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A Novel Simian Virus 40 Early-Region Domain Mediates Transactivation of the Cyclin A Promoter by Small-t Antigen and Is Required for Transformation in Small-t Antigen-Dependent Assays

ANALÍA PORRÁS,[1] JOHN BENNETT,[1] ALAN HOWE,[1] KAREN TOKOS,[1] NOËL BOUCK,[1] BERTHOLD HENGLEIN,[2] SWAMINATHAN SATHYAMANGALAM,[1] BAYAR THIMMAPAYA,[1] AND KATHLEEN RUNDELL[1]*

Department of Microbiology-Immunology and Lurie Cancer Center, Northwestern University Medical Center, Chicago, Illinois 60611,[1] and Institut Curie, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 144, F-75005 Paris, France[2]

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At least three regions of the simian virus 40 small-t antigen (small-t) contribute to the protein's ability to enhance cellular transformation. As we showed previously for rat F111 cells, one region includes sequences from residues 97 to 103 that are involved in the binding and inhibition of protein phosphatase 2A. In the present study, the role of the protein phosphatase 2A binding region was confirmed in two additional small-t-dependent transformation systems. Second, small-t was found to provide a function previously identified as a large-T transformation domain. Mutations in residues 19 to 28 of large-T affected its transforming ability, but these mutations were complemented by a wild-type small-t. A third region of small-t was also required for efficient transformation. This region, the 42-47 region, is shared by large-T and small-t and contains a conserved HPDKGG hexapeptide. The 42-47 region function could be provided by either small-t or large-T in small-t-dependent systems. Mutations in the 42-47 region reduced the ability of small-t to transactivate the cyclin A promoter, of interest because small-t increased endogenous cyclin A mRNA levels in both human and monkey cells, as well as transactivating the promoter in transient assays.

The simian virus 40 (SV40) small-t antigen (small-t) enhances the ability of SV40 to transform several growth-arrested cell types in vitro and to induce tumors in vivo, especially in nonreplicating tissues. Initial reports of studies using rodent cell lines suggested that small-t was needed primarily in stringent transformation assays, such as growth in agar, but that the need for small-t could be bypassed in others, such as focus formation (5). When infected hamster cells were allowed to divide once or twice before plating in agar, the need for small-t was greatly reduced (24), which demonstrates that the primary contribution of small-t to transformation is to promote cell cycle progression. Direct evidence for a role for small-t in stimulating cell cycling has been obtained in studies of nonpermissive infections of mouse cells (16) and permissive infections of monkey cells (9, 37). In semipermissive human cells, small-t is required for the induction of focus formation (7, 10).

Although clues to the functions of small-t were slow to emerge, it is now known that one important activity is the binding and inhibition of the cellular enzyme protein phosphatase 2A (PP2A) (26, 44, 47). Point mutations and deletions that alter the PP2A interaction have been studied in several systems. Importantly, point mutations at residues 97, 101, and 103 of small-t were shown to affect the ability of small-t to enhance transformation of rat F111 cells to anchorage-independent growth (25). Small-t mutants with deletions of sequences between residues 110 and 130 failed to bind the PP2A A subunit in vitro and were unable to overcome growth arrest of monkey kidney CV1 cells induced by limiting serum concentrations (37). In contrast to the wild-type (WT) small-t, these truncated proteins also failed to activate mitogen-activated protein kinase (MAPK) and its kinase MEK.

Activation of these kinases by small-t correlated with the transcriptional activation of AP1-driven reporter constructs (1). It was surprising, therefore, that the transcriptional activation of the adenovirus E2A promoter, another well-known function of small-t, was not affected by mutations in the PP2A binding region. Instead, point mutations at both residues 43 and 45 of small-t eliminated its transactivation of the E2A promoter (25). The precise mechanism through which small-t transactivates the E2A promoter has not been determined, but clear distinctions exist between transactivation mediated by E1A and that mediated by small-t (21). For example, deletion of E2F binding sites in the E2A promoter eliminated the response to both small-t and E1A, while deletion of an ATF site affected the E1A response to a greater extent than the small-t response (22). Potential transrepression of some promoters, e.g., that of c-*fos*, has also been described (45).

Mutations in the amino-terminal region shared by small-t and large-T have been used primarily to study the consequences of these alterations on functions of large-T (23, 28, 49). Point mutations in the conserved hexapeptide found between residues 42 and 47 of large-T and small-t had no effect on focus formation in primary rat cells, a transformation assay that is independent of small-t (28), but severely limited viral DNA replication. In contrast, amino-terminal sequences between residues 17 and 28 defined a domain of large-T that was critical both for transformation in vitro and for tumorigenesis in transgenic mice (39). The 17-28 region works together with others unique to large-T to induce the host cell (11). The current study was designed to more fully evaluate the role of the 42-47 region of small-t in transactivation and in transformation systems that require small-t and to explore possible contributions of small-t to the 17-28 region function.

* Corresponding author. Mailing address: Department of Microbiology-Immunology, Northwestern University Medical Center, 303 E. Chicago Ave., Chicago, IL 60611. Phone: (312) 503-5923. Fax: (312) 908-1372. Electronic mail address: krundell@nwu.edu.

