Simian Virus 40 (SV40) T Antigen Binds Specifically to Double-Stranded DNA but Not to Single-Stranded DNA or DNA/RNA Hybrids Containing the SV40 Regulatory Sequences

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Simian virus 40 T antigen has been shown previously to bind specifically with high affinity to sites within the regulatory region of double-stranded simian virus 40 DNA. Using competition filter binding and the DNA-binding immunoassay, we show that T antigen did not bind specifically to either early or late single-stranded DNA containing these binding sites. Moreover, T antigen did not bind these sequences present in single-stranded RNA, RNA/RNA duplexes, or RNA/DNA hybrids. T antigen did, however, bind as efficiently to single-stranded DNA-cellulose as to double-stranded DNA-cellulose. This binding was nonspecific because it was independent of the presence of T-antigen-binding sites. The implications of these observations are discussed.

Simian virus 40 large T antigen (SV40 T Ag) has been shown to be a DNA-binding protein that can be retained by both native or denatured DNA-cellulose (5, 27, 38). It has also been extensively characterized with respect to its specific interactions with viral DNA. Three tandem sites (sites 1, 2, and 3) within the viral regulatory region each contain multiple copies of the consensus pentanucleotide 5' GA/GGGC 3' to which T Ag binds (9, 20, 33, 34, 45). Methylation and ethylation interference experiments have identified the guanine and phosphate residues within the binding sites that are the likely contact points between T Ag and SV40 DNA (9, 20). The binding affinity of T Ag probably depends on several factors, including the number of and spacing between the pentanucleotides as well as their adjacent sequences (9, 20, 30, 33, 41). Binding to site 1 is the strongest (9, 13, 33, 34, 45) and is related to the autoregulatory function of T Ag (18, 29). Binding to site 2 is less strong than to site 1 by a factor of between 3 and 10, depending on the binding assay used (9, 15, 33, 34, 41, 45), and is related to the role of T Ag in the initiation of viral DNA replication in this region (10, 24, 35, 44). Binding to site 3, whose function is unknown, is the weakest and is not detected in some assays (9, 33, 41, 44, 45).

It was recently observed that T Ag can unwind DNA containing wild-type but not mutant viral origin DNA sequences (7). The initial stage in this process is very likely the specific binding of T Ag to site 2, which is part of the core replication origin. The unwinding reaction must result in the generation of single strands in this region. This is supported by the requirement of the reaction for single-stranded binding protein (7). Because the helicase activity of T Ag (7, 39) is most likely an integral part of the unwinding reaction, it can be suggested that the origin, once unwound, no longer serves as a specific binding substrate for T Ag and that the

protein may then either dissociate from or move along the single strands in order to further unwind the double helix. However, evidence was provided (38) that T Ag binds preferentially to single-stranded DNA versus double-stranded DNA. It was therefore of interest to compare the specific and nonspecific binding of T Ag single-stranded and double-stranded DNA.

The SV40 T Ag that was used in these experiments was derived from human 293 cells (16) infected with the helper-independent adenovirus vector AdSVR111 (14) that contains SV40 early-region sequences controlled by the major late promoter of adenovirus (14). T Ag was purified by immunoaffinity by using the T Ag-specific monoclonal antibody PAb 419 (19) cross-linked to protein A Sepharose by previously described procedures (11, 36). This routinely yielded substantial quantities (approximately 250 to 500 µg/10⁸ 293 cells) of highly purified viral protein that stimulated the *in vitro* synthesis of viral DNA to extents comparable to those previously described (21, 40, 46; E. Wang and C. Prives, unpublished data). The DNAs or RNAs containing T Ag-binding sites that were used in these experiments were derived from M13 bacteriophage clones mp8SVO and mp9SVO that were prepared by inserting the *Hind*III-*Kpn*I fragment of SV40 DNA from nucleotides 5171 to 294 (termed SVO) in opposite orientations into the M13 vectors mp8 and mp9 replicative forms. This 366-nucleotide fragment contains binding sites 1, 2, and 3 and extends into the late-region *Kpn*I site past the 72-base-pair repeat enhancer region. The *Kpn*I site was changed to an *Eco*RI site by using *Eco*RI linkers. To examine the interaction of T Ag with single strands containing binding sites 1, 2, and 3, several binding assays were used.

