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Mutational Analysis of Simian Virus 40 Small-t Antigen

PRATIMA JOG,[1] BANSI JOSHI,[1] VANDANA DHAMANKAR,[1] MICHAEL J. IMPERIALE,[2] JOAN RUTILA,[2]† AND KATHLEEN RUNDELL*

Department of Microbiology and Immunology and the Cancer Center, Northwestern University Medical and Dental Schools, 303 East Chicago Avenue, Chicago, Illinois 60611–3008,[1] and Department of Microbiology and Immunology, The University of Michigan Medical School, Ann Arbor, Michigan 48109–0620[2]

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Several point mutations in the simian virus 40 (SV40) small-t antigen have been analyzed for their effects on protein stability, transformation, transactivation, and binding of two cellular proteins. All mutations which affected cysteine residues in two cysteine clusters produced highly unstable small-t antigens. Four point mutations outside these clusters and one in-frame deletion mutant, *dl*890, produced stable proteins but reduced transformation efficiency. These were able to transactivate the EII promoter and bind the cellular proteins, suggesting that these activities are not sufficient for small-t-mediated enhancement of transformation.

The simian virus 40 (SV40) small-t antigen is a 17-kilodalton polypeptide found predominantly in the cytoplasm of infected cells (8, 23). The protein shares 82 amino acids at its amino terminus with the large-T antigen, the key transforming protein, while the remaining 92 amino acids are unique. By comparing small-t deletion mutants with the wild-type (WT) virus, several functions have been assigned to small-t. Of particular interest is the ability of small-t antigen to enhance transformation efficiency of rodent cells (2, 3, 4, 11, 16, 22). It has been suggested that small-t may be of particular importance in transformation when nongrowing cells are used (16) and when concentrations of the viral large-T antigen are limiting (2). Properties of the small-t antigen which may be related to its enhancement of transformation are its association with two cellular proteins (18, 25) and its ability to overcome the growth-arresting effects of theophylline in CV1 cells (19) and to promote continued cell cycle progression in some cell types (13). Recently, it has been shown that small-t antigen, in the absence of large-T antigen, has the ability to activate transcription from certain polII and polIII promoters (15), raising the possibility that expression of key cellular genes may be enhanced by small-t.

It is not yet clear which of the biologic activities attributed to small-t are related to its ability to enhance transformation and whether separate domains exist within the small-t protein. The unique region of small-t antigen is cysteine rich and contains a unique organization of cysteines into two CysX CysXXCys clusters (10), separated by a stretch of 21 amino acids. These clusters are located at amino acids 111 to 116 and 138 to 143 of the SV40 small-t antigen and are conserved in other papovaviruses, such as polyomavirus and BKV (6, 10). The importance of the residues in these cysteine clusters was suggested by studies of one mutant in which replacement of Cys-111 with tryptophan abolished the ability of small-t to transform BALB/c 3T3 cells in an abortive transformation assay (1).

Recently, a series of point mutations was introduced into a fragment of SV40 (nucleotides 3808 to 4826) by bisulfite mutagenesis. This fragment contains sequences of large-T antigen and those which encode the carboxy-terminal portion of small-t (amino acids 112 on). Mutations affecting the large-T antigen have been described previously (20, 21). In the study described here, plasmids containing point mutations in small-t antigen were used to produce viruses with which effects of the mutations on transformation could be correlated with other activities of small-t. Mutations of the cysteine residues in the two clusters to tyrosine residues had pronounced effects on protein stability, making it difficult to assess the contribution of these residues to the various activities. Mutations in two cysteine residues not organized in the clusters did not affect protein stability, but one of these in which Cys-161 was changed to a tyrosine residue showed reduced transformation efficiency. A mutation at Val-134 and a double mutation at Met-120 and Glu-123 also showed reduced transformation efficiency. Both of these mutations affect residues in the spacer region between the two cysteine clusters. The reductions in transformation ability were not associated with inability of the mutant small-t antigens to associate with the cellular proteins, render CV1 cells theophylline resistant, or to transactivate the adenovirus EII promoter, suggesting that these activities alone cannot account for the enhancement of transformation by small-t antigen.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (CV1) cells and Fischer rat F111 cells were propagated in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum. All virus stocks were harvested from lysates initiated with a low multiplicity of virus and the titers were determined on CV1 cells.