MATERIALS AND METHODS

Cells. Permanent cell lines used in this study include African green monkey kidney (CV1) cells and baby hamster kidney (BHK) subline SN-10 (4). Primary human diploid fibroblast (HDF) cells were prepared from foreskin. CV1 and HDF cells were maintained in Dulbecco modified Eagle (DME) medium containing 10% fetal bovine serum. BHK SN-10 cells were grown in DME containing 10% tryptose phosphate broth and 10% donor calf serum. Serum concentrations were increased to 15 to 20% for cloning of cells in plastic dishes. Stable small-t-expressing cell lines were obtained following cotransfection of pw2t WT or 43/45 plasmids, described below, with pSVneo. Colonies were selected in medium containing 400 to 600 ng of geneticin (GIBCO/BRL) per ml. Small-t levels in each clone were determined by immunoprecipitation with antitumor sera as described previously (18).

Plasmids. All mutations were introduced into the basic plasmids pw2, which expresses both large-T and small-t under the control of SV40 regulatory sequences, and pw2t, which expresses only small-t. These plasmids were used extensively in previous studies (8, 18, 25, 29). The mutant plasmid 5002 was obtained from J. Pipas (University of Pittsburgh). Sequences between the *Kpn*I site at SV40 nucleotide 294 and the *Bst*XI site at 4759 were used to replace those found in pw2 and pw2t. Important control plasmids, pw2*dl*888 and pw2t*dl*888, fail to express small-t because they contain a 100-bp deletion that eliminates the splice donor sequence necessary for small-t expression (29, 40). To generate constructs that also contained defective SV40 origins of replication (ori$^-$ plasmids), sequences between the SV40 *Kpn*I site at 294 and the *Stu*I site at 5190 were taken from a pMK-based ori$^-$ plasmid (31) and used to replace WT sequences. A new construct containing the double mutation D44E/G47R was constructed by PCR-based mutagenesis as described previously (13). The mutations present in various constructs are defined by using the single-letter code for amino acids. For example, the designation G47R indicates that glycine (G), the 47th residue of small-t, has been replaced by arginine (R).

Transformation. BHK subline SN-10 was transfected with 5 to 10 μg of plasmids expressing WT and mutant small-t in a total of 20 μg of DNA prepared with carrier salmon sperm DNA. Transformation was then assayed by colony formation in soft agar as described previously (4). In all experiments, transfections included 1 μg of a plasmid that encodes resistance to the drug hygromycin, and portions of each transfection containing 2×10^5 cells were plated for colony formation in plastic tissue culture dishes in the presence of 400 μg of hygromycin per ml. Four equivalent aliquots were plated in soft agar in the absence of hygromycin. Relative transformation efficiency, i.e., the proportion of hygromycin-resistant colonies (stably transfected cells) that were able to clone in soft agar, was determined for each experimental point.

Transformation of HDF cells was determined by a focus formation assay. Triplicate dishes of subconfluent cells (2×10^5 to 4×10^5 cells per 6-cm-diameter dish) were transfected with a total of 10 μg of ori$^-$ plasmid DNA. When pw2-based plasmids were used, both large-T and small-t were expressed from a single plasmid. In some experiments, cells were cotransfected with 10 μg each of a plasmid that expressed small-t (pw2t derivatives) and one that expressed large-T (pw2*dl*888 derivatives). Two days following transfection, cells were trypsinized and distributed equally to four 6-cm-diameter dishes. Once cells reached confluence, they were refed every 4 to 5 days with DME containing 1% fetal bovine serum until foci appeared at 4 to 8 weeks.

Transactivation. Transactivation of pE2CAT was performed at 48 h posttransfection as described previously (18, 25), using cotransfection of the reporter plasmid and a pw2t-based plasmid in the presence of Lipofectin. The calcium phosphate procedure was used for cotransfections of PALUC, which expresses luciferase under the control of a 7-kb cyclin A promoter sequence (15), and a pw2t-based plasmid. Cells were extracted 48 h posttransfection, and equivalent amounts of extracted protein were assayed for luciferase activity as measured in a luminometer by using standard procedures.

Construction of adenoviruses. Plasmid pCMV-t is a pUC18-based plasmid that contains the following elements: (i) 450 nucleotides of the left end of adenovirus type 5 (Ad5), sequences necessary for viral replication and packaging as well as for efficient transcription; and (ii) the cytomegalovirus (CMV) promoter followed by (iii) the small-t coding region from a cDNA derivative of pw2t (29) and (iv) SV40 transcription termination and polyadenylation sequences, as found in pw2t. Cleavage of pCMV-t with the polylinker sites for *Cla*I and *Xba*I releases a 2.8-kb fragment containing adenovirus, CMV, and SV40 small-t sequences. Details of the construction of recombinant viruses from this plasmid are provided elsewhere (38). To construct the virus Ad-t, the 2.8-kb fragment containing either the WT or the P43L/K45N small-t sequence was ligated to a 35-kb fragment isolated from *Xba*I-digested Ad5*dl*309, a phenotypically WT variant that has a single *Xba*I site at map unit 4.0 (32). Ligation mixtures were introduced into 293 cells by using Lipofectin, and the resulting virus, Ad-t, was plaque purified before use. To confirm the presence of the desired mutations, viral DNA was isolated by Hirt extraction and then used as a template in PCRs using a forward primer from SV40 nucleotide 5163 and a reverse primer from SV40 nucleotide 3950. The reaction product was used in sequencing reactions with a primer located within 100 nucleotides of the expected mutations. Control viruses included an adenovirus that expresses the E4orf6/7 protein from the CMV promoter (Ad-E4) or a virus that contained the CMV promoter but expressed no recombinant protein (AdCMV).