Competition of double-stranded DNA with single-stranded SVO-containing DNA measured by the filter-binding assay. Competitive filter binding was used to compare the ability of single-stranded or double-stranded DNAs containing or lacking the binding sequences to compete with ³²P-labeled double-stranded DNA for binding to T Ag. DNAs that lacked binding sites were single-stranded circular M13mp9 and double-stranded supercoiled pBR322. DNAs that contained binding sites were single-stranded mp8SVO and mp9SVO and double-stranded circular mp9SVO replicative

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form. pSVO1ΔEP (46), a derivative of pBR322 containing the 311-nucleotide *Eco*RII G SV40 fragment, that includes binding sites 1, 2, and 3, was cleaved with *Acc*I that cuts once in the pBR322-derived region of the plasmid and end labeled by using Klenow DNA polymerase I (New England BioLabs, Inc.). 32 P-labeled pSVO1ΔEP DNA (20 ng) was bound to T Ag in the presence of increasing quantities of unlabeled double- and single-stranded DNAs with or without SVO for 20 min at 20°C in binding buffer (100 mM NaCl, 20 mM sodium phosphate [pH 6.8], 100 mM EDTA, 3 mM dithiothreitol) and 100 ng of pBR322 DNA. The reaction mixture was filtered through 25-mm-diameter nitrocellulose filters, which were then washed repeatedly with binding buffer. The radioactivity retained on the filters was determined by scintillation counting. The only DNA that efficiently competed with the labeled DNA over the range of concentrations tested was double-stranded DNA containing SVO sequences (mp9SVO replicative form DNA) (Fig. 1). At the highest concentration, one of the single-stranded DNAs, ss mp9, did appear to compete slightly for specific binding. However, the fact that this DNA lacked the SV40 origin sequences makes it unlikely that any of the single-stranded DNAs are capable of significant competition for binding of T Ag specifically to double-stranded DNA.

Binding of T Ag to nucleic acids containing SV40 regulatory sequences by the DNA-binding immunoassay. The ability of T Ag to bind to a variety of nucleic acid molecules containing SVO sequences, namely, double- and single-stranded DNA, double- and single-stranded RNA, and DNA/RNA hybrids, was tested by using a modification of the DNA-binding immunoassay (23, 33). Single-stranded SVO DNA was synthesized by primer extension with 32 P-labeled nucleotides from mp8SVO or mp9SVO templates, followed by cleavage with the appropriate enzyme (*Hind*III or *Eco*RI) and isolation from denaturing gels by previously described procedures (2, 22). The single-stranded nature of these probes was confirmed by their complete degradation by S1 nuclease and altered electrophoretic mobility compared with the mobility of the double-stranded fragment (data not shown). RNA transcripts of the SVO DNA sequences were synthesized by using the GEM plasmid vector (Promega Biotec) containing SP6 and T7 RNA polymerase promoters in opposite orientations, between which the SVO fragment was inserted, creating pGEMSV0. The late strand was synthesized by transcribing the DNA with SP6 RNA polymerase after the plasmid was linearized with *Hind*III, and the early strand was synthesized with T7 RNA polymerase after the plasmid was linearized with *Eco*RI. Transcription reactions were carried out at 37°C for 30 min in 40-μl reaction mixtures containing 10 mM NaCl, 40 mM Tris (pH 7.9), 6 mM MgCl₂, 10 mM dithiothreitol, 2 mM spermidine, 0.5 mM each of ATP, CTP, GTP, and UTP, 2.5 μCi of [α - 32 P]CTP (specific activity, 6,000 Ci/mol), 1 μg of template DNA, and 7 U of enzyme. Enzymes and reaction specifications were obtained from Promega Biotec. RNA/RNA duplexes were created by heating equal quantities (as estimated by determination of radioactivity) of early and late RNA SVO strands in hybridization buffer {40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 6.4], 1 mM EDTA, 0.4 M NaCl, 80% formamide}, first at 72°C for 15 min and then at 52°C for 3 h. DNA/RNA hybrids were made by the same procedure. The formation of stable duplexes or hybrids was verified by resistance (>90%) to S1 nuclease (data not shown). Purified T Ag (0.5 μg) was bound to 10 to 50 ng of labeled nucleic acids for 1 h at 4°C in buffer (0.1 M NaCl, 0.01 M HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 0.01

