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DNA-Binding Properties of Simian Virus 40 T-Antigen Mutants Defective in Viral DNA Replication

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Three simian virus 40 (SV40)-transformed monkey cell lines, C2, C6, and C11, producing T-antigen variants that are unable to initiate viral DNA replication, were analyzed with respect to their affinity for regulatory sequences at the viral origin of replication. C2 and C11 T antigens both bound specifically to sequences at sites 1 and 2 at the viral origin region, whereas C6 T antigen showed no specific affinity for any viral DNA sequences under all conditions tested. Viral DNA sequences encoding the C6 T antigen have recently been cloned out of C6 cells and used to transform an established rat cell line. T antigen from several cloned C6-SV40-transformed rat lines failed to bind specifically to the origin. C6 DNA contains three mutations: two located close to the amino terminus of T antigen at amino acid positions 30 and 51 and a third located internally at amino acid position 153. Two recombinant SV40 DNA mutants were prepared containing either the amino-terminal mutations at positions 30 and 51 (C6-1) or the internally located mutation at position 153 (C6-2) and used to transform Rat 2 cells. Whereas T antigen from C6-1-transformed cells bound specifically to sequences at the origin, T antigen from C6-2-transformed cells lacked any specific affinity for these sequences. Therefore, the single mutation at amino acid position 153 (Asn→Thr) is sufficient to abolish the origin-binding property of T antigen. A T antigen-specific monoclonal antibody, PAb 100, which had been previously shown to immunoprecipitate an immunologically distinct origin-binding subclass of T antigen, recognized wild-type or C6-1 antigens, but failed to react with C6 or C6-2 T antigens. These results indicate that viral replication function comprises properties of T antigen that exist in addition to its ability to bind specifically to the SV40 regulatory sequences. Furthermore, it is concluded from these data that specific viral origin binding is not a necessary feature of the transforming function of T antigen.

The simian virus 40 (SV40) *A* gene product, large T antigen, is required for both viral replication in productive infection of permissive hosts and oncogenic transformation of nonpermissive cells. This suggests that either both roles can be attributed to a single property of the protein or that T antigen can provide more than one function. That Gluzman et al. (10) were able to transform permissive monkey cells with UV-irradiated virus, producing a T antigen that was unable to complement *tsA* mutants for viral DNA replication, was evidence for the separation of transforming and replication functions of the viral *A* gene product. More recently, additional mutants have been isolated in different laboratories, which supports this conclusion (2, 3, 16, 19). The concept that T antigen has more than one role is borne out by biochemical and biological analysis of this protein. T antigen has been shown to be a DNA-binding protein, with specific and high affinity for discrete sites at the viral origin of replication (25, 31). It has also been shown to have ATPase activity, to form oligomers, and to bind to the cellular p53 protein (for a review, see reference 32). Many of the known biological activities of T antigen have been determined by using temperature-sensitive mutants. Experiments with these conditional mutants have demonstrated that T antigen is required for initiation of new rounds of viral DNA replication, induction of cell DNA synthesis, repression of E strand-specific RNA transcription, and induction of viral L strand-specific transcription (32). More recently, T antigen has been shown to regulate cellular rRNA transcription (29) and induction of specific cellular mRNAs in transformed cells (24). In addition, both the initiation and maintenance of transformation of nonpermissive cells have been ascribed to the SV40 *A* gene product (32). The

plethora of biochemical and biological properties associated with T antigen provides an interesting system with which to sort out which properties are associated with which functions. To approach this, we have undertaken an analysis of the specific DNA-binding properties of mutant T antigens which have lost or impaired replication functions, but which have retained their ability to transform cells.

MATERIALS AND METHODS

Materials. *Bst*NI, *Rsa*I, and *Dde*I restriction enzymes were purchased from New England Biolabs, Beverly, Mass. Anti-T antigen antiserum was obtained from the Research Resources Branch, National Cancer Institute, Bethesda, Md. Monoclonal antibody cells secreting PAb 100 were obtained from the American Type Culture Collection, Rockville, Md. Normal hamster serum was purchased from Cappel Laboratories, Downingtown, Pa. Inactivated *Staphylococcus aureus* was purchased as Pansorbin from Calbiochem, La Jolla, Calif., ^{32}P-labeled deoxyribonucleoside triphosphates, $^{32}P_i$, and [^{35}S]methionine were bought from the Radiochemical Centre, Amersham, England.