Construction of mutant viruses and plasmids. The generation of M13mp8 clones containing point mutations in the SV40 *Nde*I B fragment by bisulfite mutagenesis was described previously (20). The M13 clones used in this study are shown in Table 1. The SV40 *Nde*I fragment from each M13 clone was inserted into the plasmid pJYM *Nde*I-B, which contains all of SV40 except for the *Nde*I B fragment. When more than one mutation was present in either small-t or large-T sequences, these were separated by using the two SV40 PflMI sites (nucleotides 4558 and 1007) or the unique *Bst*XI site (SV40 4759). In one case, it was necessary to separate mutations by using the *Taq*I site (SV40 4739), and this was done directly at the virus level, producing virus 21-6 (Table 2). In all other cases, viral sequences were excised

* Corresponding author.
† Present address: Department of Biology, Brandeis University, Waltheim, MA 02254.

TABLE 1. Mutations present in M13mp8

Mutant	Map position[a] of mutation	Amino acid substituted	Mutated protein
3221	4764	Val-134 to Met	t
	4706	Cys-153 to Tyr	t
3224	4682	Cys-161 to Tyr	t
	4645	(173)[b]	t
3231	4804	Met-120 to Ile	t
	4797	Glu-123 to Lys	t
	4537	Trp-94-term	T
3233	4810	(118)	t
	4736	Cys-143 to Tyr	t
	4616	(IVS)[c]	t
3237	4819	(115)	t
	4751	Cys-138 to Tyr	t
3239	4758	Val-136 to Ile	t
	4472	Ala-116 to Thr	T
3245	4706	Cys-153 to Tyr	t
3252	4817	Cys-116 to Tyr	t
	4745	Cys-140 to Tyr	t
	4483	Ser-112 to Asn	T

[a] All mutations resulted from G to A changes (20) at the SV40 nucleotide number shown.
[b] Parentheses indicate silent mutation.
[c] Mutation in the intervening sequence.

from the plasmid, ligated, and used to transfect CV1 cells. The initial lysate was used to produce plaques which were picked and expanded. Viral DNA was sequenced by using a Sequenase kit (United States Biochemical Corp.) to confirm the presence of a single mutation.

The actual virus stocks produced are shown in Table 2. All viruses contain single mutations in small-t sequences, with the exception of virus 31-2, in which two close mutations could not be separated from one another. For clarity, viruses will be referred to by altered amino acids (e.g., Cys-140) in the remainder of this report. Also used were deletion mutants *dl*890, which contains a small in-frame deletion in the first cysteine cluster, and *dl*888, which produces no small-t antigen because it lacks the splice donor for small-t mRNA (3).

Plasmids used in transactivation assays were derivatives of pw2t (4, 17) or pw2tBg, in which the SV40 *Hin*dIII site at 5171 was converted to a *Bgl*II site. No large-T fragment has been detected by using pw2t (17), but to confirm that transactivation resulted only from small-t, pw2t-*dl*888 was constructed. The plasmid pw2t-*dl*888 is totally defective in transactivation.

Immunoprecipitation. Confluent 3.5-cm dishes of CV1 cells were infected with equal multiplicities of the various mutant viruses and were labeled with [^{35}S]methionine either from 40 to 68 h or from 40 to 41 h postinfection. For chase experiments, isotope was removed and cells were incubated with DMEM for various periods of time. Immunoprecipitation with polyclonal sera from hamsters bearing tumors induced with SV40-transformed cells and gel electrophoresis were performed as described previously (17, 25).

TABLE 2. Virus constructs

Virus no.	Mutation[a]
21-6	Val-134 to Met
24	Cys-161 to Tyr
31-2	Met-120 to Ile
	Glu-123 to Lys
33	Cys-143 to Tyr
37	Cys-138 to Tyr
39	Val-136 to Ile
45	Cys-153 to Tyr
116	Cys-116 to Tyr
140	Cys-140 to Tyr

[a] All mutations are in the small-t antigen and have been separated from any other mutations present in the original M13mp8 clone.

