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NOTES

The DNA-Binding Domain of Simian Virus 40 Tumor Antigen Has Multiple Functions

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The DNA-binding domain of simian virus 40 tumor antigen has been previously shown to participate in a number of different activities. Besides being involved in binding to sequences at the viral replication origin, this domain appears to be required for nonspecific DNA binding, for structurally distorting origin DNA (melting and untwisting), and possibly for oligomerization of the protein into hexamers and double hexamers. We now provide evidence that it also takes part in unwinding origin DNA sequences, contributes a function specifically related to in vivo DNA replication, and perhaps supports the assembly of the virus or release of the virus from the cell. This 100-amino-acid domain appears to be an excellent model system for studying how a small region of a protein could have a number of distinct activities.

The DNA-binding domain of simian virus 40 (SV40) tumor (T) antigen has been extensively studied at biochemical and genetic levels (1, 17–21, 24). The structure of this 100-amino-acid region (residues 137 to 246) is not known, nor is the mechanism by which it binds to DNA understood. We have obtained some information on the organization of the domain by analyzing over 90 mutants with conservative single amino acid substitutions throughout this region. In this systematic analysis, the domain has been nearly saturated with mutations. Almost half of the conservative mutations that we made have no effect on virus multiplication in monkey cells. About 15% of them result in the appearance of small plaques, and the remainder (38%) totally block the production of plaques (19; unpublished data).

By studying the biochemical activity of replication-negative mutants, we identified several amino acid residues that are important for origin-specific or nonspecific DNA binding (21). These amino acids map exclusively to discrete regions of the domain which we term elements A (residues 147 to 159), B1 (183 to 187), B2 (203 to 207), and B4 (226 to 227) (20, 21). Interestingly, conservative mutations at a number of other sites within, as well as outside, these elements have no apparent effect on DNA binding but prevent virus multiplication in permissive monkey cells (19–21; this study).

In a recent publication (20), we described three distinct classes of DNA-binding domain mutations. T-antigen mutants in class 1 are defective for binding to the SV40 DNA replication origin but bind normally to nonorigin DNA. Because most of these mutants are able to oligomerize normally in the presence of ATP to form hexamers and double hexamers, it appears that their defect lies in the failure to directly or indirectly recognize origin sequences. Mutants in class 2 are unable to bind specifically or nonspecifically to DNA. Unlike the mutants in class 1, they are unable to oligomerize normally (20) and are impaired in DNA helicase activity (21), indicating that they have a structural defect which prevents them from recognizing any DNA (i.e., they may be unable to make proper contacts with the sugar-phosphate backbone). Class 3 mutants bind origin and nonorigin DNA at near wild-type (WT) levels, structurally distort the origin like the WT protein does, and perform the helicase reaction normally (20). However, they are repressed in the ability to unwind an origin-containing double-stranded DNA fragment and fail to form correct oligomers in response to ATP. The properties of these mutants led us to postulate that proper oligomerization to correctly position the molecules on the DNA appears to be more important for initiating unwinding than for either efficient DNA binding, inducing structural changes in the DNA, or carrying out the helicase reaction (see Table 2 for a summary of the activities of mutants in classes 1 to 3).

We report here on the characterization of 13 other virus replication-defective mutants of T antigen, which fall into three additional classes (Table 1). All of the mutants described here and in the previous study (20) were expressed in insect cells infected with recombinant baculoviruses. T antigen was purified by immunoaffinity chromatography using monoclonal antibody PAb419 (16) and tested for its ability to perform various biochemical reactions associated with the replication of viral DNA. The experimental conditions used in many of these assays were detailed in a recent publication (20).

Table 1 lists all of the mutants described in this study and summarizes the results of these assays. We found that all 13 mutant proteins bound to origin and nonorigin DNA like WT T antigen did. This result was obtained by using a quantitative DNA binding assay under DNA replication and nonreplication conditions and by a DNase footprinting assay performed under replication conditions (20). In all cases, DNA binding was indistinguishable from that of WT T antigen. The purified proteins were then tested for the ability to melt the imperfect palindrome on the early side of the T antigen site 2 binding region and to untwist the AT-rich sequence on the late side (2, 6). These structural changes can be monitored by sensitivity of the T-antigen-bound DNA to $KMnO_4$ oxidation (2, 9, 13).

