JOURNAL OF VIROLOGY, July 1982, p. 73–82
0022-538X/82/070073-10$02.00/0

Vol. 43, No. 1

Discrete Regions of Simian Virus 40 Large T Antigen Are Required for Nonspecific and Viral Origin-Specific DNA Binding

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Received 24 November 1981/Accepted 25 March 1982

The nondefective adenovirus type 2 (Ad2)-simian virus 40 (SV40) hybrid viruses, $Ad2^+ND_2$ and $Ad2^+ND_4$, have been used to determine which regions of the SV40 genome coding for the large tumor (T) antigen are involved in specific and nonspecific DNA binding. $Ad2^+ND_2$ encodes 45,000 M_r (45K) and 56,000 M_r (56K) T antigen-related polypeptides. The 45K polypeptide did not bind to DNA, but the 56K polypeptide bound nonspecifically to calf thymus DNA. $Ad2^+ND_4$ encodes 60,000 M_r (60K), 66,000 M_r (66K), 70,000 M_r (70K), 74,000 M_r (74K), and 90,000 M_r (90K) T antigen-related polypeptides, all of which bound nonspecifically to calf thymus DNA. However, in more stringent assays, where tight binding to viral origin sequences was tested, only the 90K protein specified by $Ad2^+ND_4$ showed specific high affinity for sequences at the viral origin of replication. From these results and previously published experiments describing the SV40 DNA integrated into these hybrid viruses, it was concluded that SV40 early gene sequences located between 0.39 and 0.44 SV40 map units contribute to nonspecific DNA binding, whereas sequences located between 0.50 and 0.63 SV40 map units are necessary for specific binding to the viral origin of replication.

Since their isolation, the adenovirus type 2 (Ad2)-simian virus 40 (SV40) hybrid viruses have proved to be extremely useful in several areas of molecular virology. Five nondefective Ad2-SV40 hybrid viruses ($Ad2^+ND_1$ through $Ad2^+ND_5$) were isolated and characterized by Lewis and colleagues (14, 20, 25). These viruses contain overlapping segments of SV40 DNA integrated at a common point on the adenovirus genome (0.86 Ad2 map units [mu]) and extending from a common point on the SV40 genome (0.11 SV40 mu) for varying extents through the SV40 early region. It has been shown that SV40-containing mRNAs are transcribed in cells infected by these viruses, and they are initiated with Ad2 sequences at the 5′ end of the transcripts (10, 12, 24, 40, 41). These hybrid mRNAs are translated into polypeptides containing small amounts of Ad2 sequences at the NH_2 terminus fused to SV40 T antigen sequences (15, 17). This family of SV40 T antigen-related polypeptides encoded by the different $Ad2^+ND$ hybrid viruses all contain common COOH termini that are similar if not identical to the COOH terminus of the authentic T antigen (15, 17). The existence of this overlapping set of T antigen-containing polypeptides has proved opportunities to ask questions about the different functional regions of the T antigen protein. The best characterized property of the SV40 T antigen is its ability to bind to DNA (2, 9, 22, 26, 31) with specific affinity for sequences at the viral origin of replication (18, 28, 32, 37; A. Scheller, L. Covey, B. Barnet, and C. Prives, Cell, in press). This specific binding is clearly related to the role of the T antigen in initiating rounds of viral DNA synthesis in infected monkey cells (34) as well as in the autoregulation of early mRNA synthesis (29). As T antigen is a multifunctional protein (39) it would be useful to be able to assess which portions of the T antigen polypeptide are required for DNA binding and to determine whether different regions of the molecule may be involved in specific and nonspecific binding. For this purpose the T antigen-related polypeptides from different nondefective Ad2-SV40 hybrid viruses were tested for their DNA binding properties.

MATERIALS AND METHODS

Materials. [^{35}S]methionine and ^{32}P-labeled deoxynucleoside triphosphates were purchased from the Radiochemical Center, Amersham, England. ^{14}C-labeled molecular weight markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from New England Nuclear Corp., Boston, Mass. *Bst*NI was bought from New England Biolabs, Bever-

ly, Maine. Native calf thymus DNA-cellulose was obtained from P-L Biochemicals, Milwaukee, Wis. Anti-SV40 tumor sera and normal preimmune hamster sera were obtained from the Research Resources Department of the National Cancer Institute. *Staphylococcus* A bacteria were purchased as Pansorbin from Calbiochem, La Jolla, Calif.