Northern (RNA) blot analysis. Ad-t infections were performed with 10 to 20 PFU of serum-free Ad-t stocks. These stocks were prepared by infecting 293 cells with Ad-t for 16 h, changing medium on these cells to serum-free DME, and then collecting only the extracellular virus once maximum cytopathic effect was evident. To look for the induction of endogenous cyclin A expression by Ad-t infection, CV1 cells were grown to confluence and then placed in DME lacking serum for 24 h. These arrested cultures were then infected with Ad-t for 2 h, medium was replaced, cultures were incubated for 18 h, and RNA was extracted by using guanidinium isothiocyanate. Ten to 20 μg of total cellular RNA was separated on formalin-agarose gels, transferred to nitrocellulose, and hybridized with the *Eco*RI fragment of the cyclin A gene (14) labeled by using the Rediprime DNA labeling system (Amersham). The *Eco*RI fragment was isolated from plasmid pLXSNCyclinA, kindly provided by J. Roberts (Fred Hutchinson Cancer Center, Seattle, Wash.). Uniformity of RNA loaded was monitored by ethidium bromide staining of the gel and/or methylene blue staining of the filter. All procedures were based on routine protocols (2, 33). Briefly, hybridizations were performed at 65°C in the presence of 1% sodium dodecyl sulfate, (SDS), 10% dextran sulfate, and 1 M NaCl, and washes were done in 0.5 to 2× SSPE buffer (33) at the same temperature.

RNase protection analysis. Early-passage HDF cells were grown to confluence and serum starved for 36 to 48 h. Cells were infected with 20 PFU per cell of standard, serum-containing adenovirus stocks, and total cellular RNA was prepared 18 h postinfection by using guanidinium isothiocyanate. RNase protections were performed as described elsewhere (2). Basically, 30 μg of total cellular RNA was used for each sample; tRNA was used as a negative control. The cyclin A *Eco*RI fragment specified above was cloned into pGEM4, and then a *Sma*I fragment was deleted such that a 150-nucleotide antisense cyclin A transcript could be produced, using T7 polymerase and [α^{32}P]CTP. A total of 5×10^5 cpm of the probe was added to each reaction mixture, and hybridizations were carried out overnight at 60°C. Each reaction mixture was digested with 350 U of RNase T_1 (GIBCO/BRL) at 16°C for 1 h. Protected fragments were visualized following electrophoresis on 8% polyacrylamide-urea gels and autoradiography. Where appropriate, densitometry was performed on a Molecular Dynamics Personal Densitometer SI.

RESULTS

Domains of small-t and corresponding mutations. The SV40 small-t shares its amino-terminal sequence (positions 1 to 82) with large-T and then contains a unique carboxy terminus. As shown in Fig. 1, the unique C-terminal region of small-t contains at least two domains necessary for its function (Fig. 1). The two Cys-X-Cys-XX-Cys clusters (residues 111 to 116 and 138 to 143) coordinate the binding of two metal ions per molecule of small-t (13, 18, 42) that are essential for the stability of the protein. Sequences in the unique region of small-t are also responsible for its binding and inhibition of PP2A. In particular, point mutations affecting residues 97, 101, and 103 (C97S, C103S, and P101A) of small-t greatly reduced its inhibition of PP2A but not its stability (25). These mutations, which eliminate the ability of the virus to transform rat F111 cells to anchorage-independent growth, define a domain referred to in this study as the 97-103 region. Deletions of sequences between residues 110 and 130 also affect the binding of small-t to the PP2A A subunit (37), but point mutations within this region that affect PP2A binding without altering protein stability have not been described.

We also wished to study sequences of small-t in regions that are shared with large-T (T/t regions). The first of these regions, residues 42 to 47, contains a hexapeptide sequence, HPDKGG, that is completely conserved in all papovaviruses (28, 41). In previous studies of this region, it was found that viral replication was affected, presumably because of the presence of the mutations in large-T. However, small-t with mutations in the 42-47 region can be studied at the plasmid level or as stable cell lines. Studies with one double mutation (P43L/K45N) have shown that the mutant small-t is stable (Fig. 2). Interestingly, the 43/45 mutant small-t was shown previously to be unable to transactivate the adenovirus E2A promoter when expressed in monkey cells (25). This finding has now been extended to a second double-mutant protein, D44E/G47R (Fig. 3). The role of the 42-47 region in PP2A-independent transactivation raised the question of whether this region might also influence