Theophylline sensitivity. Confluent 3.5-cm dishes of CV1 cells were infected with equal multiplicities of the various mutant viruses, and then the infection was stopped by adding serum-free medium containing zero or 2 mM theophylline. After 40 h at 37°C, cells were pulsed with 0.5 μCi of [^{3}H]thymidine per ml for 6 h. Cellular DNA was extracted by the method of Hirt (12), sonicated in water, and counted. Radioactivity in Hirt supernatants and Hirt pellets was always parallel, and data are shown only for cellular (Hirt pellet) DNA.

Transformation. Rat F111 cells passaged less than 25 times were used for transformation assays as described previously (3). Briefly, cells were trypsinized and suspended in calcium- and magnesium-free Trizma-buffered saline containing 1% filtered fetal bovine serum. Infection was carried out in suspension for 1 h. Cells were passed through drawn Pasteur pipettes to produce single-cell suspensions and were then plated in DMEM containing 5% fetal bovine serum, 5% dog serum, and 0.5% agar. Cells (6×10^4) were plated in each 6-cm dish. Colonies were counted after incubation at 38°C for 4 to 6 weeks, and the efficiency of transformation was expressed as the number of colonies per 100 cells plated.

Transactivation. Monkey kidney CV1-P cells were used in transactivation experiments according to procedures described by others (15). A plasmid, EIIccCAT, was made by replacing the cytochrome *c* promoter of plasmid pRC4CATB (9) with the adenovirus 5 EII promoter. This construction places an intron between the EII promoter and the chloramphenicol acetyltransferase (CAT) coding region.

RESULTS

Growth of mutant viruses. As expected, all viruses with mutations in the small-t antigen grew in CV1 cells. The virus yields (plaque-forming units) were at least as high as those of small-t antigen deletion mutants. Mutants which grew routinely to lower titers than WT viruses included those in which cysteines of the second cysteine cluster were mutant. The titers and plaque sizes of these mutants were similar to those of known small-t deletion mutants such as *dl*888 and *dl*890 and to mutants which contained termination codons in small-t antigen. These titers were 5- to 10-fold lower than titers of WT virus.

Stability of the mutant small-t antigens. Replicate cultures of CV1 cells were infected with equal multiplicities of WT and mutant viruses and then were labeled overnight with [^{35}S]methionine from 40 to 68 h postinfection to measure steady-state levels of the small-t antigens produced. The amount of small-t antigen which could be detected varied among the mutants, with two clear phenotypes emerging (Fig. 1). First, several of the mutants accumulated small-t antigen to levels approximately that of the WT infections. These included the double mutant Val-134 Cys-153, mutant Cys-161, and mutant Cys-153. In the second class, very low levels of small-t antigen accumulated in infected cells. Among these mutants were Cys-143 and the double mutant Cys-116 Cys-140. Although not shown here, when the two cysteine mutations were separated, individual viruses containing mutations at either Cys-116 or Cys-140 also showed