Cells and viruses. $Ad2^+ND_2$ and $Ad2^+ND_4$ stocks were grown in monolayer cultures of human KB cells, infected at a multiplicity of 5 PFU/cell, and purified by twice banding in CsCl gradients after extraction with 1% *n*-butanol from sonic extracts of infected cells (8). The stock viruses were plaque-titrated on monolayers of primary human embryonic kidney cells. The $Ad2^+ND_{4del}$ stock was a kind gift from Andrew Lewis, Jr. To analyze SV40 T antigen-related polypeptides, human KB cells in monolayer cultures (150-cm^2 bottles) were infected with the appropriate $Ad2^+ND$ hybrid virus at a multiplicity of 20 PFU/cell. Twenty-four hours postinfection, the monolayers were rinsed with phosphate-buffered saline and incubated at 37°C in Eagle minimal essential medium containing 2% fetal calf serum and 100 μCi of [^{35}S]methionine per ml (specific activity of >1,000 Ci/mmol). After 2 h the cells were rinsed again with phosphate-buffered saline and lysed with RIPA buffer (50 mM Tris-hydrochloride, pH 7.5; 150 mM NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS). Cell lysates were centrifuged at 2,000 rpm for 5 min to remove debris and were stored at −80°C until used. To analyze T antigens isolated directly from infected cell extracts or after binding to DNA, 100-μl samples were incubated with either 10 μl of anti-SV40 T serum or 10 μl of nonimmune hamster control serum for 1 h at 0°C. A 50-μl volume of a 10% suspension of formaldehyde-inactivated *Staphylococcus* A bacteria was added for a further 30 min, and then the immunoprecipitates were centrifuged and washed as previously described (26). The proteins bound to the immune complexes were released by heating in sample electrophoresis buffer (26) and were analyzed by SDS-PAGE followed by autoradiography with Kodak SB-5 film.

Tryptic peptide analysis. T antigen polypeptides resolved on SDS-polyacrylamide gels were eluted and subjected to digestion with trypsin as previously described (7). The tryptic peptides were analyzed by two-dimensional electrophoresis and chromatography on thin-layer cellulose plates, followed by autoradiography of the labeled peptides.

Binding of SV40 T antigen-related polypeptides from $Ad2^+ND$-infected cells to DNA-cellulose. DNA-cellulose columns were either obtained from commercial sources or prepared as previously described (22). Generally columns contained approximately 300 μg of DNA per g of cellulose. Nonspecific DNA binding to native calf thymus DNA-cellulose was measured by a modification of published procedures (26). Extracts (0.5 ml) of $Ad2^+ND$-infected cells, which had been adjusted to pH 6.0 with 2-μl volumes of 0.1 M acetic acid, were applied to calf thymus DNA-cellulose which had been preequilibrated with 20 column volumes of buffer containing 0.15 M NaCl, 0.01 M sodium phosphate, 0.001 M $MgCl_2$, and 0.5% Nonidet P-40, pH 6.0 (pH 6.0 buffer). Binding was allowed to occur for 60 min, and the nonbound material was then collected in 10 300-μl fractions. The column was washed with 5-ml portions of pH 6.0 buffer until no further decrease in radioactivity was detected. The DNA column was then similarly eluted with buffer containing 0.15 M NaCl, 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 0.5% Nonidet P-40 at pH 8.5 (pH 8.5 buffer), followed by a final elution step in pH 8.5 buffer which contained 1.0 M NaCl. Aliquots (5 μl) of column fractions were counted by liquid scintillation to determine the peak fractions eluted at each step. Peak fractions were then pooled, adjusted to pH 8.5 and 0.15 M NaCl, and immunoprecipitated with either anti-SV40 T serum or normal hamster serum.

For specific binding to F-161 multiorigin variant (MOV) DNA-cellulose columns, procedures were as previously described by Oren et al. (22). Cell extracts (500 μl) were adjusted to pH 7.0 with 2-μl volumes of acetic acid and bound to MOV DNA-cellulose columns which had been preequilibrated with buffer containing 5 mM sodium phosphate, 0.1 M NaCl, and 0.5% Nonidet P-40 at pH 7.0. The proteins that did not bind in these conditions were collected, and the columns were extensively washed to remove all nonbound material and then successively eluted with pH 7.0 buffers containing 0.2, 0.4, 0.8, and 1.0 M NaCl. Peak fractions of each step were adjusted to 0.2 M NaCl and immunoprecipitated with either anti-SV40 T serum or normal hamster serum.

Binding of SV40 T antigen-related polypeptides to specific viral DNA sequences in solution. The binding of T antigen-related polypeptides to specific viral DNA sequences in solution was performed according to published procedures (18; Scheller et al., in press). Briefly, 100-μl extracts of KB cells infected with $Ad2^+ND$ viruses were incubated with 5 μg of sheared herring sperm DNA and 5 ng of *Bst*NI-digested SV40 DNA that had been labeled with ^{32}P-labeled deoxynucleoside triphosphates to a specific activity of 2×10^3 to 20×10^3 cpm/ng by the method of Rigby et al. (30). After binding at 0°C for 1 h, 10 μl of either anti-SV40 T serum or normal preimmune hamster serum was added for 30 min, followed by addition of 50 μl of formaldehyde-fixed *Staphylococcus* A bacteria for a further 15 min. The immune complexes were pelleted by centrifugation and washed three times with NET buffer (pH 7.0; 0.15 M NaCl, 0.01 M Tris, 0.1 mM EDTA, 0.05% Nonidet P-40). DNA fragments were dissociated from the immune complex by incubation in 60 μl of buffer containing 0.01 M Tris (pH 8.5), 0.25 M β-mercaptoethanol, and 1% SDS and subjected to electrophoresis in 2% agarose gels. The gels were fixed in 3 volumes of ethanol, dried, and autoradiographed with Kodak XR5 films.