Cell lines. C2, C6, and C11 (10) and Cos 7 (8) transformed CV-1 cell lines were maintained in Dulbecco modified Eagle medium containing 10% calf serum as were the SV40- and C6-SV40-transformed rat cell lines recently isolated by Gluzman and Ahrens (9). These latter transformants were produced by subcloning isolated foci that overgrew normal cells after transfection with wild-type or mutant SV40 sequences. pK1, which contains the SV40 genome inserted into the *Eco*RI site of the pMK16#6 (9) vector, was used to produce wild-type SV40-transformed cells. C6 mutant DNA, which was obtained by rescue from fused C6-Cos 1 heterokaryons by the Hirt procedure (15), was cloned into pBR322 and then cleaved with *Bgl*I-*Bam*HI to isolate the mutated early-region fragment. By ligation of this fragment to the larger fragment produced by *Bgl*I-*Bam*HI digestion of pK1, a plasmid pC6, containing a complete copy of the SV40 genome consisting of wild-type late region and mutated C6 early region, was produced. After identification of the mutations in the C6 early region, their partial segregation was achieved by digestion of pC6 with *Taq*I-*Bgl*I (0.56 to 0.66 map units) or with *Bam*HI-*Taq*I (0.14 to 0.56 map units), yielding two fragments which, when ligated to complementing subfragments from the early region, produced two new C6 submutations containing wild-type DNA substituted with C6 sequences, mapping between 0.66 and 0.56 (C6-1), or with C6 sequences mapping between 0.56 and 0.14 (C6-2).

Extraction of cells for DNA-binding and immunoprecipitation studies. Cells were grown in 90-mm culture dishes to confluence, yielding ca. 4×10^6 cells per culture. After the cells were washed three times with cold phosphate-buffered saline, all subsequent operations were performed at 4°C. Nuclear extracts were prepared by scraping monolayers in 1 ml of hypotonic buffer, pH 6.0 (0.01 M NaCl, 0.01 M PIPES [1,4-piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)], 0.001 M magnesium acetate, and 1-1-tosylamide-2-phenylethyl chloromethyl ketone [TPCK] and phenylmethylsulfonyl fluoride [PMSF] to final concentrations of 250 μg/ml), into a 15-ml Dounce homogenizer. After 10 strokes with a tight-fitting pestle, the homogenate was centrifuged for 5 min at 2,000 rpm in a clinical centrifuge. For DNA-binding studies, the pellet, containing mainly cell nuclei, was suspended in 0.4 ml of nuclear extraction buffer, pH 8.5 (0.3 M NaCl, 0.02 M HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], 0.001 M $MgCl_2$, 0.5% Nonidet P-40 and TPCK and PMSF, both at 250 μg/ml). The nuclear extract was then centrifuged as above, and the supernatant was either directly used or stored in 100-μl aliquots at −80°C. For experiments requiring radioactively labeled proteins, the cells were incubated in medium either lacking methionine or phosphate plus 2% dialyzed fetal calf serum for 1 h before being labeled with [^{35}S]methionine (250 μCi/ml) or $^{32}P_i$ (300 μCi/ml), respectively, for 2 h before extraction as above. For immunoprecipitation studies, the nuclear extraction buffer contained 0.15 M NaCl. T antigens extracted from these cell lines by nuclear extraction buffer containing 0.15 M or 0.3 M NaCl performed similarly both in the DNA-binding immunoassay and in immunoprecipitation studies with either heterospecific or monospecific antisera.

DNA-binding immunoassay. Immunoprecipitation of T antigen bound to DNA was performed essentially as described previously by Scheller et al. (23). SV40 DNA was nick translated to a specific activity of 2×10^3 to 20×10^3 cpm/ng (22) either before or after digestion with the appropriate restriction enzyme. After preadjustment of cell extracts to pH 6.8, 5 ng of ^{32}P-labeled DNA fragments and 5 μg of sheared herring sperm DNA were added, and the reaction was kept at 4°C for 1 h. Anti-SV40 tumor antiserum (10 μl) was added for an additional 30 min, followed by 50 μl of a 10% suspension of formaldehyde-fixed *S. aureus* in NET buffer (0.15 M NaCl, 0.01 M EDTA, 0.05% Nonidet P-40, 0.01 M Tris-hydrochloride [pH 7.0]). After centrifugation of the immune complexes for 1 min at 8,000 rpm, the pellets were washed three times in NET buffer containing 0.1 mM EDTA. DNA fragments were dissociated from the immune complex by incubation in 60 μl of TMS buffer (23) for 30 min at 20°C. After deproteinization and concentration, the DNA fragments bound were analyzed by electrophoresis in 2% agarose gels.

Immunoprecipitation of T antigen from transformed cells. Extracts of [^{35}S]methionine- or ^{32}P-labeled cells were centrifuged at 38,000 rpm for 40 min. Supernatants were incubated with antiserum or culture medium from monoclonal antibody-producing cells for 1 h, followed by the addition of 50 μl of formaldehyde-fixed *S. aureus* for an additonal 30 min. After centrifugation at 8,000 rpm for 2 min, the pellets were washed three times in NET buffer followed by the addition of 50 μl of electrophoresis sample buffer (21). The pellets were heated to 90°C for 1 min before being loaded onto 12.5% polyacrylamide gels.