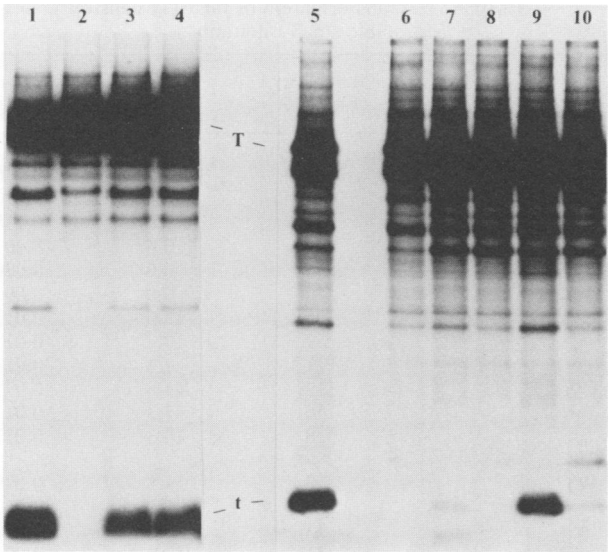

FIG. 1. Steady-state levels of mutant small-t antigens. Confluent dishes of CV1 cells were infected with equivalent multiplicities of virus for 1 h at 37°C and then were incubated for 24 h. Plates were then labeled from 24 to 36 h postinfection with 50 μCi of [^{35}S]methionine in medium containing 1/18 the normal concentration of methionine. Extracts were prepared by using Nonidet P-40 and immunoprecipitated. Patterns shown are sodium dodecyl sulfate-polyacrylamide gels of immunoprecipitates from cells infected with WT SV40 (lane 1), *dl*888 (lane 2), Val-134 Cys-153 (lane 3), Cys-161 (lane 4), WT SV40 (lane 5), *dl*888 (lane 6), *dl*890 (lane 7), Cys-143 (lane 8), Cys-153 (lane 9), and Cys-116 Cys-140 (lane 10). Lanes 1 through 4 represent one experiment, lanes 5 through 10 another.

reduced levels of accumulated small-t antigen. Notably, mutations in cysteines which formed part of the two clusters showed the reduced small-t levels, while those at Cys-153 and Cys-161 did not.

Reduced small-t levels had been reported previously (14) for the deletion mutant *dl*890, also shown in Fig. 1. In this report, it was postulated that the deletion present might have altered splicing patterns, possibly because of the structure of the RNA precursor. To determine whether the reduced levels of small-t observed with the point mutations resulted from reduced rates of synthesis, short-pulse analyses were performed.

Viruses containing mutations of individual cysteine residues of the two cysteine clusters produced nearly WT levels of small-t antigen during a 1-h pulse, but the proteins produced were very unstable. In the experiment shown in Fig. 2, replicate cultures were infected with equal multiplicities of the mutant viruses for 40 h and were then pulsed for 1 h with [^{35}S]methionine. One plate was extracted immediately, and the rest were washed and incubated with fresh DMEM for 0.5, 1, 2, and 3 h. The WT small-t antigen is very stable under these conditions, and previous experiments not shown here had shown that small-t antigen has a half-life of approximately 10 to 12 h. In contrast, mutant small-t antigens turned over rapidly during the 3-h chase. By using densitometric analyses of the autoradiograms, half-lives of about 30 min were calculated for cysteine mutations at 138, 140, and 143, with a half-life of 60 min for the cysteine mutation at 116. Two mutations at the remaining cysteines in the first cluster (Cys-111 and Cys-113) were obtained from David Livingston (Dana-Farber Cancer Institute), and these also showed greatly reduced stabilities relative to the WT small-t antigen (data not shown). In these two mutants, the Cys-111 residue was changed to tryptophan and the Cys-113 residue to serine.

Pulse-chase analyses were performed on all mutants described in Table 2. Mutants which accumulated levels of small-t antigen similar to those present in WT infections after overnight pulses were shown to be stable, as expected from the accumulation data. One double mutant showed a different pattern (Fig. 3). This mutant has mutations at Met-120 and Glu-123, and reduced amounts of small-t antigen were found even during pulse-labeling. Interestingly, even though reduced amounts of small-t antigen were expressed, the protein produced was relatively stable. The same pattern was observed for the in-frame deletion, *dl*890 (Fig. 3).