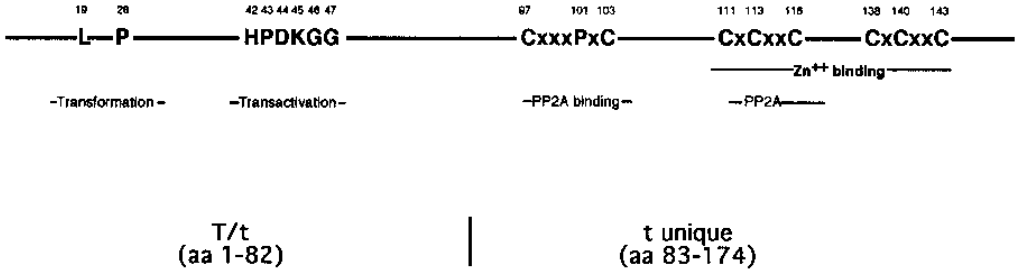

FIG. 1. Important regions of small-t. The locations of mutations in the SV40 early region used in this study are indicated. The line represents the 174-residue small-t which shares its first 82 amino acids (aa) with large-T (T/t).

small-t-dependent transformation. Because these mutations prevented isolation of viable virus (reference 28 and data not shown), this region was studied in the plasmid-based assays described below.

For comparison with the 42-47 region mutations, we also included plasmids that contained 5002 mutant sequences (L19F/P28S), kindly provided by J. Pipas. Sequences in the T/t region have been explored by several laboratories (23, 28, 49). In particular, a region between residues 17 and 28 was shown to be required for efficient large-T-mediated transformation (39), but the role of this region in small-t-dependent systems was not examined. To compare effects of mutations in the 17-28 and 42-47 regions on either large-T or small-t, we used systems in which transformation could be achieved efficiently by plasmid transfection of small-t alone or by cotransfections with plasmids that separately encoded the two SV40 proteins.

Transformation of SN-10 cells. Clone SN-10, a subclone of BHK21/cl13, is susceptible to transformation by a series of traditional agents, such as large-T and activated *ras*. Of importance to this study, however, is that it is the only cell line available that can be transformed to anchorage independence by small-t alone (4). Plasmids encoding WT or mutant small-t were cotransfected with a constant amount of a plasmid encoding hygromycin resistance into early-passage SN-10 cells by using calcium phosphate precipitation. Transformation was measured by colony formation in soft agar. To standardize for transfection efficiency, a relative transformation efficiency was calculated in these experiments. This number is the ratio between the average number of colonies formed per agar plate and the average number of hygromycin-resistant colonies per dish.

The experiment represented in Table 1 is representative of three separate experiments performed with mutant small-t. In this experiment, about one-third of the stably transfected cells gave rise to colonies in agar. A similar relative transformation efficiency was found with a plasmid that encodes an activated *ras* gene, EJ-*ras*, while the SV40 large-T was severalfold more efficient (data not shown). When mutant C103S, a PP2A-binding mutant, and the transactivation-defective mutants P43L/K45N and D44E/G47R were tested, all three mutants were found to be defective in inducing transformation. This result indicated that both the small-t transactivation function and the inhibition of PP2A are required for the transformation of SN-10 cells by small-t. In agreement with the pulse-chase experiment shown in Fig. 2, equal levels of WT and mutant proteins were found when pools of hygromycin-resistant colonies were labeled to steady state with [^{35}S]methionine (16 h), extracted, and immunoprecipitated, indicating that the differences in transformation did not result from altered protein stability of the mutant proteins (data not shown).

Transformation of HDF cells. Although small-t is frequently required to induce anchorage-independent growth, it is not required for focus formation in several cell lines (5, 24). In contrast, both small-t and large-T are required for focus formation by primary HDF cells (7, 10). In initial experiments with this small-t-dependent system, single plasmids that expressed both small-t and large-T (pw2) were used to determine whether the mutations described above had any effect in this assay. All plasmids also contained mutations at the origin of replication (31) because the low levels of plasmid replication that occur in semipermissive HDF cells when large-T is present are lethal to transfected cells.

The interaction of small-t with PP2A (97-103 region) was required for induction of focus formation in HDF cells (Fig.

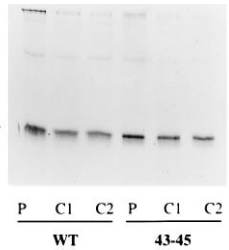

FIG. 2. Stability of the 43/45 mutant small-t. Stable cell lines that express either WT or 43/45 mutant small-t were incubated for 1 h in methionine-free (Met-free) medium and then pulsed for 1 h with 50 μCi of [^{35}S]Met per dish in Met-free medium. For each cell type, one plate was extracted immediately after the pulse (P). Isotope was washed off the remaining plates with phosphate-buffered saline, and then plates were incubated for 1.5 h (C1) or 3 h (C2) in DME before extraction. Proteins were immunoprecipitated with hamster anti-tumor serum and resolved by SDS-gel electrophoresis as described previously (18).