RESULTS

T antigen-related polypeptides encoded by the Ad2-SV40 hybrid viruses. The Ad2-SV40 hybrid virus $Ad2^+ND_2$ consists of a mixture of two classes of hybrid DNAs: one population (40%) contains SV40 sequences mapping between 0.11 and 0.39 SV40 mu, and the other population (60%) contains sequences mapping between 0.11 and 0.44 SV40 mu (10). T antigen-related polypeptides encoded by $Ad2^+ND_2$ have molecular weights estimated by SDS-PAGE analyses in these studies of 45,000 (45K) and 56,000 (56K)

(Fig. 1). These are the same two polypeptides described as 42K and 56K in earlier publications (17, 41). Although it has not yet been conclusively demonstrated, it is likely, due to size constraints, that the 0.11–0.39 SV40 DNA insert encodes the 45K T antigen and the 0.11–0.44 SV40 DNA insert encodes the 56K T antigen.

$Ad2^+ND_4$ expresses a series of T antigen-related polypeptides estimated in these analyses to be 60,000 (60K), 66,000 (66K), 70,000 (70K), 74,000 (74K), and 90,000 (90K) daltons (Fig. 1). These are the same polypeptides whose molecular weights were estimated in previous studies to be 60K, 64K, 70K, 72K, and 90K (17, 41). Like $Ad2^+ND_2$, $Ad2^+ND_4$ is also a heterogeneous population consisting of major (85%) and minor (15%) classes of hybrid DNA molecules (10, 41). The abundant class contains SV40 sequences mapping between 0.11 and 0.63 SV40 mu but lacking sequences between 0.50 and 0.60 SV40 mu, thereby missing the normal splice donor and acceptor sequences located at 0.59 and 0.54 SV40 mu (10). The minor DNA class present in $Ad2^+ND_4$ isolates contains undeleted SV40 sequences extending from 0.11 to 0.63 mu containing the normal SV40 0.59–0.54 splice donor and acceptor junctions, which are used. Recently a population of $Ad2^+ND_4$ ($Ad2^+ND_{4del}$) has been isolated which lacks the minor class containing undeleted 0.11–0.63 SV40 DNA and contains only the major class of SV40 molecules deleted between 0.50–0.60 (40). Cells infected with $Ad2^+ND_{4del}$ express the 60K, 66K, 70K, and 74K T antigen-related proteins, but lack the 90K T antigen-related polypeptide (Fig. 1), leading to the conclusion that the 90K protein is encoded by the undeleted (0.11 to 0.63) DNA present in $Ad2^+ND_4$ isolates, but absent from $Ad2^+ND_{4del}$ isolates.

To further analyze this series of T antigen-related polypeptides, two-dimensional tryptic peptide analysis was performed on a series of T antigen-related polypeptides encoded by $Ad2^+ND_1$, $Ad2^+ND_2$, and $Ad2^+ND_4$. From the [^{35}S]methionine-labeled tryptic peptides generated from the T antigen-related proteins of each of these viruses, it was possible to construct a map showing the regions of SV40 DNA that encode specific tryptic peptides (17). Some relevant tryptic peptide maps are shown in Fig. 2, and the map positions of [^{35}S]methionine-containing peptides derived from the SV40 large T and small t antigens, as well as the $Ad2^+ND$ T antigen-related polypeptides, are summarized in Fig. 3. By comparing the protein and DNA analyses of these three classes of $Ad2^+ND$ hybrid viruses with data obtained in DNA binding experiments, it has been possible to assess which regions of the T antigen protein contribute to specific and nonspecific DNA binding.

Nonspecific DNA binding properties of T antigens encoded by $Ad2^+ND_2$ and $Ad2^+ND_4$. DNA binding experiments measuring nonspecific affinities for DNA were performed by binding T antigen to DNA immobilized on cellulose (26). Extracts of [^{35}S]methionine-labeled cells were applied to native (double-stranded) DNA-cellulose columns under salt and pH conditions (pH 6) which have been shown to favor maximal (especially nonspecific) DNA binding. After sufficient time was allowed for binding to occur, nonbound proteins were collected. This was followed by washing the DNA columns with buffers of increased pH or increased pH and salt concentrations, which cause dissociation of proteins from DNA. The nonbound and bound portions of cell extracts were then immunoprecipitated with anti-T serum to determine which T antigen-related polypeptides bind DNA. By binding extracts of SV40-infected cells to calf thymus DNA at pH 6.0 and eluting the bound proteins in buffers at pH 8.5, or pH 8.5 contain-