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TABLE 1. Properties of mutants in classes 4 through 6

Mutant class	Mutant[a]	Oligomerization	% or level of replication by WT T antigen	
			In vitro	In vivo
4	148HN	Normal	2	
4	167KR	Slightly abnormal	33	
4	213QH	Normal	12	
4	215LV	Normal	13	
4	216CG	Normal	28	
4	219ST	Normal	47	
4	220FY	Normal	5	
4	246ED	Normal	50	
5	151FY	Normal	130	Negative
5	217TS	Normal	118	Negative
5	227NT	Normal	89	Low
5	245ED	Normal	100	Low
6	237TS	Normal	150	WT

[a] Mutants are named according to the position of the altered amino acid, followed by the one-letter designations of the WT amino acid and then the mutant amino acid. All mutants exhibited WT DNA-binding, melting, and fragment-unwinding properties.

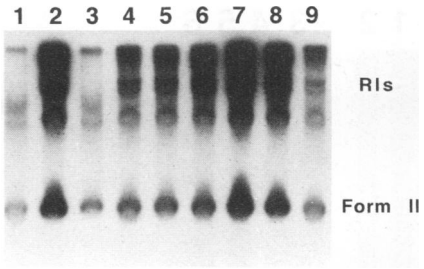

FIG. 1. DNA replication in vitro by mutant T antigens. Equal amounts of immunoaffinity-purified T antigens were incubated in a cell-free DNA replication system in the presence of [α-^{32}P]dATP. The DNA was extracted, precipitated, and applied to a 1% agarose gel. The gel was dried and exposed to X-ray film. Lanes: 1, control without T antigen; 2 through 9, reactions with WT, 204KR, 213QH, 215LV, 216CG, 217TS, 219ST, and 220FY T antigens, respectively. The positions of replicative intermediates (RIs) and form II DNA are shown (23). To quantitate results for Table 1, the radioactivities in gel slices containing both of these DNAs were determined by scintillation counting.

Again, all 13 mutants behaved like WT T antigen in this assay (Table 1). This finding adds support to our previously stated correlation between WT levels of DNA binding and normal structural distortion of origin sequences (20).

Third, all 13 mutant proteins were tested for the ability to oligomerize into hexamers and double hexamers in the presence of ATP (11, 14). In this assay, immunoaffinity-purified, labeled T antigen was incubated at 37°C under replication conditions and cross-linked with glutaraldehyde (20). The proteins were then resolved on a 4 to 25% acrylamide gradient gel. With the exception of mutant 167KR, which was only mildly defective, all others oligomerized normally (Table 1). Fourth, the mutant proteins were examined for the ability to unwind an origin-containing linear DNA fragment (8, 20) in the presence of *Escherichia coli* single-stranded-DNA-binding protein, and again all mutants behaved normally. The 13 new mutants were therefore distinguished from those in class 3 at least by being able to oligomerize normally and to unwind a linear origin-containing DNA fragment like WT T antigen did. These findings indicate that their virus replication defect is in a step after local unwinding at the origin.

We then tested whether these mutants could support the replication of SV40 DNA in vitro. Equal amounts (600 ng) of immunoaffinity-purified T antigen were added to a DNA replication system consisting of a plasmid DNA (pSV0Δ2792) (26) with an SV40 replication origin, an extract of adenovirus-transformed 293 cells, deoxynucleoside triphosphates (dNTPs), ribonucleoside triphosphates, [α-^{32}P]dATP, and an ATP-regenerating system as described previously (23). After 1 h at 37°C, the DNA was purified by phenol-chloroform extractions, precipitated with ethanol, and subjected to electrophoresis on an agarose gel. The extent of DNA replication was quantitated by cutting out the bands corresponding to replicative intermediates and form II (nicked circular DNA) (23) from the dried gels and counting for radioactivity in a scintillation counter. Figure 1 shows the results of one such experiment with several representative mutants, and Table 1 lists all quantitative results. As a negative control, we used mutant 204KR (Fig. 1, lane 3), which belongs to class 1 (20) and cannot bind to origin DNA. The small amounts (~1% of WT) of radioactive DNA in the reaction without T antigen (Fig. 1, lane 1) and in the one with 204KR (Fig. 1, lane 3) are presumably due to repair synthesis. Many of the mutants described here (213QH, 215LV, etc.) were defective at supporting DNA replication in vitro, some severely (Fig. 1 and Table 1). Several other mutants (217TS, 227NT, etc.) behaved normally.

