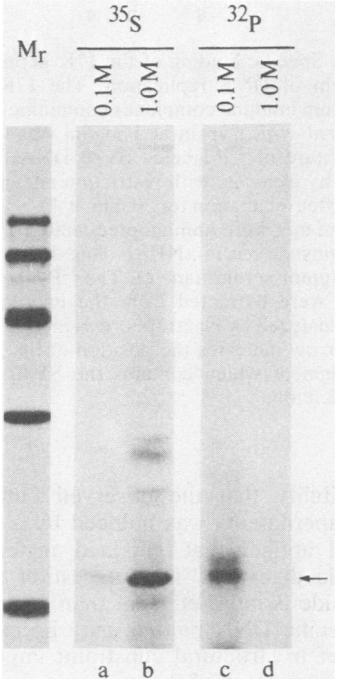

FIG. 5. Binding of the 17K peptide to calf thymus DNA. The 17K peptide released from labeled T antigen with trypsin was tested for binding to calf thymus DNA immobilized on cellulose. The 0.1 M NaCl flow-through material (lanes a and c) was concentrated by acetone precipitation and saved, and the bound material was eluted with either 1.0 M NaCl or 2% SDS (lanes b and d). The molecular weight markers (M_r) are described in the legend to Fig. 2. The arrow indicates the position of the 17K peptide.

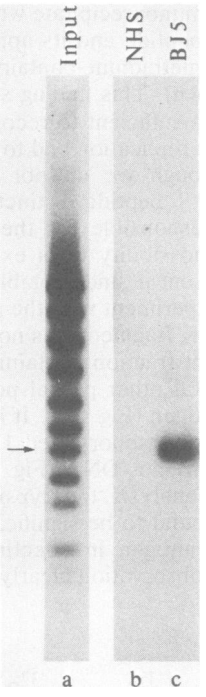

FIG. 6. Specific binding of the 17K peptide to the SV40 origin of DNA replication. The 17K peptide released from immune complexes containing T antigen by treatment with trypsin at 1 mg/ml was incubated with a mixture of ^{32}P-labeled SV40 DNA fragments generated by cleavage with restriction enzyme *Bst*N1 (lane a). After incubation for 30 min at 4°C, samples of the reaction mix were immunoprecipitated with either normal hamster serum (NHS) (lane b) or hamster SV40 antitumor serum (lane c). The ^{32}P-labeled DNA fragments were extracted from the immunoprecipitates and analyzed by electrophoresis on a 2% agarose gel. The arrow indicates the position of the 311-base-pair G fragment which contains the SV40 origin of DNA replication.

the possibility that the observed binding by tryptic supernatants was induced by a residual level of T antigen that remained uncleaved by proteolytic digestion. The suggestion that the 17K peptide is more efficient than T antigen in origin-specific DNA binding may be explained by a relief in structural constraint imposed by the adjoining region of the molecule. In support of this possibility is the finding that whereas only a fraction of the total immunoprecipitable T antigen bound calf thymus DNA, virtually all of the 17K peptide released from the T antigen bound it efficiently (Fig. 5b). Alternatively, the apparent increase in binding efficiency after trypsin digestion may be due to a difference in the stability of the DNA-protein complex after binding with antibody.

DISCUSSION

Although it is well established that the SV40 T antigen can interact specifically with the viral origin of replication, the region(s) of the polypeptide required to effect this specific binding has not been defined. Dissection of the T antigen molecule and localization of the DNA-binding domains are pertinent to a clear understanding of the roles and mechanisms of action of this multifunctional antigen.

We previously suggested, from analysis of truncated T antigen-related proteins encoded by nondefective Ad2-SV40 hybrids, that sequences located between 0.39 and 0.44 SV40 map units contribute to nonspecific DNA binding and that sequences between 0.50 and 0.63 (minus the intervening sequence between 0.54 and 0.59 map units, which are not represented in the polypeptides) are necessary for specific binding to the SV40 origin of replication (26). It was not possible to determine in that study whether (i) binding at the nonspecific site between 0.39 and 0.44 map units is a prerequisite for origin-specific binding between 0.50 and 0.63, (ii) sequences between 0.50 and 0.63 are involved directly in origin-specific DNA binding or are required indirectly to induce a conformationally active binding site elsewhere in the molecule, or (iii) origin-specific binding betwen 0.50 and 0.63 can be affected by posttranslational modifications at other regions of the molecule.