M MES [morpholineethanesulfonic acid], 0.1% Nonidet P-40 [pH 6.8]) in the presence of 1 U of RNasin (Promega Biotec) and 100 ng of herring sperm DNA. PAb 419 cross-linked to protein A Sepharose (11, 36) (approximately 7.5 μg of cross-linked antibody) was added to the binding reaction for 2 h. After centrifugation of the immune complexes, nucleic acids in the supernatant, constituting the nonbound fraction, were purified by phenol extraction and ethanol precipitation, and the T Ag-bound nucleic acids remaining in the pellet were released in buffer containing 10 mM Tris (pH 8.0) and 1 mM EDTA by heating at 60°C for 15 min, followed by phenol extraction and ethanol precipitation. The double-stranded DNA SVO fragment was specifically bound to T Ag (Fig. 2B) as has been previously shown (12, 23, 33). The other nucleic acids containing SVO sequences were not detected in the immunoprecipitates. All species survived the binding reaction and were detected in the unbound supernatants with relatively little degradation (Fig. 2C). Although not shown in Fig. 2, E strand DNA synthesized from mp9SVO template also did not bind to T Ag in the immunoassay under any conditions tested. In addition, the early and late single-stranded fragments of SVO were tested in the DNA-binding immunoassay by using another T Ag monoclonal antibody, PAb 416 (18), that has also proved to be effective in detecting binding to double-stranded DNA fragments, yielding results similar to those with PAb 419 (data not shown). A variety of pH conditions (from 6.2 to 7.2) and salt concentrations (from 0.05 to 0.225 M) were tested, and none resulted in T Ag binding to either single-stranded fragment, although the double-stranded fragment was consistently bound by the viral protein (data not shown). Finally, we also tested whether T Ag binds to single-stranded DNA provided with a double-stranded region to load T Ag onto the DNA. A 50-nucleotide oligomer 5' to the multiple cloning site of M13 and complementary to the single-stranded M13mp8 bacteriophage genome was synthesized,

FIG. 1. Competition of double- and single-stranded DNAs for binding of T Ag to double-stranded SV40 DNA. T Ag (200 ng) and 32 P-labeled pSVO1ΔEP DNA (20 ng) were mixed with the indicated amounts of unlabeled DNAs for 20 min at 20°C, followed by binding of reaction mixtures to nitrocellulose filters. [32 P]DNA retained on filters was quantified by liquid scintillation. Radioactivity bound in the absence of T Ag (consistently less than 10³ cpm) was subtracted from the values shown. Competing DNAs were single stranded with SVO sequences (ss mp8SVO and ss mp9SVO) or without SVO (ss mp9) and double stranded with SVO (ds SVO) or without SVO (ds w/o SVO).

FIG. 2. Binding of T Ag to double-stranded DNA, single-stranded DNA, RNA, RNA/DNA, and RNA/RNA containing the T-Ag-binding sequences. ^{32}P -labeled double- and single-stranded SVO DNA fragments and SVO RNA transcripts were prepared as described in the text, as were RNA/RNA duplexes and RNA/DNA hybrids. (A) Aliquots of the input nucleic acids. Nucleic acids were incubated with T Ag, followed by immunoprecipitation with PAb 419-protein A Sepharose. (B) The bound material was released from the immune complexes by denaturation, purified, and analyzed by agarose gel electrophoresis and autoradiography. (C) Nucleic acids that remained unbound in the supernatant were purified and similarly analyzed. The input labeled nucleic acids are double-stranded SVO DNA (a), single-stranded L strand SVO DNA (b), single-stranded early RNA SVO transcript (c), RNA/RNA SVO duplex (d), and RNA/DNA hybrid (e).

radiolabeled, and annealed to mp8SVO or mp9SVO. The duplex was used in the DNA-binding immunoassay to determine if it could bind to T Ag. Although this could theoretically serve as a substrate for the helicase activity of T Ag (7, 38), the binding reaction was performed under conditions that are incompatible with helicase activity (i.e., no ATP, incubation at 4°C). Under conditions in which binding to double-stranded SV40 was demonstrated, no binding to the partial duplex was detected (data not shown).