RESULTS

Origin-specific DNA binding of T-antigen mutants defective in viral DNA replication. When permissive cells, such as the CV-1 monkey cell line, are infected with wild-type SV40 virus, the cells are ultimately killed. By UV-irradiating

SV40 virus before infection, Gluzman et al. (10) were able to isolate three transformed CV-1 cell lines, C2, C6, and C11, that constitutively expressed T antigen as determined by immunofluorescence (10) and by immunoprecipitation (21). These cell lines exhibited various alterations in growth properties, such as the ability to overgrow normal cells or to grow in low serum and in soft agar. However, although fully permissive to lytic superinfection with wild-type virus, they were unable to support the replication of SV40 *tsA* mutants at the restrictive temperature. The lack of complementation by the C-series T antigens of *tsA* mutants led to the suggestion that these proteins may be defective in some aspect of viral DNA replication. The most well-studied biochemical property of T antigen linked to its role in viral DNA replication is its specific affinity for sequences at the replication origin. A DNA-binding immunoassay was developed recently (17, 23), making it possible to analyze the origin-binding activity of T antigen present in small quantities in crude or partially purified preparations from infected or transformed cells. When T antigen-containing cell extracts are bound to ^{32}P-labeled SV40 DNA restriction fragments under suitable conditions, followed by immunoprecipitation of the T antigen-DNA complex with anti-T antigen antibody, analysis of the labeled DNA fragments released from the immune complexes has shown that fragments containing sequences spanning the viral origin of replication are preferentially retained. It was previously shown that T antigen from lytically infected CV-1 cells or transformed Cos cell line cells (23) binds specifically and semiquantitatively to the 311-base-pair (bp) SV40 *Bst*NI G fragment which contains T-antigen binding sites 1 and 2. In addition, some extracts specifically bind smaller quantities of the *Bst*NI D fragment which contains sequences mapping in the late leader region of the viral genome (23). Using the immunoassay, we tested the T antigens from the C2, C6, and C11 cell lines for their affinity for sequences at the viral origin. After presentation of ^{32}P-labeled *Bst*NI DNA fragments to extracts of these cells, both C2 and C11 T antigens preferentially bound to the 311-bp G fragment (Fig. 1). In addition, C2 but not C11 T antigen showed specific affinity for the *Bst*NI D fragment. T antigen from C6 cells, as previously shown (23), showed no specific retention of any viral sequences in the immune complex. The inability of the C6 T antigen to bind specifically to the origin was observed under a wide variety of experimental conditions including salt concentration, pH, assay of various poly- and monoclonal antibodies, as well as the presence or absence of carrier DNA.

It has been shown that the binding region on SV40 DNA for T antigen consists of two discrete contiguous sites, 1 and 2, as well as a minor site, 3 (31). Cleavage of the SV40 regulatory region with *Dde*I separates site 1 and a small portion of site 2 (site 2a) from the remainder of site 2 (site 2b). Site 2b contains the replication origin core sequence (1, 18, 28), as well as the sequences shown to be the main contact points for T antigen in that region (4). Therefore, cleavage of the SV40 regulatory region with *Dde*I separates discrete binding sequences for T antigen. It has been observed that most preparations of T antigen bind 3- to 10-fold more site 1- and 2a-containing fragments in the immunoassay than site 2b-containing fragments (6) (A. Scheller, unpublished data). Since binding of T antigen to fragments containing both sites 1 and 2, such as the *Bst*NI G fragment, generally largely reflects site 1 binding, it is possible that a defect specifically in site 2b binding would not be detected. Failure to bind site 2b could be correlated with a

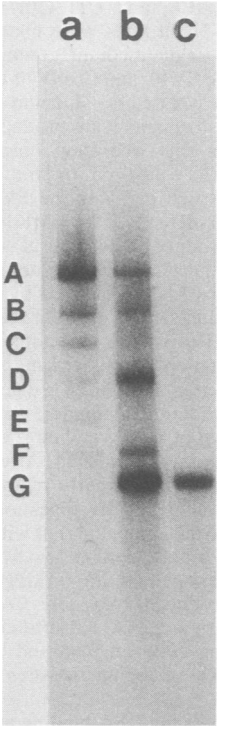

FIG. 1. Specific SV40 fragment binding of SV40 T antigen from transformed CV-1 cell lines. Nuclear extracts of 10^6 cells from C6 (a), C-2 (b), and C-11 (c) CV-1 lines were bound to 5 ng of ^{32}P-labeled *Bst*NI SV40 DNA fragments plus 5 μg of sheared herring sperm DNA, followed by immunoprecipitation with anti-SV40 tumor antiserum. Bound DNA was released and analyzed by agarose gel electrophoresis and autoradiography. A, B, C, D, E, F, and G are *Bst*NI SV40 DNA fragments.