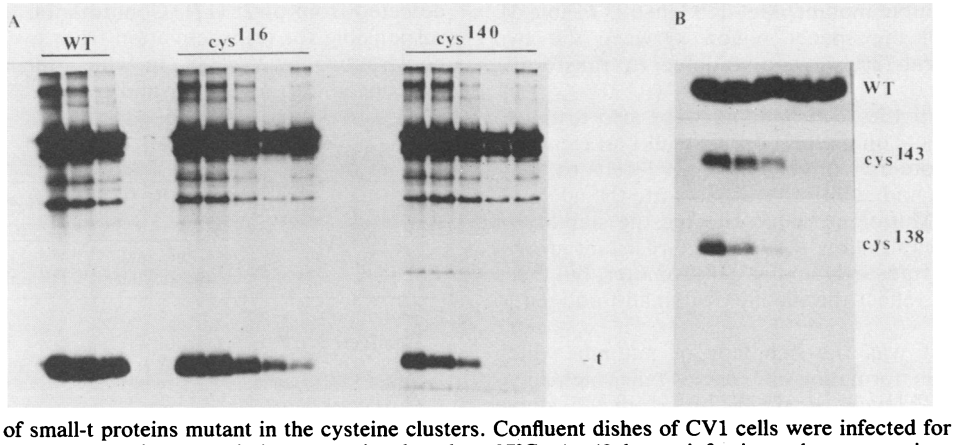

FIG. 2. Stability of small-t proteins mutant in the cysteine clusters. Confluent dishes of CV1 cells were infected for 1 h with equivalent multiplicities of WT or mutant viruses and then were incubated at 37°C. At 40 h postinfection, plates were incubated for 1 h with methionine-free medium and then were pulsed for 1 h with 50 μCi of [^{35}S]methionine in methionine-free medium. For each virus, one plate was extracted immediately after the pulse. Isotope was removed from the remaining plates, and cells were washed twice with phosphate-buffered saline and then incubated for various periods of time with DMEM before extraction. Patterns shown are sodium dodecyl sulfate gel electrophoresis profiles of immunoprecipitated proteins. Pulse-chase analyses from two separate experiments are shown in panels A and B, with a WT pattern shown for each. WT-infected cells were extracted immediately after the pulse (lane 1) or following 1- or 3-h chase periods (lanes 2 and 3) (A). Patterns shown for the WT (B) and for all mutants are immunoprecipitates of pulsed cells (lane 1) or cells chased for 0.5, 1, 2, and 3 h (lanes 2 through 5).

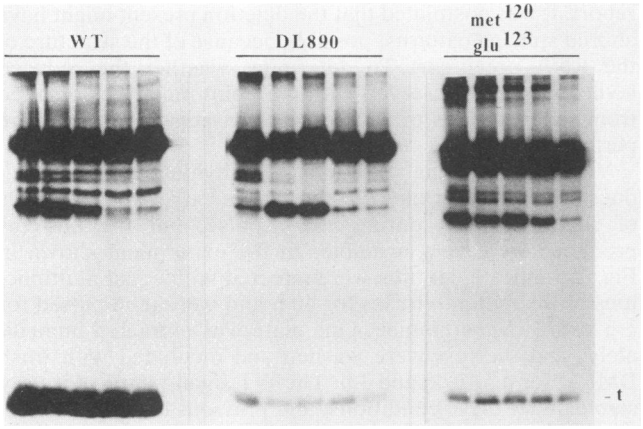

FIG. 3. Stability of small-t antigens produced by *dl*890 and Met-120 Glu-123. Cells were infected and labeled as described in the legend to Fig. 2. Patterns shown are immunoprecipitates of pulsed cells (lane 1) or cells chased for 0.5, 1, 2, or 3 h.

Transformation of rat F111 cells. The ability of the mutant viruses to transform rat F111 cells was tested by using growth in soft agar as an assay. Optimal transformation efficiencies of this cell type depend on the presence of small-t antigen, and it has been shown previously (3, 4) that in the absence of small-t, transformation efficiencies were reduced 10- to 100-fold. All viruses with mutations in the cysteine residues of the two cysteine clusters were as defective as *dl*890 in this assay (data not shown). However, the failure of small-t antigen to accumulate following infection makes it impossible to distinguish the need for a given cysteine residue in transformation from a role for that residue in protein stability.

Of the mutants which produced stable small-t antigens, four had effects on transformation efficiency (Table 3). Mutation of Cys-153 had a small but reproducible effect on transformation which was most pronounced at lower multiplicities. At higher multiplicities, transformation efficiency was reduced about two-fold. Greater reductions in transformation efficiency were observed for mutant Cys-161, mutant Val-134, and the double mutant, Met-120 Glu-123 (Table 3). Another mutant in the spacer region between the two cysteine clusters, Val-136, showed no defect in transformation.

Other biologic activities. All mutants were also tested to determine their effects on interaction of small-t antigen with the two cellular proteins, conversion of CV1 cells to theophylline resistance, and ability to transactivate the adenovirus EII promoter. Mutations which affected the stability of small-t antigen failed to allow theophylline-resistant growth of CV1 cells and to transactivate the EII promoter, but these results may simply reflect the inability of small-t antigen to accumulate.