Mutants listed under class 4 (Table 1) are those which are seriously or partially compromised in the ability to support DNA replication in vitro. Although these mutants can unwind an origin-containing DNA fragment normally, it is possible that they are deficient in unwinding closed circular DNA. Both activities require the presence of single-stranded-DNA-binding protein, but the latter activity is much more easily detectable in the presence of human replication protein A than with *E. coli* single-stranded-DNA-binding protein (4). The closed circular unwinding assay also requires topoisomerase I and gives rise to a highly unwound form of DNA called form U (3–5). Representatives of the class 4 mutants (213QH, 215LV,

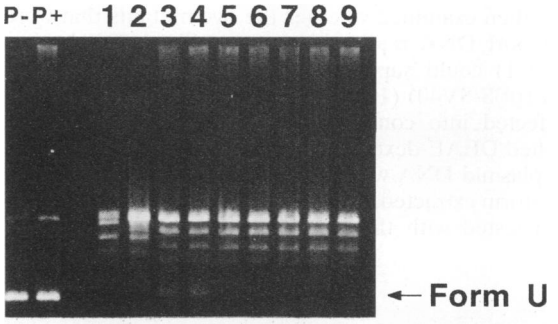

FIG. 2. Form U unwinding assays of mutant T antigens. Immunoaffinity-purified T antigen was added to 100 ng of topoisomerase I-relaxed pSV011 − (ori$^-$) (25) or pSV011 (ori$^+$) (15) DNA for 30 min at 37°C under replication conditions, but without dNTPs, rCTP, rGTP, or rUTP, as previously described (25). The deproteinized DNA was applied to a 0.8% agarose gel, stained with ethidium bromide, and photographed under UV light. Lanes: P− and P+, input, unrelaxed ori$^-$ and ori$^+$ plasmids, respectively; 1 and 2, relaxed pSV011 − DNA; 3 through 9, relaxed pSV011 DNA; 1 and 9, no T antigen; 2 through 4, reactions with 2.8, 2.8, and 1.4 μg of WT T antigen, respectively (the T antigen used in lane 4 was prepared concurrently with the mutants); 5, 1.4 μg of 213QH T antigen; 6, 1.4 μg of 215LV T antigen; 7 and 8, 2.3 and 4.5 μg of 216CG T antigen, respectively. The position on the gel of the unwound form U is indicated.

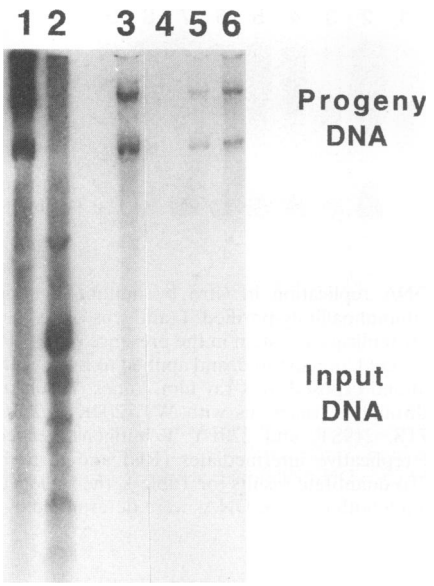

FIG. 3. DNA replication in vivo by mutant T antigens. pBS-SV40 coding for WT or mutant T antigen was transfected into monkey cells. At 60 h posttransfection, DNA was isolated by Hirt extraction, treated with *Dpn*I, and applied to a 1% agarose gel. The DNA was transferred to a nylon membrane and probed with nick-translated ^{32}P-labeled WT pBS-SV40 DNA. Lanes: 1, marker of uncut pBS-SV40 DNA; 2, marker of *Dpn*I-cleaved pBS-SV40; 3 through 6, DNA extracted from monkey cells transfected with DNA coding for WT, 217TS, 227NT, and 245ED T antigens, respectively. The positions of cut, input DNA and uncut, progeny DNA are shown.

and 216CG) were examined for this activity as described previously (25) and were found to be defective (Fig. 2). These results suggest that T antigen's topological requirements are different for unwinding superhelical DNA than for unwinding short linear DNAs or, alternatively, that these mutants are unable to properly associate with a cellular protein to unwind closed double-stranded DNA.