defect in the viral DNA replication function of T antigen (18). Therefore, the binding of C2 and C11 T antigens to *Rsa*I-*Dde*I-digested SV40 DNA was examined (Fig. 2). As a control, extracts of Cos 7 cells which contain similar quantities of wild-type viral T antigen were also bound to these fragments. It was observed that C2 and C11 T antigens bound restriction fragments containing site 1 plus 2a and site 2b. Although C11 T antigen, as previously observed, bound DNA considerably less efficiently, the same proportion of these two fragments was specifically immunoprecipitated as seen with either Cos or C2 T antigen. The reduced binding of C11 T antigen was correlated with greatly decreased levels of T antigen in these cells (L. Covey, unpublished data). C6 T antigen, as expected, did not bind specifically to these DNA fragments (data not shown). Although it cannot be ruled out that the binding of C2 and C11 T antigens to the viral regulatory sequences is not identical to that of wild-type T antigen, these results suggest that their lack of viral DNA replication function is due to a mutation affecting another stage of T-antigen activity in this process. It was concluded from these experiments that viral origin binding is not the sole component of the *A* gene function related to viral DNA replication function.

Origin-specific DNA binding of T antigen from cells transformed by C6-SV40 DNA. That the C6 cell extracts failed to bind specifically to viral DNA fragments suggested that the origin-binding property of T antigen is not a necessary component of the transforming function of SV40 T antigen. However, the formal possibility remained that C6 cells are transformed by another mechanism unrelated to T-antigen function and that C6 T antigen may not have any role in transformation. To test this, SV40 sequences from C6 cells have recently been rescued and cloned (9). As the DNA from this mutant efficiently transforms both permissive and nonpermissive cell lines, it was concluded that C6 T antigen has retained transformation function. To examine whether the T antigen from rat cells transformed with C6-SV40 DNA still lacks origin binding, several clones of rat cells transformed with C6-SV40 DNA were tested in the DNA-binding immunoassay (Fig. 3). It was found that rat cells transformed with a recombinant plasmid bearing wild-type SV40 DNA produced T antigen that bound specifically to the

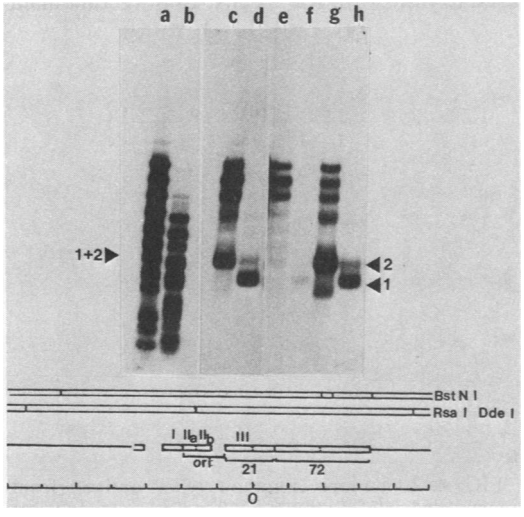

FIG. 2. Binding of T antigen from transformed CV-1 lines to DNA fragments containing separated binding sites. Extracts of 10^6 Cos 7 (c and d), C11 (e and f), or C2 (g and h) cells were bound to 5 ng of ^{32}P-labeled *Bst*NI (c, e, and g)- or *Rsa*I-*Dde*I (d, f, and h)-digested SV40 DNA. The 311-bp *Bst*NI G fragment spans binding sites 1 and 2 (a), whereas the 549-bp *Rsa*ID fragment containing sites 1 and 2 has been cleaved by *Dde*I to produce 300-bp and 240-bp fragments containing sites 2b and 1 + 2a, respectively (b).

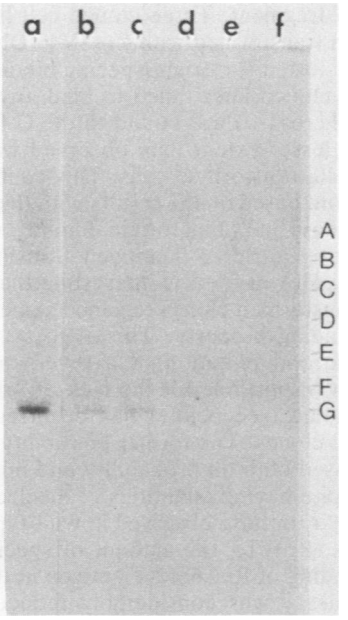

FIG. 3. Specific SV40 fragment binding of SV40 T antigen from transformed rat cells. Extracts of 10^6 cloned rat cells transformed by wild-type SV40 DNA (RpWT-2 [a]) or extracts of 10^6 cells of subclones of cells transformed by C6-SV40 DNA (RpC6c12-2 [b], RpC6c12-5 [c], RpC6c14-1 [d], RpC6c14-3 [e], and RpC6c15-1 [f]) were bound to 5 ng of ^{32}P-labeled *Bst*NI-digested SV40 DNA, followed by immunoprecipitation and analysis as described in the legend to Fig. 1. RpC6c12-2 and RpC6c12-5 are subclones of RpC6c12 transformants, whereas RpC6c14-1 and RpC6c14-3 are subclones of RpC6c14 transformants. A, B, C, D, E, F, and G are *Bst*NI SV40 DNA fragments.