Of more interest was the behavior of mutants which showed reduced transformation efficiencies but which accumulated nearly WT levels of small-t or produced stable mutant proteins at reduced levels. No correlations were found between the effects of the mutations on transformation and any of these other assays for small-t. As shown in Table 4, all these point mutants increased the theophylline resistance of infected CV1 cells to nearly the same extent as WT-infected cells. Although not shown here, the deletion mutant *dl*890 reproducibly induced intermediate or low levels of thymidine incorporation in theophylline-treated cultures. However, thymidine incorporation was always greater than in cultures in which no small-t antigen was present (*dl*888). The reason for this is not known but may reflect the reduced rate of synthesis of small-t and the low levels of this protein which accumulate in infected cells.

Transactivation by the mutant proteins was measured by cloning each mutation into the vector pw2t, which expressed small-t as the sole viral protein. No large-T fragment can be detected from pw2t (17). Confirmation that small-t alone is responsible for transactivation from pw2t was obtained by constructing pw2t-*dl*888, in which the *dl*888 deletion was introduced into pw2t. Plasmid pw2t-*dl*888 failed to transac-

TABLE 3. Transformation of rat F111 cells by stable small-t mutants

Virus	Multiplicity of infection	Average no. of colonies	% Transformation
Expt 1			
WT	100	71.0	0.110
	50	71.5	0.120
	20	71.0	0.118
*dl*890	100	0.6	0.001
	50	0.0	0.000
	20	0.0	0.000
Cys-161	105	10.0	0.017
	52	10.7	0.018
	21	7.4	0.012
Cys-153	112	44.8	0.075
	56	35.3	0.017
	28	10.3	0.017
Expt 2			
WT	39	53.8	0.089
	36	36.0	0.060
	13	25.2	0.040
*dl*890	41	3.0	0.005
	22	1.6	0.003
	15	0.6	0.001
Met-120 Glu-123	40	4.2	0.007
	25	3.3	0.005
	15	1.6	0.003
Val-136	40	50.3	0.084
	25	33.0	0.055
	15	29.2	0.048
Val-134	42	5.7	0.009
	28	3.5	0.005
	14	1.7	0.003

TABLE 4. Sensitivity of infected cells to theophylline

Virus	[^{3}H]Thymidine uptake (cpm, 10^3)		% Decrease
	Zero mM theophylline	2 mM theophylline	
Expt 1			
Uninfected	4.7	1.2	74
WT	8.6	11.3	
*dl*888	5.7	1.5	74
Val-134 Cys-153	6.5	9.3	
Cys-161	7.6	10.4	
Expt 2			
Uninfected	13.5	2.7	80
WT	14.7	21.3	
*dl*888	13.5	3.4	75
Val-134	15.4	18.4	
Val-136	16.2	22.9	
Met-120 Glu-123	15.4	17.8	

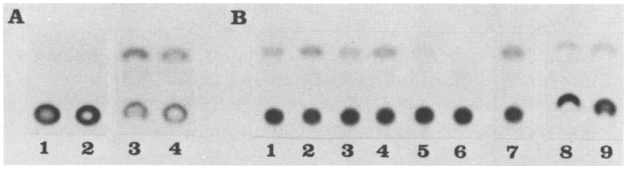

FIG. 4. Transactivation of the adenovirus EII promoter by small-t mutants. Subconfluent CV1 cells were transfected with pEIIccCAT and pw2t or mutant pw2t derivatives by using the calcium phosphate procedure (15). At 48 h posttransfection, extracts were made and analyzed for CAT activity by using [^{14}C]chloramphenicol. Acetylated forms of chloramphenicol were analyzed by thin-layer chromatography. (A) Patterns shown are the CAT activities induced from the EII promoter by pw2t-*dl*888 (lanes 1 and 2) and parental plasmid pw2t (lanes 3 and 4). No transactivation occurs when small-t is deleted. (B) In a separate experiment, patterns shown are the CAT activities induced by small-t antigen mutant at Val-134 and Cys-153 (lane 1), Val-134 (lane 2), Cys-153 (lane 3), Cys-161 (lane 4), Cys-116 (lane 5), Cys-140 (lane 6), Met-120 and Glu-123 (lane 7), and *dl*890 (lane 8). WT small-t expressed from pw2t is shown in lane 9.