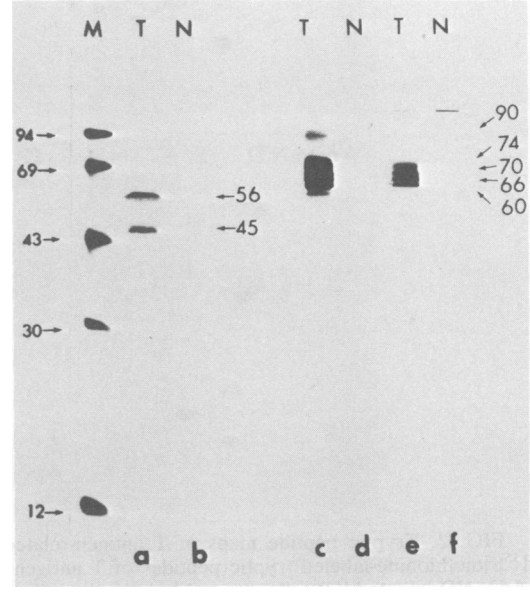

FIG. 1. T antigen-related polypeptides in cells infected with Ad2-SV40 hybrid viruses. Human KB cells infected with $Ad2^+ND_2$ (a, b), $Ad2^+ND_4$ (c, d), or $Ad2^+ND_{4del}$ (e, f) were labeled with [^{35}S]methionine. Samples (100 μl) of cell extracts were immunoprecipitated with anti-T serum (T) or normal hamster nonimmune serum (N), and immunoprecipitates were subjected to SDS-PAGE and autoradiography. ^{14}C-labeled markers (M) were run in the same gel. Numbers refer to molecular weights ($\times 10^3$) as estimated by relative mobility compared to the molecular weight markers. The 90K T antigen polypeptide encoded by $Ad2^+ND_4$ but not $Ad2^+ND_{4del}$ frequently appears as a doublet.

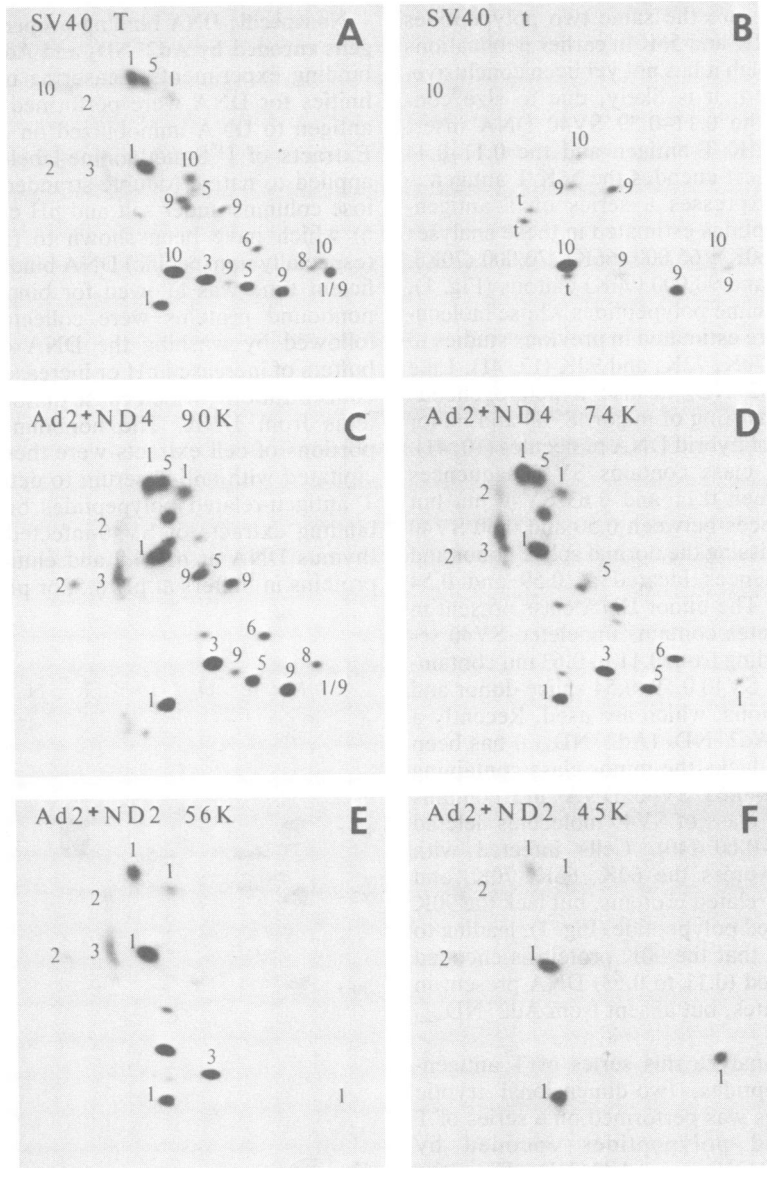

FIG. 2. Tryptic peptide maps of T antigen-related polypeptides isolated from Ad2-SV40 hybrid viruses. [^{35}S]methionine-labeled tryptic peptides of T antigens immunoprecipitated from SV40-infected CV-1 cells or Ad2$^+$ND-infected KB cells were subjected to two-dimensional thin-layer electrophoresis and chromatography as described in the text. Peptide numbers refer to regions of the genome delineated in Fig. 3 and are derived from data obtained from these and from tryptic peptides of the Ad2$^+$ND$_1$ 28K and Ad2$^+$ND$_2$ 60K, 66K, and 70K T antigen polypeptides (not shown).

ing 1.0 M salt, it has been shown that a considerable portion of authentic SV40 T antigen binds to DNA (26). When extracts of Ad2$^+$ND$_2$-infected cells were bound to calf thymus DNA at pH 6.0, followed by elution at pH 8.5 and then at pH 8.5 with 1.0 M NaCl, it was shown that, although a portion of the 56K T antigen protein bound to DNA at pH 6.0 and was eluted at pH 8.5, no 45K T antigen protein was bound under these conditions (Fig. 4). In a separate experiment, the Ad2$^+$ND$_1$ 28K polypeptide also did not bind (data not shown). The presence of a nonbinding population of 56K T antigen is not unexpected; similar observations have been made with authentic SV40 T antigen (26). Thus DNA sequences encoding the 56K T antigen, which