*Bst*NI G fragment. Three cloned cell lines that had been transformed with C6-SV40 DNA were similarly tested for origin-specific binding. Two of these cloned lines failed to bind any G fragment, whereas a third bound to the G fragment but to a lesser extent than observed with wild-type SV40-transformed cells. This confirms the suggestion, based on the results with the original C6 monkey line, that origin binding is not a necessary feature of T-antigen transformation function. However, it is interesting that one of three transformed clones regained some specific origin-binding property. This suggests that either a second mutation or a reversion of the mutation responsible for the lack of origin-specific binding (see below) has occurred in the RpC6c12 clones. The former possibility is more likely based both on probability and on the fact that despite having quantities of labeled T antigen similar to those observed in wild-type transformants (RpWT), the amount of specific fragment binding of RpC6c12 T antigen in the DNA immunoassay was considerably reduced when compared with that of RpWT T antigen.

The levels of labeled T antigen in rat cells transformed by wild-type and C6 mutant SV40 DNA were analyzed by immunoprecipitation of [^{35}S]methionine-labeled cell extracts with anti-SV40 tumor serum (Fig. 4). When equal quantities of [^{35}S]methionine-labeled cell extracts were immunoprecipitated with antitumor serum, similar amounts of T antigen were detected in cells transformed by wild-type SV40 DNA (lines RpWT and RpWT-2) as in those C6-SV40 transformants (lines RpC6cl2-2 and RpC6cl2-5) that had demonstrated some origin-binding activity. However, lines transformed by C6-SV40 DNA (RpC6c14-1 and RpC6c15-1 in Fig. 4, as well as RpC6c15-2, not shown), in which origin-specific binding was totally lacking, consistently produced greater quantities (at least twofold) of labeled T antigen. This suggests that origin-binding activity detected in this in vitro assay may also occur in vivo and may result, to some extent, in autoregulation of T-antigen production. However, it should be noted that in an earlier study (10), as well as in ongoing experiments in our laboratory (C. Prives, unpublished data), no significant and reproducible difference in the amount of immunoprecitable [^{35}S]methionine-labeled T antigen from C2 (orgin binding) or C6 (nonorigin binding) has been observed. Thus, either T-antigen autoregulation may function from integrated viral sequences in rodent but not simian cells or the increased T-antigen production in rat lines expressing an origin-binding defective T antigen is coincidental. Furthermore, neither copy number of integrated genomes nor virus-specific transcription levels in these C6-SV40-transformed rat lines have yet been established. T antigens isolated from the C6 mutant or wild-type SV40-transformed cells were associated with the p53(NVT) host protein, because the anti-SV40 tumor antiserum used in these experiments had no detectable intrinsic reactivity with the host antigen (Fig. 4).

Origin-specific DNA binding of C6-1 and C6-2 mutants. C6 DNA contains three point mutations which are transversions (G to T at nucleotide 5074, A to T at nucleotide 5011, and A to C at nucleotide 4360) that result in three predicted amino acid changes on the basis of the known SV40 DNA sequence and codon usage (32): Met→Ile at position 30, Lys→Asn at position 51, and Asn→Thr at position 153. The substitutions at amino acid positions 30 and 51 occur in the NH_2-terminal region of the large-T-antigen polypeptide that is common to the small t antigen, and therefore, it is expected that both early polypeptides are mutant in these lines. The NH_2-terminal-located mutations (positions 30 and 51) were separated from the mutation at position 153, producing two fragments (0.14 to 0.56 and 0.56 to 0.66 map units). Each of these fragments was ligated to wild-type SV40 DNA containing the remainder of the genome. This yielded two recombinant mutants, C6-1 and C6-2, containing the NH_2-terminial mutations at positions 30 and 51 and the internally located mutation unique to large T antigen at position 153, respectively. These two DNAs were then used to transform Rat 2 cells. Both recombinant

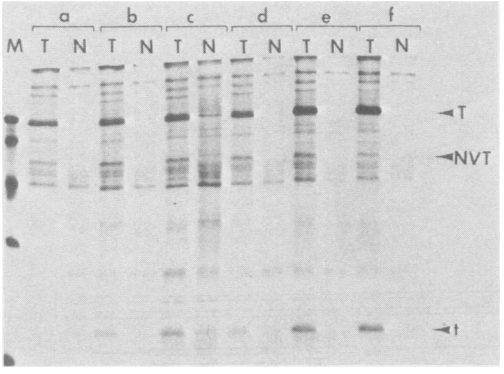

FIG. 4. Immunoprecipitation of T antigen from transformed cells. Extracts of 10^6 cells from cloned rat lines transformed by wild-type SV40 DNA (RpWT [a] and RpWT-2 [b]) or by C6-SV40 DNA (RpC6c12-2 [c], RpC6c12-5 [d], RpC6c14-1 [e], and RpC6c15-1 [f]) which had been labeled with [^{35}S]methionine (250 μCi/ml) for 2 h before extraction were immunoprecipitated with 10 μl of hamster anti-SV40 tumor antiserum (T) or 10 μl of hamster nonimmune serum (N). M, ^{14}C-labeled marker polypetides with estimated molecular weights, from top, in kilodaltons: 94, 69, 45, 25, and 12.5. T, large T antigen; NVT, p53; t, small t antigen.