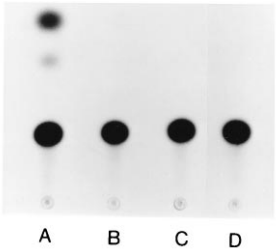

FIG. 3. Transactivation of the adenovirus E2A promoter by mutant small-t. CV1 monkey kidney cells were cotransfected with the reporter plasmid pE2ACAT and a plasmid that expressed WT small-t (pw2t; lane A), small-t double mutants P43L/K45N (lane B) and D44E/G47R (lane C), or no small-t (pw2*tdl*888; lane D). Cells were extracted 48 h posttransfection, and extracts were assayed for chloramphenicol acetyltransferase activity, using [^{14}C]chloramphenicol as described in Materials and Methods. The extract shown in lane A gave enzyme activity that fell within the linear range of the assay (9.7% substrate conversion).

TABLE 1. Anchorage-independent growth of SN-10 cells induced by WT and mutant small-t

Small-t plasmid[a]	No. of hygromycin-resistant colonies[b] (avg ± SD)	No. of colonies in soft agar[b] (avg ± SD)	Relative transformation efficiency[c]
WT	53 ± 10	18 ± 7	0.34
C103S	132 ± 4	0.25 ± 0.9	0.01
P43L/K45N	23 ± 2	0.25 ± 0.9	0.01
D44E/G47R	83 ± 15	0.75 ± 2.6	0.01

[a] All plasmids used were derivatives of pw2t and expressed small-t but not large-T.

[b] Transfected cells were trypsinized, counted, and plated in triplicate (2×10^5 cells per dish) for hygromycin selection or in quadruplicate (2×10^5 cells per dish) for colony formation in soft agar as described previously (4).

[c] Ratio between the average number of colonies formed per agar plate and the average number of hygromycin-resistant colonies per dish.

4A to D). No foci were found up to 8 weeks following transfection with plasmids that encoded WT large-T and either P101A or C103S mutant small-t (Fig. 4C and D). Foci induced by WT pw2 were evident by 4 weeks posttransfection (Fig. 4A). The few foci that appeared following transfection with pw2C97S appeared much more slowly and were smaller than foci induced by the WT plasmid (Fig. 4B). Interestingly, the C97S mutant virus also showed reduced but detectable transformation of F111 cells under some conditions (25), and this mutation had the least effect on the interaction of small-t with PP2A (13). Overall, results with the HDF system, like the SN-10 system, confirm the important role played by the interaction of small-t with PP2A in cellular transformation.

No foci were detected following transfection with plasmids that carried the P43L/K45N (Fig. 4E) or the 5002 (L19F/P28S) (Fig. 4F) mutation. The failure of 5002 to transform HDF cells was consistent with studies of others with this mutant construct and others that carried deletions in this region (39). In contrast, the requirement for the 42-47 region in HDF cell transformation was unexpected because mutations in this region did not affect focus formation of REF52 cells by large-T, an assay that does not require small-t (28). Thus, HDF cell transformation reveals a requirement for a novel region of the SV40 early region, not previously recognized in other transformation systems.

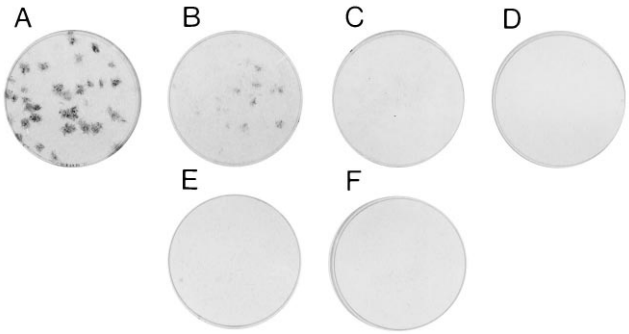

FIG. 4. Focus formation in HDF cells transfected with mutant plasmids. HDF cells were transfected with derivatives of ori$^-$ pw2, a plasmid that encodes both large-T and small-t. Foci appeared 6 to 8 weeks following transfection as described in Materials and Methods. Stained plates shown were representative of at least three independent experiments in which no foci were ever detected in the absence of small-t. Plates shown represent foci from 5×10^4 cells per plate following transfections with WT pw2 (A) and small-t mutants C97S (B), P101A (C), and C103S (D). Both large-T and small-t were mutant when plasmids P43L/K45N (E) and L19F/P28S (5002; F) were used.