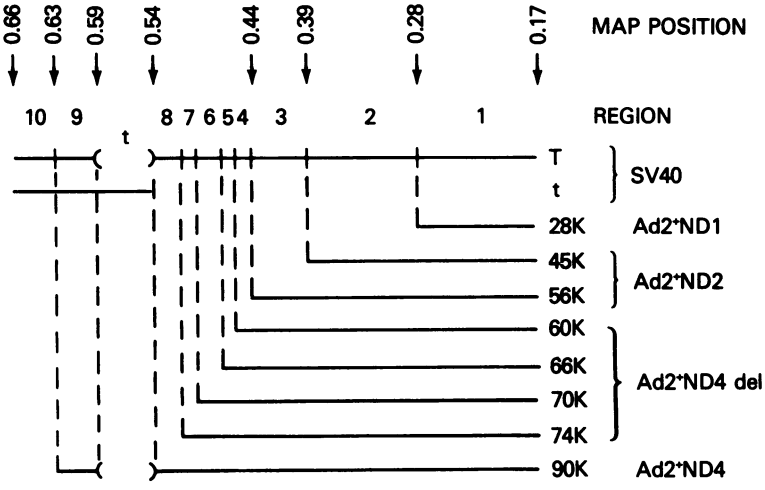

FIG. 3. Location of tryptic peptide regions on the SV40 genome by using overlapping tryptic peptide maps of SV40 and Ad2$^+$ND hybrid virus T antigen polypeptides.

most likely map between 0.11 and 0.44 SV40 mu, contribute to pH 6.0 calf thymus DNA binding. As the 45K protein did not bind DNA under any of the conditions tested, and as this protein is most likely derived from the Ad2$^+$ND$_2$ hybrid DNA-containing sequences mapping between 0.11 and 0.39 SV40 mu, it is suggested that sequences mapping between 0.39 and 0.44 (~262 nucleotides) may contribute to the general DNA binding property of SV40 T antigen (see Fig. 3, region 3).

When extracts of [^{35}S]methionine-labeled Ad2$^+$ND$_4$-infected cells were similarly tested for binding to calf thymus DNA-cellulose, the results were not surprising in that the entire series of polypeptides from 60K through 90K bound to DNA (Fig. 5). Whereas the 60K–74K series of T antigen proteins eluted both with pH 8.5 and with pH 8.5 plus salt, a considerable portion of each of these proteins did not bind even at pH 6.0. In contrast, the 90K protein bound very efficiently to DNA under these conditions; virtually no labeled 90K T antigen was detected in the pH 6.0 (nonbound) column eluates. In addition, the 90K T antigen that was bound at pH 6.0 was relatively more sensitive to elevated pH than the other T antigen-related polypeptides, with virtually complete elution at pH 8.5.

High-affinity DNA binding of SV40 T antigen-related polypeptides. DNA-cellulose columns can also be used to test for specific (high-affinity) and nonspecific (low-affinity) DNA binding properties. Previously it was shown that higher salt concentrations are required to elute SV40 T antigen from SV40 DNA-cellulose than from calf thymus DNA-cellulose, and even greater salt concentrations are required to elute SV40 T antigen from a multiorigin variant (MOV) of SV40 DNA (22). These data suggested that the viral genome has sequences for which T antigen has higher affinity than does calf thymus DNA. This is consistent with previous studies in which T antigen was found to bind specifically

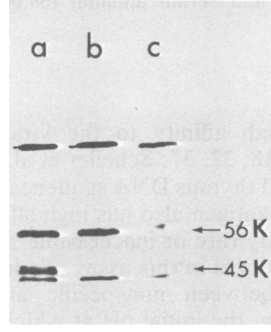

FIG. 4. Binding of T antigen-related polypeptides expressed by Ad2$^+$ND$_2$ to calf thymus DNA. Extracts of Ad2$^+$ND$_2$-infected cells were adjusted to pH 6.0 and bound to calf thymus DNA-cellulose. Proteins that did not bind to the column and were eluted at pH 6.0 (a), or that did bind to the column and were eluted at pH 8.5 (b) or pH 8.5 plus 1.0 M NaCl (c), were immunoprecipitated with anti-SV40 T serum and then subjected to SDS-PAGE and autoradiography. Polypeptides with slower and more rapid electrophoretic mobility than 56K and 45K T antigens were also consistently immunoprecipitated with normal hamster serum and are considered to be the adenovirus hexon polypeptide and cellular actin, respectively, because of their abundance, molecular weights, and reported DNA binding properties (13).