mutant DNAs were able to transform Rat 2 cells with an efficiency of transformation comparable to that of wild-type SV40 DNA. The T antigens from wild-type-, C6-, C6-1-, and C6-2-transformed rat cells were tested in the DNA-binding immunoassay (Fig. 5). As before, T antigen from wild-type SV40-transformed rat cells bound specifically to the origin-containing G fragment, whereas C6 transformants did not. However, when C6-1 and C6-2 T antigens were examined, C6-1 T antigen demonstrated origin-specific DNA binding, whereas C6-2 T antigen showed no specific affinity for sequences at the origin. Therefore, it is likely that a single base change causing a single amino acid change (Asn→Thr) at position 153 obliterates the unique viral origin-specific DNA-binding property of SV40 T antigen. However, it cannot be rigorously excluded that mutations in other regions of C6-2 DNA exist that affect specific DNA binding (see below). It is noteworthy that we consistently observed less of the *Bst*NI G fragment immunoprecipitated in C6-1 extracts than in wild-type SV40-transformed cell extracts. This is consistent with the observation that C6-1 T antigen

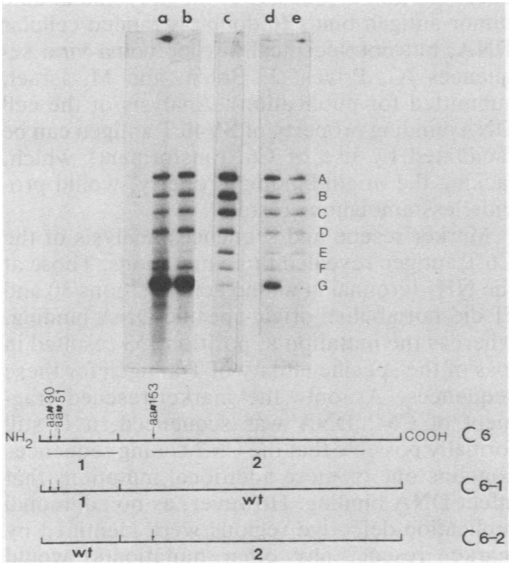

FIG. 5. Specific SV40 fragment DNA binding of C6-1 and C6-2 mutant T antigens. Extracts of 10^6 Rat 2 cells transformed by wild-type SV40 (a and b), C6-SV40 (c), C6-1 (d), and C6-2 (e) DNA were bound to ^{32}P-labeled *Bst*NI SV40 DNA fragments. After immunoprecipitation of the T antigen-DNA complex with hamster anti-SV40 tumor antiserum, bound DNA was released and analyzed as described in the legend to Fig. 1. The positions of the mutations in C6 and in the recombinant mutants C6-1 and C6-2 are depicted below the autoradiogram. The predicted amino acid changes are Met→Ile at position 30, Lys→Asn at position 51, and Asn→Thr at position 153 (9).

was significantly impaired in viral DNA replication function (9).

Binding of monoclonal antibody PAb 100 to C6 T antigens. The DNA-binding immunoassay used in these studies has proven useful not only as a means to assess the specific affinities of mutant T antigens for viral origin sequences, but also as a means to isolate immunologically distinct subpopulations of T antigen that differ in their DNA-binding properties. Using a monoclonal antibody (PAb 100) isolated by Gurney and colleagues (13), we showed that although this antibody immunoprecipitates less than 10% of the T antigen from transformed or infected cells, the majority of the orgin-binding activity in extracts containing wild-type T antigen is immunoprecipitated in the DNA-binding immunoassay (23). This suggests that only a small proportion of T antigen in a given population is active in origin-specific binding. The notion that a small, immunologically distinct subpopulation of T antigen binds to the viral origin was supported by the observation that PAb 100 fails to immunoprecipitate any labeled C6 T antigen (23). As C6 T antigen failed to bind to the viral origin, this observation suggested that the unique origin-binding subpopulation of T antigen was absent in C6 cells. To further test the idea that there is a correlation between origin binding and reactivity with PAb 100, the reactivity of T antigens in C6-1- and C6-2-transformed cells was tested (Fig. 6). It had been observed that PAb 100 reacts somewhat better with ^{32}P-labeled T antigen than with [^{35}S]methionine-labeled T antigen (23). Accordingly, ^{32}P-labeled T antigens from C6 cells, Cos cells, and C6-1- and C6-2-transformed rat cells were immunoprecipitated with excess quantities of PAb 100. As observed earlier, PAb 100 recognized ca. 10% of the ^{32}P-labeled T antigen from Cos cells that contain a wild-type T antigen, but failed to react with any C6 T antigen whatever. The complete inability of this monoclonal antibody to recognize C6 T antigen has been consistently observed in several experiments in which a wide range of labeling, extraction, and immunoprecipitation conditions were tested. When T antigens from C6-1- and C6-2-transformed Rat 2 cells were similarly tested, it was found that whereas C6-1 T antigen reacts with PAb 100, virtually no T antigen from C6-2 cells was immunoprecipitated with this antibody. This further supports the suggestion that the origin-binding subclass of T antigen is unique.