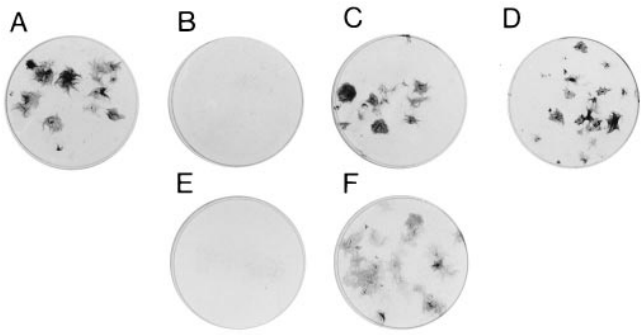

FIG. 5. HDF cell transformation by cotransfection of large-T- and small-t-expressing plasmids. HDF cells were cotransfected with derivatives of pw2t, which encodes only small-t, and derivatives of pw2*dl*888, which encodes only large-T. Foci appeared 6 to 8 weeks following transfection as described in Materials and Methods. Plates shown represent foci from 5×10^4 cells per plate following cotransfections with plasmids that expressed WT large-T and small-t (A), 43/45 mutant large-T and small-t (B), 43/45 mutant large-T and WT small-t (C), WT large-T and 43/45 mutant small-t (D), 5002 mutant large-T and small-t (E), and 5002 mutant large-T and WT small-t (F).

Separation of mutations in small-t and large-T. The 5002 mutations and those in the 42-47 region were present in both small-t and large-T in the experiments shown in Fig. 4. To determine whether these mutations affected small-t, large-T, or both proteins, cotransfections with plasmids that separately encoded the proteins were performed (Fig. 5). Foci appeared following cotransfection of HDF cells with two WT plasmids (Fig. 5A) at about the same efficiency as in single-plasmid transfections. No foci appeared when both large-T and small-t were mutant (Fig. 5B). When the P43L/K45N double mutation was in small-t, foci were apparent when large-T was WT (Fig. 5C). Interestingly, foci were also observed when large-T carried the 43/45 mutation and small-t was WT (Fig. 5D). These data suggest that the 42-47 region required for transformation of HDF cells can be provided by either small-t or large-T.

We performed the same kind of analysis for the 5002 double point mutation (residues 19 and 28). When large-T and small-t both carried the 5002 mutation, no foci were produced (Fig. 5E). Surprisingly, transformation was as efficient as for the WT proteins when a mutant large-T was combined with WT small-t (Fig. 5F). The apparently less dense foci observed in this experiment were not found reproducibly in studies of 5002; rather, variations in the density of foci occurred in WT transfections as well and may reflect transfection efficiencies and levels of proteins expressed on a per-cell basis. This experiment is the first indication that small-t may provide the 17-28 region function, a known large-T transformation domain. We also cotransfected WT large-T with a 5002 mutant small-t. No foci were observed in these cells, a finding that suggested that the 5002 mutant small-t may be unstable (not shown). Reduced stability would interfere with all critical small-t functions such as PP2A inhibition, a function that is clearly required to complement WT large-T. We have also been unable to detect WT levels of small-t in cells transfected with the 5002 mutant small-t (not shown).

The 42-47 region of small-t is required for transactivation of the cyclin A promoter. The transactivation of the adenovirus E2A promoter by small-t suggested that small-t might also activate transcription from relevant cellular promoters. Several lines of evidence suggest that small-t promotes cell cycle progression, and so studies of the cyclin genes became of interest. One good candidate was cyclin A, the cyclin expressed at the G_1-S border which is necessary for the maintenance of S phase (12, 30).

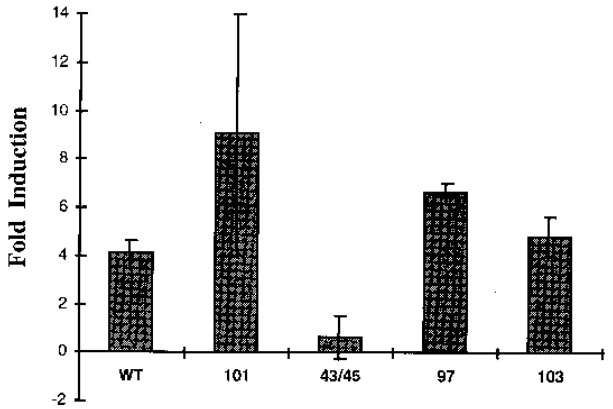

FIG. 6. Transactivation of the cyclin A promoter by small-t. CV1 cells were cotransfected with the reporter plasmid PALUC and a plasmid that expressed either WT small-t or the mutant 101, 43/45, 97, or 103. Cells were extracted and assayed 48 h after transfection. Activation of PALUC is expressed as fold induction relative to cells transfected with PALUC and the negative control, *dl*888. Results of three separate experiments were analyzed.