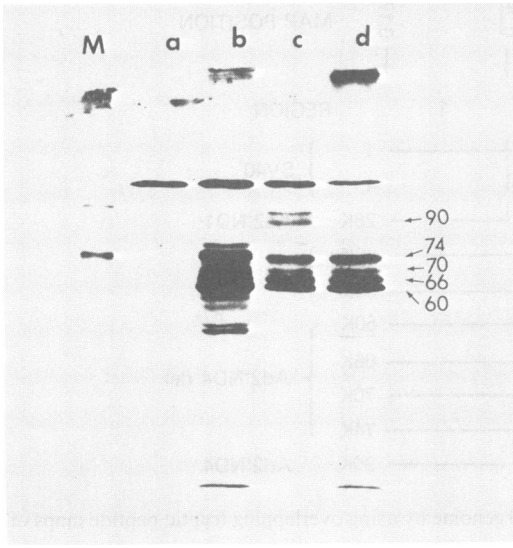

FIG. 5. Binding of T antigen-related polypeptides expressed in $Ad2^+ND_4$-infected KB cells to calf thymus DNA. Extracts of $Ad2^+ND_4$-infected cells were adjusted to pH 6.0 and bound to calf thymus DNA-cellulose columns which were equilibrated with pH 6.0 buffer as described for Fig. 4. Proteins that did not bind (b) and proteins that did bind and were eluted at pH 8.5 (c) or at pH 8.5 plus 1.0 M NaCl (d) were immunoprecipitated with anti-SV40 T serum. Samples of nonbound proteins (pH 6.0) were also immunoprecipitated with normal hamster serum (a). M lane, Marker polypeptides: phosphorylase A (95,000 molecular weight) and serum albumin (68,000 molecular weight).

and with high affinity to the viral origin of replication (18, 32, 37; Scheller et al., in press). Although calf thymus DNA sequences may exist for which T antigen also has high affinity, these are sufficiently rare or inaccessible so that they are not functional in this assay. To increase the distinction between nonspecific and specific DNA binding, the initial pH at which T antigen was bound to the DNA-cellulose was raised to 7.0. Under these conditions a considerable portion of T antigen does not bind to any DNA. However, the fraction that does bind has high affinity for DNA and requires a high salt concentration (0.5 to 1.0 M) for elution from MOV DNA. In contrast, under these same conditions, all of the bound SV40 T antigen was eluted from calf thymus DNA by 0.3 M NaCl (22). The SV40 T antigen-related proteins present in $Ad2^+ND_2$- and $Ad2^+ND_4$-infected cells were tested for their ability to bind tightly to MOV DNA cellulose. When $Ad2^+ND_2$-infected [^{35}S]methionine-labeled cell extracts were bound to MOV DNA cellulose at pH 7.0 and eluted with increasing NaCl concentrations, it was observed that neither the 45K nor the 56K protein bound to SV40 MOV DNA under these conditions (Fig. 6A). This confirmed earlier experiments (22) suggesting that binding to calf thymus DNA at pH 6.0 measures a reduced affinity binding, whereas salt elution at pH 7.0 from MOV DNA measures high-affinity binding.

When the T antigens synthesized in $Ad2^+ND_4$-infected cells were similarly analyzed, it was found that the only polypeptide that bound tightly to the MOV column at pH 7.0 was the 90K protein (Fig. 6B). The identity of this protein as the $Ad2^+ND_4$-encoded 90K T antigen-related polypeptide was shown by the fact that this protein was not immunoprecipitated by the serum of nontumored hamsters (data not shown). The binding of this 90K hybrid protein to MOV DNA was similar to that of wild-type T antigen since both protein populations contain a class of T antigen which can bind tightly to MOV DNA at pH 7.0 and which requires NaCl concentrations up to 0.8 M for elution from the DNA column. Of the other $Ad2^+ND_4$ T antigen-related polypeptides, only the 60K protein bound loosely to MOV DNA under these more stringent binding conditions, eluting from the column at 0.2 M NaCl. It was therefore concluded that the 90K protein possesses tight DNA binding properties that are absent from the other $Ad2^+ND$ T antigen-related polypeptides. As all of these except the 45K polypeptide can bind nonspecifically to calf thymus DNA, it was concluded that T antigen sequences contributing to specific (tight) and nonspecific (loose) binding to DNA are located in discrete regions of the T antigen polypeptide.

Binding of T antigens from $Ad2^+ND$-infected cells to specific DNA sequences in solution. Although binding of T antigens to DNA-cellulose columns provides useful information about which polypeptides do and do not bind under different conditions, few data can be obtained regarding the DNA sequences for which the T antigens have affinity. More recently an alternative assay was developed (18; Scheller et al., in press) to address this question. This alternative approach also has the advantage that it can be used to test crude extracts, avoiding the necessity of preparing highly purified T antigen. In this system, when radioactively labeled restriction fragments of SV40 DNA are incubated with extracts of SV40-infected or transformed cells containing T antigen, the restriction fragments that contain sequences at the viral origin of replication are selectively bound to T antigen. The T antigen-DNA complex can be isolated and effectively purified by immunoprecipitation with anti-T antibodies (either tumor serum or monoclonal antibodies). For example, it was shown