DISCUSSION

It is becoming increasingly clear that SV40 *A* gene product is a functionally complex protein. Isolation of the C-series of T antigens indicated that the *A* gene has separable functions in viral

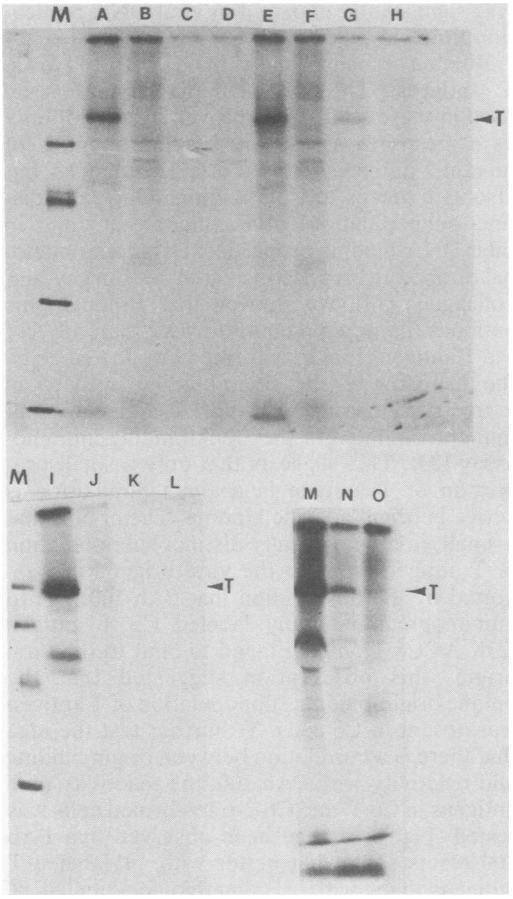

FIG. 6. Immunoprecipitation of T antigen from transformed cells with monoclonal antibody PAb 100. Nuclear extracts of 10^6 ^{32}P-labeled C6 (A to D), Cos 7 (E to H), C6-2 (I to L), or C6-1 (M to O) cells were incubated with 10 μl of hamster anti-SV40 tumor antiserum (lanes A, E, I, and M), 10 μl of nonimmune hamster serum (lanes B, F, and J), 200 μl of medium from monoclonal antibody-secreting mouse cells (lanes C, G, K, and N), or 200 μl of DME plus 10% fetal calf serum (lanes D, H, L, and O). After incubation with 50 μl of Formalin-fixed *S. aureus*, immune complexes were washed and polypeptides were released and subjected to polyacrylamide gel electrophoresis and autoradiograpy. Large-M lanes contain marker polypeptides with molecular weights, from top, in kilodaltons: 94, 69, 46, 25, and 12.5. Autoradiograms of C6-1 and C6-2 immunoprecipitates (lower panels) were exposed to film three times longer than those of Cos 7 and C6 to unequivocally determine lack of reactivity of C6-2 T antigen with PAb 100. T, T antigen.

replication and oncogenic transformation. The C6 mutant provides insight into T-antigen function in that, as a result of the experiments we have described, it can be stated that the characteristic specific affinity of this protein for regulatory sequences at the viral origin of replication is not a necessary feature of the *A*-gene transforming function. It is noteworthy that we have previously shown that C6 T antigen binds efficiently and tightly to calf thymus DNA-cellulose (23). This indicated that origin-specific and cellular DNA binding are separate properties of the SV40 T antigen. This was also demonstrated by analyzing the affinities of T antigens from the nondefective adenovirus type 2 (Ad2)-SV40 hybrid viruses Ad2+ND_2 and Ad2+ND_4, in which it was shown that sequences contributing to origin binding are mapped between 0.50 and 0.54 map units, whereas cell DNA binding requires sequences mapping between 0.39 and 0.44 map units (20). Thus, T antigen may possess two different DNA-binding activities, one of which is retained by the C6 mutant. It is possible to speculate that T antigen may interact with cellular DNA sequences, consequently affecting cellular gene expression in a manner that leads to the altered growth properties characteristic of transformed cells. Our data with the C6 T antigen would argue that if this is so, this interaction would be mediated by a property that is independent of origin binding. Intriguingly, it has been observed that a subclass of the polyoma middle tumor antigen binds to double-stranded cellular DNA, but not specifically to polyoma viral sequences (C. Prives, J. Bolen, and M. Israel, submitted for publication). Analysis of the cell DNA-binding property of SV40 T antigen can be facilitated by use of C6 transformants which, lacking the origin-binding property, would provide less-ambiguous results.