In the present study, we attempted to answer some of these questions. We identified antisera which would not only bind intact T antigen but would also specifically release the N-terminal fractional peptide of ~17K from the immune complex after treatment with trypsin. We showed that this 17K peptide could not only bind to calf thymus DNA, but was sufficient to induce SV40 origin-specific binding.

Previous studies showed that this 17K peptide extends from the extreme N-terminus approximately to amino acid 130 (29). The coding sequence for this peptide would be located between 0.51 and 0.67 SV40 map units (minus the intron between 0.54 and 0.59). Since the first 82 amino acids of the large T antigen are shared with small t antigen, which does not bind DNA (27), it can be tentatively concluded that the sequence between isoleucine 83 and arginine 130 is important (although not necessarily sufficient) for origin-specific binding by T antigen. Whether the N-terminal 82 residues play an indirect role in maintaining an active conformation for binding is not clear. The present study also showed that sequences downstream from amino acid 130 are not required for binding, although we cannot exclude the possibility that the downstream sequences play an active role in modulating the

binding affinity. For example, release of the 17K peptide from the T antigen might conceivably relieve a conformational constraint imposed by other parts of the molecule and allow efficient DNA binding. As a multifunctional protein, perturbation at one region of the T antigen molecule may well affect its properties at a different region.

It has been reported that newly synthesized T antigen molecules are poorly phosphorylated, sediment predominantly as dimers and tetramers, and bind efficiently to SV40 DNA. As these T antigen molecules mature in the cell, they become heavily phosphorylated, form large oligomers, and bind poorly to SV40 DNA (6, 8, 10, 21, 24). Although most of the phosphorylation sites of the T antigen have been identified, it is not clear whether the level of phosphorylation plays a role in inducing oligomerization or in the loss of DNA-binding activity.

In the present study, we observed that phosphorylation of the T antigen within the 17K peptide resulted in its increased susceptibility to trypsin digestion and that the released 17K peptide became sensitive to further cleavage by trypsin. These results are therefore consistent with the suggestion that phosphorylation (at least in this region of the T antigen molecule) led to a change in conformation as detected by an alteration in trypsin sensitivity. More important, we found that phosphorylation in this region of the molecule completely abolished the ability of the 17K peptide to bind DNA. Whether phosphorylation of the intact molecule at the downstream sites also affects DNA binding is not clear. Based on the present findings, we suggest that the consequence of phosphorylation at the N-terminus of the T antigen is the loss of DNA-binding activity. This modification may be characteristic of an aging population of T antigen molecules. In light of this finding, analysis of DNA binding by truncated T antigens should not be carried out with ^{32}P-labeled molecules, which clearly represent a selected subpopulation of the T antigen (3).

In the course of these studies, we also observed that the 17K fractional peptide had a tendency to form apparent dimers, which were resistant to disruption by SDS and reducing agents. These dimers were generated reproducibly either after immunoprecipitation with different antibodies or by binding to DNA. It is not clear whether the process was induced simply by juxtaposing the 17K peptide molecules or what functional significance this interaction might have. Apparent oligomers of T antigen that are resistant to disruption by SDS and reducing agents have been detected previously (14). The increase in efficiency for the dimerization of the 17K peptide may have resulted from a relief in structural constraint after proteolytic release of the adjoining region of the molecule. It is interesting to note that the only molecular forms of T antigen that can bind SV40 DNA seem to be dimers and tetramers (1, 25); it is not clear what induces such dimerization.

In conclusion, the experiments presented here provide a basis for future studies to determine the specific sites of interaction between the T antigen and SV40 DNA.

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