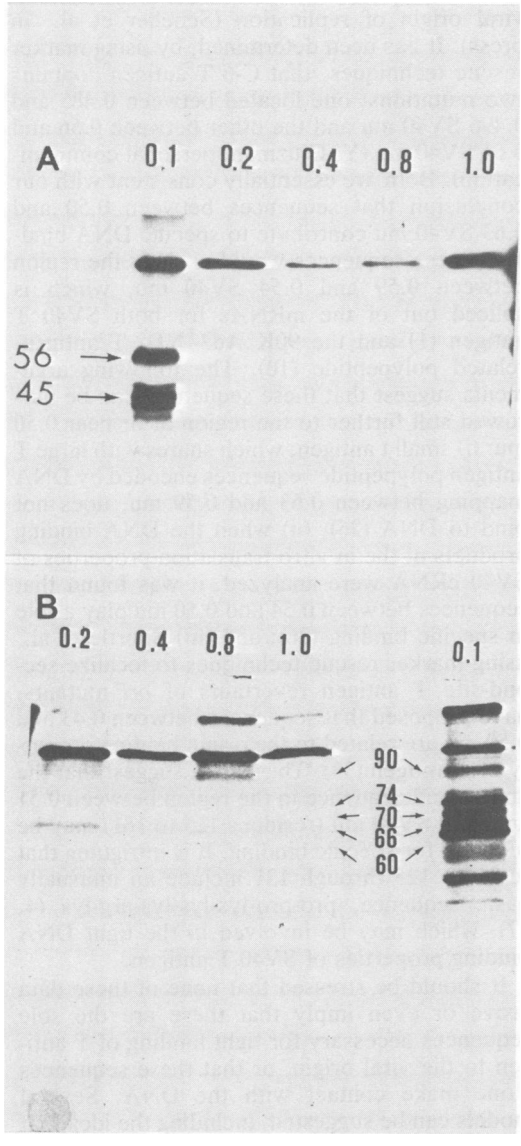

FIG. 6. Binding of T antigen-related polypeptides from $Ad2^+ND_2$- and $Ad2^+ND_4$-infected cells to SV40 MOV DNA-cellulose. (A) Extracts of [^{35}S]methionine-labeled $Ad2^+ND_2$-infected KB cells were adjusted to pH 7.0 and 0.1 M NaCl and applied to MOV DNA-cellulose which had been preequilibrated with the same buffer. After collection of proteins not bound at 0.1 M NaCl, bound proteins were eluted successively with buffers containing 0.2, 0.4, 0.8, and 1.0 M NaCl as shown. All fractions were adjusted to 0.2 M NaCl, immunoprecipitated with anti-SV40 T serum, and subjected to SDS-PAGE and autoradiography. (B) Polypeptides from $Ad2^+ND_4$-infected KB cells were adjusted to pH 7.0 and 0.1 M NaCl and applied to MOV DNA-cellulose which had been preequilibrated with the same buffer. After collection of proteins not bound to the column at 0.1 M NaCl, fractions were eluted successively with buffers containing 0.2, 0.4, 0.8, and 1.0 M NaCl as shown. All fractions were adjusted to 0.2 M NaCl and immunoprecipitated with anti-SV40 T serum. In this gel system the protein that migrated slightly more slowly than the 90K protein was considered to be adenovirus hexon because it had the same electrophoretic mobility as this protein, and it bound DNA.

that when ^{32}P-labeled, *Bst*NI-digested SV40 fragments were incubated with extracts of SV40-infected CV-1 cells, the 311-base pair G fragment which spans the viral origin was selectively bound to the T antigen and immunoprecipitated with anti-T serum but not normal (preimmune) serum (Scheller et al., in press). With this approach, extracts of $Ad2^+ND_2$-, $Ad2^+ND_4$-, and $Ad2^+ND_{4del}$-infected cells were bound to ^{32}P-labeled *Bst*NI-digested SV40 DNA (Fig. 7). By immunoprecipitating the DNA bound to the T antigen-related polypeptides, it was found that only extracts of cells infected with $Ad2^+ND_4$ demonstrated specific G-fragment binding. Neither $Ad2^+ND_2$, or, more significantly, $Ad2^+ND_{4del}$-infected cells demonstrated any specific G-fragment binding. These results confirmed and extended the data obtained when $Ad2^+ND_4$-infected cell extracts were bound to MOV DNA cellulose, in which only the 90K T antigen-related polypeptides bound tightly to the DNA. By combining the data on the nature of the DNA inserts in the Ad2-SV40 hybrid viruses with the DNA binding studies, it can be concluded that only the longest product of the nondeleted SV40 DNA insert in $Ad2^+ND_4$ virus isolates is capable of viral origin-specific high-affinity binding. As this product must be the 90K T antigen-related polypeptide (see Fig. 1), it can be inferred that sequences mapping between 0.5 and 0.63 on the viral genome (Fig. 3, regions 8 and 9) are required for the unique and tight binding of T antigen to the origin.

DISCUSSION

The overlapping set of T antigen-related polypeptides synthesized in some of the nondefective Ad2-SV40 hybrid viruses have been used to determine which regions of the T antigen polypeptide are necessary for its DNA binding properties. It can be concluded from these experiments that two distinct regions of the molecule contribute to tight and loose DNA binding. Although we have shown that nonspecific binding can occur in the absence of sequences necessary for specific binding, there is no evidence from these or other studies that tight or specific binding can occur in the absence of sequences required for nonspecific binding. Nor can it be concluded that the sequences contributing to nonspecific binding, delineated in this study as between 0.39 and 0.44 mu, are the only sequences on the molecule that contribute to this