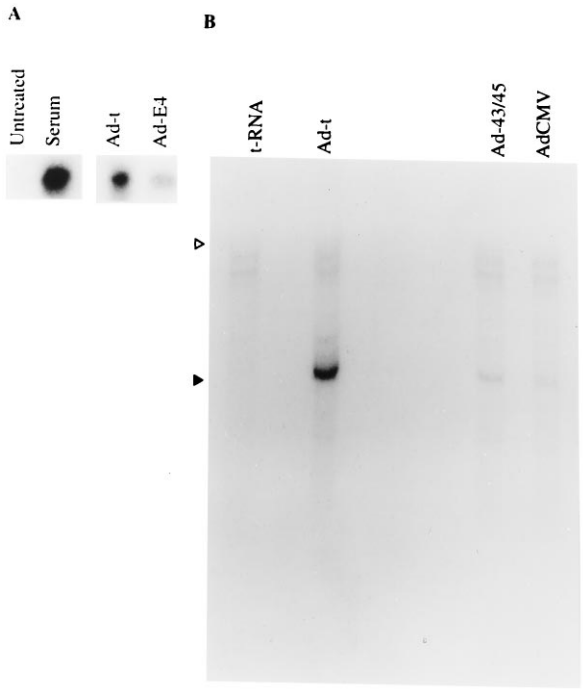

FIG. 7. Induction of endogenous cyclin A levels by small-t. (A) Northern blot analysis was used to measure levels of cyclin A mRNA in infected and uninfected CV1 cells. Patterns shown are from untreated cells, cells stimulated with 10% serum, or cells infected with Ad-t or Ad-E4 in the absence of serum. After 18 h, RNA was prepared as described in Materials and Methods. (B) RNase protection was used to measure levels of cyclin A mRNA in infected HDF cells. Patterns shown are for RNA prepared from cells infected with Ad-t, Ad-43/45, or AdCMV for 18 h; no protected fragment was observed following hybridization of the probe with tRNA. The open arrowhead indicates the position of the intact probe, and the filled arrowhead shows the position of the specific protected fragment.

Small-t was able to activate transcription from the cyclin A promoter following transient cotransfections of CV1 cells with pw2t and PALUC, a plasmid that contains 7 kb of the cyclin A promoter cloned upstream of a luciferase reporter gene (15). To investigate which region of small-t was responsible for the transactivation of PALUC, we tested the series of point mutations in the 97-103 and 42-47 regions of small-t. As shown in Fig. 6, small-t mutants containing one of the three mutations in the 97-103 region (P101A, C97S, and C103S) induced transactivation of PALUC equal to or better than that induced by WT small-t. This was of particular interest for the P101A mutation because it was the most defective in the inhibition of PP2A (25). In contrast, the P43L/K45N mutant small-t was unable to significantly transactivate the cyclin A promoter. These data suggested that the ability of small-t to transactivate the cyclin A promoter is independent of PP2A inhibition but, rather, correlates with the transactivation of E2A by small-t.

Expression of small-t in CV1 cells increases levels of the endogenous cyclin A gene. Having found that small-t could transactivate the cyclin A promoter in transient assays, we decided to examine the effect of small-t on cyclin A mRNA levels in infected cells. To eliminate contributions of large-T, we used a recombinant E1A-defective adenovirus that expresses small-t under the control of the CMV promoter (Ad-t). As controls, we used Ad-E4 and AdCMV (see Materials and Methods). Preliminary experiments showed that the levels of small-t produced by Ad-t by 8 to 18 h postinfection were similar to those produced 48 to 72 h postinfection with WT SV40, the peak period of small-t accumulation (data not shown).

In the experiment shown in Fig. 7A, CV1 cells were grown to confluence and then deprived of serum for 48 h. Cells were then refed with medium containing 10% serum or infected with a recombinant virus at 10 to 20 PFU per cell. Total cellular RNA was prepared after infection, fractionated on a formalin gel, transferred to nitrocellulose, and probed with a ^{32}P-labeled *Eco*RI fragment of the human cyclin A gene (14). Cells infected with Ad-t or stimulated with serum for 18 h showed far greater levels of cyclin A mRNA than the Ad-E4 control-infected cells, although levels of cyclin A in Ad-E4-infected cells were slightly greater than those in untreated cells. Cyclin A was not detected until at least 12 h postinfection, and peak levels were found at 18 h (data not shown).

To establish a stronger correlation between small-t transformation functions and the transactivation of cyclin A, we performed cyclin A mRNA analyses on HDF cells as well. We also wanted to test the effect of the 43/45 mutation on expression of the endogenous cyclin A gene. Accordingly, we infected confluent early-passage, serum-starved HDF cells with 20 PFU of Ad-t, Ad-43/45, or the control virus AdCMV per cell. For greater sensitivity, we performed RNase protection assays with RNA prepared 18 h postinfection. As shown in Fig. 7B, levels of cyclin A mRNA in Ad-t-infected cells were much higher than in AdCMV-infected cells. Importantly, the 43/45 small-t mutant failed to stimulate levels of endogenous cyclin A expression to levels found in Ad-t-infected cells. By densitometric analysis, Ad-43/45 increased cyclin A mRNA levels less than 2-fold over AdCMV, while the induction by Ad-t was 10- to 12-fold. This finding with Ad-43/45 is in agreement with the behavior of the mutant in transient transactivation assays and suggests that this property may be related to the role that this region plays in transformation of HDF cells.

DISCUSSION

The best-understood biochemical activity of SV40 small-t is its ability to bind and inhibit PP2A. This activity, assessed by using mutations in the 97-103 region of small-t, is now known to be essential for activity in three distinct small-t-dependent transformation systems. In addition to the induction of anchorage-independent growth of rat F111 cells, reported previously (25), these systems now include anchorage-independent growth

of BHK SN-10 cells and focus formation by human fibroblasts. The actual consequences of PP2A inhibition are not completely defined, although the finding that small-t can activate MAPK and its kinase MEK (37), resulting in increased cell growth, is a key observation in this regard. It seems unlikely, however, that activation of these key kinases alone can completely explain the small-t function in transformation. These kinases are activated efficiently by high serum concentrations, but we have found that high levels of serum are not sufficient to support focus formation by HDF cells (data not shown).