We then examined whether the five mutants that were able to support DNA replication in vitro like WT T antigen did (Table 1) could support DNA replication in vivo. Plasmid DNA (pBS-SV40) (10) bearing the appropriate mutation was transfected into confluent BSC-1 monkey cells by using a modified DEAE-dextran procedure (22). At 60 h posttransfection, plasmid DNA was recovered by Hirt extraction, phenol-chloroform extracted, and precipitated with ethanol. The DNA was digested with the methylation-dependent enzyme *Dpn*I and subjected to electrophoresis in a 1% agarose gel. DNA was blotted to a Hybond-N membrane (Amersham) and probed with nick-translated pBS-SV40. In this assay (7), replicated progeny DNA is unmethylated and is resistant to digestion by the endonuclease *Dpn*I. Figure 3 shows the results with three of the five mutants, and all data are summarized in Table 1. Two of these mutants (217TS and 151FY) gave rise to no detectable DNA replication in BSC-1 cells. Two others (227NT and 245ED) gave some level of replication, but this was clearly lower than the WT level. These four mutants are listed under class 5. The one mutant (237TS) which supported DNA replication in vivo normally was then placed in class 6. These results were confirmed by polymerase chain reaction amplification of *Dpn*I-digested DNA (data not shown).

There is no simple explanation for the existence of the class 5 mutants. They must be defective in some aspect of DNA replication specific to conditions within cells. Three interesting but yet untested possibilities are an inability to support multiple rounds of DNA replication, a defect in their interaction with a cellular protein needed specifically for DNA replication in monkey cells, and a failure to activate DNA replication factors in quiescent cells. This last possibility has been suggested by Maulbecker et al. (12) to explain the properties of an NH_2-terminal mutant of T antigen which behaves similarly to our class 5 mutants.

The single class 6 mutant appears to be proficient at DNA replication in vitro and in vivo. This mutant can transactivate the late viral promoter to give rise to viral structural proteins (data not shown), but no virus plaques are made in transfected monkey cells. The existence of this mutant may indicate a role of this region in virus assembly or release of virus from the cell, although this interpretation will require the identification of additional mutants with this property.

The results presented here and in a recent publication (20) indicate that the DNA-binding domain of SV40 T antigen participate in numerous functions. This domain is involved in origin-specific (class 1 mutants) and nonspecific (class 2) DNA binding, in structurally distorting origin DNA (classes 1 and 2), possibly in oligomerization (classes 2 and 3), in unwinding linear and closed circular DNA (classes 3 and 4), in supplying some function needed during in vivo DNA replication (class 5), and perhaps in virus production (class 6). These various classes of mutants are summarized in Table 2.

These results also provide further information about the organization of the DNA-binding domain. Previous work (21) has suggested that origin DNA binding is mediated mostly by two discrete regions (or elements) in the domain called A and B2 (residues 147 to 159 and 203 to 207). These elements are the only regions in T antigen where a mutation leads to a class

TABLE 2. Categories of mutants[a]

Mutant class	Binding		Melting	Unwinding		Oligomerization[b]	DNA replication	
	Origin	NS[c]		Fragment	CC[d]		In vitro	In vivo
1	−	+	−	−	ND	+	−	ND
2	−	−	−	−	ND	−	−	ND
3	+	+	+	−	ND	−	−	ND
4	+	+	+	+	−	+	−	−
5	+	+	+	+	ND	+	+	−
6	+	+	+	+	ND	+	+	+

[a] Consensus activities of each class of mutants with changes in origin-binding domain. −, reduced compared with WT; +, at or near WT level; ND, not done.
[b] ATP-induced oligomerization into hexamers and double hexamers.
[c] NS, Nonspecific double-stranded DNA.
[d] Closed circular DNA containing an SV40 origin of replication.

1 phenotype (origin binding negative but nonspecific DNA binding positive and oligomerization positive). As summarized previously (20, 21), our interpretation is that these two elements may lie side by side in the major groove to contact the GAGGC pentanucleotide recognition sequence. Certain sites in elements A and B2 as well as in elements B1 (residues 183 to 187) and B4 (226 to 227) appear to be important for nonspecific DNA binding. Mutations at these residues (class 2) seem to affect the proper contact of DNA at the sugar-phosphate backbone. However, because these mutants appear to be structurally altered (Table 2), the mutated sites may not be the ones that are directly involved in DNA binding. The data presented in this report suggest that another region (element B3; residues 213 to 220) may function in unwinding origin-containing closed circular DNA. This region may have some specific enzymatic activity, or it may be a site of interaction with a cellular protein. Our results, therefore, indicate that the DNA-binding domain of SV40 T antigen contains a number of smaller regions (elements) that have one or more individualized function.

In summary, we found that the replication-negative mutants belong to at least six distinct categories based on the ability of the purified mutant proteins to perform a number of biochemical activities (Table 2). Further characterization of the specific defects found in these classes of mutants is in progress and may provide additional insights into the complex role of this small region.

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