Thus, by two different methods, filter binding and the DNA-binding immunoassay, we have been unable to detect any specific affinity of T Ag for single-stranded DNA containing SV40-binding sites. In addition, DNase I footprinting showed no specific protection of DNA sequences on either single strand by T Ag (K. Auburn and C. Prives, unpublished data). As all of these methods use excess quantities of nonspecific DNA, they require that T Ag bind with a higher affinity to the SVO single- or double-stranded nucleic acid than to the nonspecific carrier DNA. Under these conditions, it is clear that of all the substrates tested, only double-stranded DNA containing the T Ag-binding sites was bound specifically. These observations are consistent with computer-generated projections derived from methylation and ethylation interference experiments that show the T Ag-DNA interactions occurring mainly along the major groove of the double-stranded helix (20). Furthermore, high-affinity binding of T Ag to site 1 has been suggested to be stabilized by bending of the double helix in this vicinity (30), a phenomenon unlikely to be manifested by single-stranded DNA. As both RNA/RNA and RNA/DNA duplex molecules generally assume A-form helices (4), the inability to bind specifically to T Ag suggests that, as predicted by Jones and Tjian (20), B-form double-stranded DNA is the preferred binding substrate. Related to this is the observation that formation of a partial (43) or complete (42) cruciform structure at the viral origin region inhibits specific binding of the T Ag to sequences at this region. Earlier observations (6) describing the identification of RNA molecules bound to T Ag may result from interactions that are distinct from the

specific binding property of T Ag and may relate more to the nonspecific DNA-binding properties of the protein.

Binding of T Ag to single-stranded DNA-cellulose. Spillman et al. (38) reported that T Ag binds more efficiently to denatured than to double-stranded calf thymus DNA cellulose. To further test the relative efficiency of T Ag for the different DNAs used in these studies, we coupled single-stranded M13, mp8SVO and mp9SVO DNA, and double-stranded SV40 DNA to cellulose by using previously described procedures (1) and compared the binding of T Ag to these DNA-celluloses by using a batchwise binding and elution protocol as previously described (3). After purified T Ag (approximately $5\text{ }\mu\text{g}$) was bound to the DNA-cellulose (approximately $50\text{ }\mu\text{g}/100\text{ mg}$ of cellulose) that had been pretreated with 2 mg of bovine serum albumin in buffer containing 0.1 M NaCl , 0.01 M potassium phosphate ($\text{pH } 6.8$), 10% glycerol, and 0.5% Nonidet P-40, the T Ag was eluted in the same buffer containing increasing amounts of NaCl. Eluates at different salt concentrations were adjusted to 0.3 M NaCl and immunoprecipitated with purified PAb 416 to determine the amount of T Ag eluted at each ionic strength tested. Finally, the DNA-cellulose was heated at 90°C for 2 min in electrophoresis sample buffer (3) to elute any remaining T Ag. The immunoprecipitates and heated eluates from the DNA-cellulose batches were subjected to polyacrylamide gel electrophoresis followed by silver staining. T Ag bound similarly to the various DNA-cellulose preparations (Fig. 3a). While greater than 50% of the T Ag was found in the fraction eluted at 0.1 M NaCl , a significant portion was eluted at 0.3 M NaCl . Moreover, a substantial amount of the T Ag remained tightly bound to the cellulose and was eluted only by heating in electrophoresis sample buffer. Virtually no difference in binding was observed among the different DNA-cellulose preparations. T Ag was not appreciably bound to cellulose without DNA, as all the detectable antigen was in the fraction eluted at 0.1 M NaCl . Similar experiments using [^{35}S]methionine-labeled cell extracts of AdSVR111-infected 293 cells (Fig. 3b) provided analogous results in that no significant differences between the quantities of labeled T Ag bound to single- or double-stranded DNA-cellulose were detected. However, it is of interest that the overall interactions of the purified T Ag and the [^{35}S]methionine-labeled T Ag in crude extracts were somewhat different. Generally, all of the labeled T Ag in extracts was eluted by 0.8 M NaCl , and little or no detectable viral protein could then be eluted by boiling in electrophoresis sample buffer. Possibly, the extracts contain an abundant protein or proteins that bind nonspecifically to DNA-cellulose with higher affinity than T Ag does. Alternately, the T Ag fraction that remained tightly bound to DNA-cellulose could represent older forms of the protein synthesized prior to the 2-h labeling period. We previously reported (28) that only a very small fraction of T Ag binds with a higher salt-sensitive affinity to SV40 DNA than to calf thymus DNA-cellulose under these conditions and that identification of a specifically bound class of T Ag to viral binding sites requires DNA molecules containing multiple copies of the binding region. Thus, these conditions favor the identification of nonspecifically bound T Ag molecules. This may be related to the fact that in the DNA-cellulose-binding assay, nonspecific DNA (e.g., from pBR322 or herring sperm) was not present, in contrast to the previous assays. These results confirm that nonspecific binding to both double- and single-stranded DNA is a property of T Ag, although in contrast to Spillman et al. (38), we were unable to detect preferential nonspecific binding of T Ag to single-