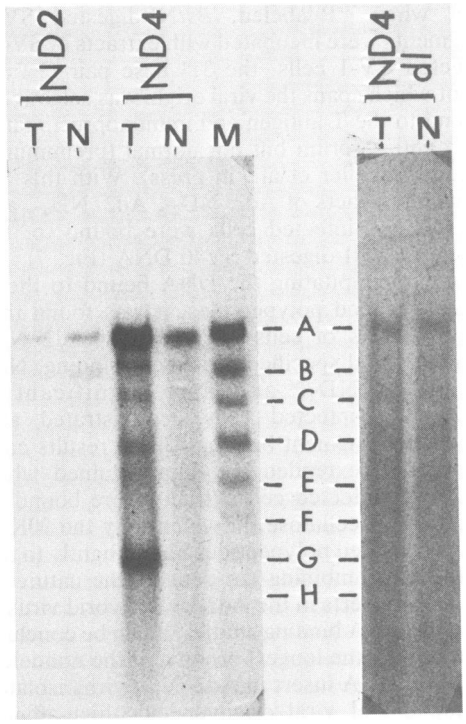

FIG. 7. Binding of SV40 T antigen-related polypeptides from Ad2$^+$ND-infected cells to specific SV40 DNA sequences. Extracts of unlabeled cells infected with Ad2$^+$ND$_2$, Ad2$^+$ND$_4$, or Ad2$^+$ND$_{4del}$ were bound to ^{32}P-labeled SV40 DNA digested with *Bst*NI, which produces 13 restriction fragments including the 311-base pair G fragment which spans sequences at the viral origin of replication. Equal quantities of extracts containing protein-DNA complexes were immunoprecipitated with anti-SV40 T serum (T) or normal nonimmune hamster serum (N), followed by binding to formaldehyde-fixed *Staphylococcus* A bacteria. DNA fragments were eluted from washed immune complexes and subjected to agarose gel electrophoresis and autoradiography.

sort of interaction with DNA. For example, Rundell et al. (31) showed that the mutant *dl*-1001 T antigen (molecular weight 33,000), which contains sequences mapping between 0.67 and 0.43 SV40 mu, can bind to calf thymus DNA. However, as *dl*-1001 can only be grown in the presence of wild-type helper large T antigen, it could not be discerned whether the *dl*-1001 T antigen was binding to DNA independently, or through association with wild-type T antigen.

The localization of sequences involved in specific binding to the viral origin is in concordance with recent data obtained using a mutant T antigen from transformed CV-1 monkey cells (C-6 cells) isolated by Gluzman et al. (5). The C-6 cell line contains a T antigen which, although it can *bind nonspecifically* to calf thymus DNA, shows no specific affinity for sequences at the viral origin of replication (Scheller et al., in press). It has been determined, by using marker rescue techniques, that C-6 T antigen contains two mutations: one located between 0.485 and 0.496 SV40 mu and the other between 0.56 and 0.64 SV40 mu (Y. Gluzman, personal communication). Both are essentially consistent with our conclusion that sequences between 0.50 and 0.63 SV40 mu contribute to specific DNA binding. These sequences would exclude the region between 0.59 and 0.54 SV40 mu, which is spliced out of the mRNAs for both SV40 T antigen (1) and the 90K Ad2$^+$ND$_4$ T antigen-related polypeptide (10). The following arguments suggest that these sequences can be narrowed still further to the region at or near 0.50 mu: (i) small t antigen, which shares with large T antigen polypeptide sequences encoded by DNA mapping between 0.63 and 0.59 mu, does not bind to DNA (26); (ii) when the DNA binding products of the in vitro translation properties of SV40 cRNA were analyzed, it was found that sequences between 0.54 and 0.50 mu play a role in specific binding (26); and (iii) Shortle et al., using marker rescue techniques to localize second-site T antigen revertants of *ori* mutants, have proposed that sequences between 0.43 and 0.50 mu are related to the origin binding property of T antigen (33). These data suggest that the amino acid sequence in the region between 0.51 and 0.49 SV40 mu (residues 125 to 161) may be required for specific binding. It is intriguing that residues 125 through 131 include an unusually basic sequence, pro-pro-lys-lys-lys-arg-lys (4, 27), which may be involved in the tight DNA binding properties of SV40 T antigen.

It should be stressed that none of these data prove or even imply that these are the sole sequences necessary for tight binding of T antigen to the viral origin, or that these sequences alone make contact with the DNA. Several models can be suggested, including the idea that these regions are required to bestow the necessary conformation upon the entire polypeptide, or that they may contribute directly or indirectly to post-translational modification of T antigen, such as phosphorylation. Alternatively, these sequences may be involved in the formation of T antigen oligomers (such as tetramers) postulated to be the bound form of T antigen (21). Other caveats include the fact that the T antigen polypeptides encoded by the Ad2$^+$ND hybrid viruses all contain adenovirus-encoded sequences at their NH_2 termini and are missing NH_2-terminal sequences present in the wild-type SV40 T antigen. Convincing evidence has been provided, however, that the D-2 hybrid T antigen-related polypeptide, which is also an Ad2-SV40 fusion product, very closely resembles authentic SV40 T antigen in its biochemical properties (36).

Despite these reservations the present experiments have provided some insight into the biochemical function of SV40 T antigen in that they have helped to define further the DNA binding regions of this important regulatory protein. T antigen has also been shown to form oligomers (2, 16, 23), to be an ATPase (38), and to form a complex with a host 53,000 M_r protein (6, 11, 19). Using the Ad2$^+$ND hybrid viruses it should be possible, with the biochemical assays now available, to make similar assessments of the regions of T antigen contributing to these functions as well.

ACKNOWLEDGMENT

This work was supported in part by Public Health Service grant CA-26905 from the National Cancer Institute to C.P.

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