Marker rescue and sequences analysis of the C6 T antigen revealed three mutations. Those at the NH_2-terminal at amino acid positions 30 and 51 did not abolish origin-specific DNA binding, whereas the mutation at position 153 resulted in loss of the specific affinity of T antigen for these sequences. As only the marker-rescued fragment of C6-2 DNA was sequenced, it is still formally possible that the C6-2 coding sequences contains one or more additional mutations that affect DNA binding. However, as no additional replication-defective regions were identified by marker rescue, any other mutation(s) would have to affect origin-specific binding without affecting replication function, which is highly unlikely. Furthermore, the position of this mutation is generally consistent with other reports suggesting that sequences mapping between 0.54 and 0.50 map units (20) or 0.44 and 0.50 map units (27) contribute to origin binding. By the substitution of a threonine residue for an asparagine residue at position 153 a potential new site for phosphorylation has been added to the protein. T antigen is a phosphoprotein (30), with several sites of phosphorylation at serine and

threonine residues (33). It has been shown that removal of the majority of the phosphate moieties does not affect its high-affinity origin-binding property (26). However, it is possible that providing a new phosphorylation site, especially in the region known to be involved in origin binding, could dramatically affect this process. Experiments to determine whether removal of phosphate residues from the C6 T antigen restores its origin-binding activity are in progress.

Two other replication-defective T-antigen mutants, C2, and C11, did not differ from wild-type T antigen in origin binding with the DNA-binding immunoassay. However, different methods of determining specific DNA binding, such as DNase protection, DNase footprinting, or filter-binding assays, might reveal some differences in these proteins from the wild type. It is likely, however, that they are lacking in some other replicative property, as mutations in C2 and C11 T antigens have been mapped to positions between nucleotide 2848 and nucleotide 3373, considerably closer to the COOH-terminal of the polypeptide (M. Manos and Y. Gluzman, unpublished data). That *A* gene function consists of T-antigen properties in addition to origin-specific binding was recently reported by Wilson et al. (34), who showed that one class of *tsA* mutants exhibits high affinity for viral origin sequences at the restrictive temperature in vitro. It has been determined that C2, C6, and C11 T antigens bind to the p53 protein (14; L. Covey and C. Prives, unpublished data) and form oligomers (D. Gidoni and C. Prives, unpublished data) and that C2 T antigen binds to calf thymus DNA-cellulose (C. Prives, unpublished data) (C11 T antigen has not yet been tested for binding to DNA-cellulose). Therefore, a defect in ATPase activity, or in other, as yet undetermined properties of T antigen, is likely to be involved in the inability of the C2 and C11 T antigens to initiate viral DNA replication.

It is interesting to speculate on the multifunctional nature of the *A* gene product. T antigen has been shown to be modified post-translationally by phosphorylation and by ADP ribosylation (11), suggesting that it may consist of different subpopulations with different functions. Evidence for T-antigen heterogeneity has been provided by both immunological (13, 23) and biochemical (1a, 12) analyses. Combining these approaches, we have recently shown that a small, immunologically distinct class of T antigen comprises the main origin-binding activity of the protein (23). Monoclonal antibody PAb 100 appears to recognize a determinant that is involved in origin-specific binding. Because PAb 100 reacts with the Ad2-SV40 hybrid virus Ad2+ ND_2-encoded 56,000-molecular-weight T antigen polypeptide that lacks sequences containing the C6-2 mutation, it is improbable that the Asn→Thr amino acid change in C6-2 cells directly affects the antigenic determinant recognized by the antibody (5). It is more likely that the C6-2 mutation results in a conformational change in the polypeptide such that the determinant is no longer recognized. That this determinant is more conformationally sensitive than most is indicated by the fact that mild denaturation treatment by a variety of agents, although causing no reduction in recognition of T antigen by antitumor serum or at least one other monoclonal antibody tested, abolishes recognition of wild-type T antigen by PAb 100 (L. Covey and C. Prives, unpublished data). This suggests that C6 T antigen may exist in a conformational state different from that of wild-type T antigen that renders it both unable to bind to the origin and unrecognizable by PAb 100. This is supported by the observation that the C6-1 (origin-binding) but not C6-2 (nonorigin-binding) T-antigen submutant is immunoprecipitated by this monoclonal antibody. We have also observed that C2 T antigen is recognized by PAb 100, suggesting that the lack of reactivity of this antibody with C6 T antigen is due to the impaired origin-binding property, as opposed to the viral replication defect (L. Covey and C. Prives, unpublished data). By combined analysis of the determinant recognized by PAb 100 and the structural alterations in C6 mutants, it should be possible to further our understanding of how T antigen binds specifically to the viral origin of replication. By further analysis of the cell DNA-binding property of wild-type and C6 T antigens, it may also be possible to understand the role of the SV40 *A* gene product in cell transformation.

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