FIG. 3. Binding of T Ag to DNA-cellulose. (a) Single-stranded DNA-cellulose was prepared by using M13 DNA with or without SVO, and double-stranded DNA-cellulose was prepared by using the replicative form of M13SVO DNA. T Ag was bound to and eluted from DNA-cellulose as described in the text. Eluates in buffers containing 0.1 (lanes b), 0.3 (lanes c), and 0.5 (lanes d) M NaCl were immunoprecipitated with PAb 416. After being washed with buffer containing 1.0 M NaCl, the cellulose was heated at 90°C for 10 min in electrophoresis sample buffer (lanes a). Samples were analyzed on 12% sodium dodecyl sulfate-polyacrylamide gels and silver stained. The arrows point to the silver-stained T Ag band; the 55-kilodalton strong band is the PAb 416 immunoglobulin heavy chain. The positions of the molecular size markers (in kilodaltons) are indicated on the left. (A) Double-stranded SV40 DNA-cellulose. (B) Cellulose only. (C) Single-stranded mp9 DNA-cellulose. (D) Single-stranded mp8SVO DNA-cellulose. (b) Extracts of 10^7 AdSVR111-infected 293 cells were labeled with [35 S]methionine (200 μ Ci/ml) for 2 h prior to lysis as previously described (28). After the extract was adjusted to pH 6.5 and applied to DNA-cellulose columns, fractions were eluted as described above. Lane T, 100 μ l of extract immunoprecipitated with PAb 416, Lane N, 100 μ l of extract immunoprecipitated with Dulbecco modified Eagle medium plus 10% fetal calf serum. Samples (100 μ l) of NaCl eluates—0.1 M (lanes a), 0.3 M (lanes b), 0.5 M (lanes c), and 1.0 M (lanes d)—were adjusted to 0.3 M NaCl and then immunoprecipitated with PAb 416. (A) Double-stranded mp9SVO replicative form DNA. (B) Single-stranded mp9M13 DNA-cellulose (without SVO). (C) mp8SVO DNA-cellulose. (D) mp9SVO DNA-cellulose. The position of T Ag is indicated by the arrow.

stranded DNA under our binding and elution conditions. However, those investigators used chromatographically purified T Ag from SV80 cells that have been shown to produce a mutant T Ag that is unable to bind specifically to the viral origin region (12, 17, 26). Our experiments also confirm previous observations (12, 27, 32) that T Ag consists of various subpopulations that vary in their interactions with DNA, ranging from nonbinding to nonspecific but tight binding. The nature of the T Ag molecules that manifest

these interactions with DNA are not yet understood, although variations in the degree of phosphorylation of T Ag molecules have been correlated with its affinity for viral and cellular DNA (25, 31, 37).

The ability of T Ag to bind to single-stranded DNA nonspecifically, as confirmed by the DNA-cellulose-binding assays, is consistent with the idea that once unwound, the viral protein stays associated with the DNA but may then be able to move along the DNA in order to unwind the replication fork. The nonspecific binding properties of T Ag have been shown to be separable from the specific binding properties of the protein. By using Ad2⁺ND hybrid virus-encoded SV40 T Ag polypeptides, different regions of the protein were shown to be required for specific and nonspecific binding to DNA (28). Furthermore, mutant T Ag that fails to bind specifically to SV40 DNA can still bind nonspecifically to DNA-cellulose columns (32; C. Prives, unpublished data). Thus, a second type of interaction of T Ag with DNA, namely, nonspecific binding, may be an important feature of the function of the protein in initiating viral DNA replication. It must be noted that the conditions for DNA replication *in vitro* that have been established (21, 40, 46) are different from those used in these and in previous binding studies. In particular, formation of the preelongation complex, in which, presumably, the initial steps in T Ag-mediated DNA replication initiation occur, namely, DNA binding and unwinding, requires temperatures at 37°C and the presence of ATP (47). Furthermore, differences in binding specificities of T Ag for DNA at elevated temperatures have been observed in some cases (11, 44) but not in others (8). To further explore the specific and nonspecific interactions of the viral A gene product with DNA, we are currently examining these properties under more varied physiological conditions than have been previously used.

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