As in several other cell transformation systems, SV40 early region sequences from the 17-28 region were also required for transformation of HDF cells. It was interesting to find that this function, previously identified as a large-T transformation domain (39), could be provided by small-t. This region has been implicated in interaction with p300 because it is required to complement E1A mutants that are defective in p300 binding (46). With the recent report that large-T can bind p300 (3), our finding that small-t can provide the 17-28 region function in a transformation assay raises the question of whether small-t has this ability as well.

A novel domain of the SV40 early region, the HPDKGG sequence between residues 42 and 47, is also required for transformation of SN-10 and HDF cells. This was a somewhat surprising finding because studies of a large number of large-T mutants for the ability to induce focus formation by REF52 cells, an assay independent of small-t, had not identified this region as a transformation domain (28). It is extremely unlikely that the HPDKGG sequence is required for binding or inhibition of PP2A because a truncated 14-kDa small-t produced in bacteria lacks this sequence but is as efficient as the full-length 17-kDa protein in binding and inhibiting PP2A (13). Furthermore, mutations in this region do not affect the ability of small-t to activate MAPK or MEK, assays that reflect inhibition of PP2A (17). Interestingly, in HDF cells, the function provided by the 42-47 region can be provided by either large-T or small-t.

Two overlapping sequence motifs are present in the HPD KGG hexapeptide. These are the HPDK motif, found in the DnaJ family of proteins, which includes several proteins known to interact with heat shock proteins (20). Recent reports have indicated that the large-T of SV40 binds hsp70 through amino-terminal sequences (34) and that the middle-T of polyomavirus binds hsp70 specifically through the HPDKGG hexapeptide (6). Binding is much more pronounced to mutant forms of these viral proteins than to the WT proteins, raising questions about the biological relevance of the hsp70 binding (43). Deletion of the HPDKGG sequence from the polyomavirus middle-T did not prevent its ability to mediate transformation, a process linked to binding of other cellular proteins, e.g., c-Src and phosphatidylinositol 3-kinase (6).

The HPDKGG hexapeptide also contains the sequence DKGG, which is the only region that small-t has in common with B subunits of PP2A (27, 36). Interestingly, mutations in the DKGG motif were reported to eliminate small-t's ability to activate expression of the human papillomavirus type 16 enhancer-promoter (36). Assuming that the sequence similarity between small-t and the B subunits has functional relevance, this must be more than simply the interaction with PP2A subunits because the DKGG region of small-t does not fall within the A-subunit binding site (13). Binding of small-t to PP2A results in the displacement of certain forms of B subunits (19), raising the possibility that released B subunits have functions that are independent of their role in PP2A, just as the transactivation function of small-t is independent of its PP2A binding. Unfortunately, the double point mutations used in this study affected both the HPDK and the DKGG motifs. We are currently trying to determine, by using single point mutations, whether the ability of small-t to transform HDF cells or to transactivate the E2A or the cyclin A promoter can be mapped to only one of these motifs.

Although the precise role of the 42-47 region in transformation of SN-10 and HDF cells is undefined, it is of interest that this region is involved in transactivation and that the cyclin A gene may represent a relevant cellular target of this activity of small-t. At least in this case, transcriptional activation does not work through small-t inhibition of PP2A. The mutations in the 97-103 region which decrease PP2A inhibition and result in decreased activation of MAPK and MEK by small-t (17) have no effect on transactivation of the cyclin A or E2A promoter in transient assays. The mechanisms through which small-t may activate expression of cyclin A are currently being explored by using truncated promoter constructs. These studies are not yet complete, but preliminary results map a small-t-responsive region to a 200-bp segment of the promoter that also responds to serum stimulation and cell cycle regulation (30a). It is of particular interest that cell cycle regulation appears to occur through a variant E2F site located in this region (35).

The possibility that small-t specifically influences the expression of cyclin A is particularly intriguing because of the recent report that some cells may be restricted in anchorage-independent growth by their inability to express cyclin A (14). With one exception (HDF cells), the assays used to assess the role of small-t in viral transformation involve the anchorage-independent growth of target cell types. It is tempting to suggest that small-t may promote growth in agar by stimulating expression of cyclin A. A different situation may exist for HDF cells. It was shown recently that some cell lines are completely unable to overcome a density-dependent block to cyclin A expression, even when stimulated with serum or other growth factors (48). HDF cells may resemble such cell lines and thus require small-t to maintain sufficient levels of cyclin A when cells reach confluence. A similar situation may exist in mouse 10T1/2 cells, which require small-t for formation of foci by monolayer overgrowth (32a, 49) but not for focus formation in cells grown from low density (49). We are currently exploring the role of small-t in overcoming restrictions to cyclin A expression that are imposed by nonadherence or high cell density. Should small-t function in this way, it may finally be clear why only some transformation systems require the presence of small-t.

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