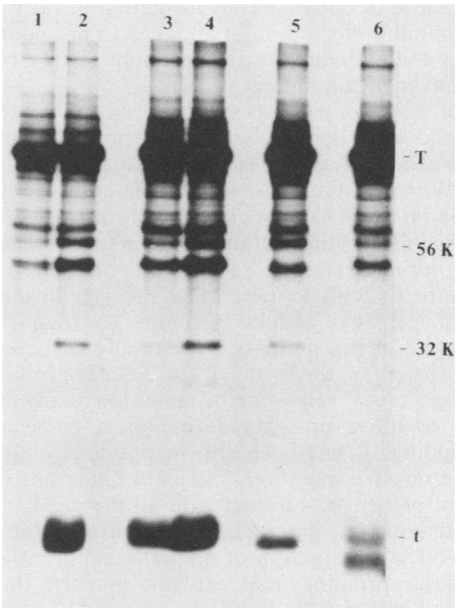

FIG. 5. Interaction of mutant small-t antigens with cellular proteins. Cells were infected with WT or mutant viruses and labeled as described in the legend to Fig. 1. Extracts were immunoprecipitated by using a hamster antitumor serum which efficiently precipitates cellular proteins associated with small-t antigen but which does not directly recognize these proteins.Patterns shown are sodium dodecyl sulfate gels of immunoprecipitates from cells infected with *dl*888 (lane 1); WT SV40 (lane 2); mutants Val-134 (lane 3), Val-136 (lane 4), and Met-120 and Glu-123 (lane 5); or deletion mutant *dl*890 (lane 6).

tivate EII-CAT, eliminating the remote possibility that undetectable large-T-related sequences were responsible for transactivation (Fig. 4A).

*dl*890 and viruses with mutations at Val-134, Met-120 Glu-123, and Cys-161 were as active as the WT small-t antigen (pw2t) in the transactivation assays (Fig. 4B). It is interesting to note that two of these mutants (*dl*890 and Met-120 Glu-123) expressed small-t at reduced levels, but the small-t antigens produced were stable. This suggests that low levels of accumulated small-t alone do not lead to a failure to transactivate. It is more likely that the conformation of small-t is appropriate for transactivation in these mutants, whereas the conformation of proteins mutant in the cysteine clusters is altered, leading both to instability and to a failure to transactivate. Of particular interest is that the reduced transformation efficiencies of these mutants do not correlate with their ability to transactivate the EII promoter.

Finally, the mutants with reduced transformation ability were capable of interaction with the two cellular proteins (Fig. 5). The small-t antigen of one mutant, Val-134, bound lower amounts of these proteins than expected based on the levels of small-t antigen which accumulated. The levels of the cellular proteins should reflect the accumulated levels of small-t if the complex is a stoichiometric one. At the present time, however, it is clear only that a failure to bind the cellular proteins is not correlated with reduced transformation efficiency.

DISCUSSION

A series of point mutations in the small-t antigen of SV40 have been analyzed, with the initial goal being to determine the effects of these mutations on various biologic systems influenced by small-t. Particular emphasis was put on mutations in the cysteine residues of the cysteine-rich unique domain of small-t. A major observation has been that alteration of cysteine residues of the two CysXCysXXCys clusters had pronounced effects on the stability of small-t in vivo. Mutation of two Cys residues outside these clusters did not show this effect.

The mutants used in this study changed cysteine residues to tyrosines, a nonconservative change. However, the general nature of the observation that these residues are required for small-t stability was supported by studies of mutants in which cysteine was exchanged for serine, kindly provided by David Livingston (V. Dhamankar and K. Rundell, unpublished observations). The Ser mutants, like the Tyr mutants, turned over rapidly in infected cells, although the half-lives were slightly longer for the Ser mutants (1 h versus 30 min). It also appeared that mutants in the second Cys cluster (138 to 143) were more defective than those in the first cluster, as indicated by greater instability and reduced titer of viral stocks. However, both sets of mutants were transfomation defective.

The basis for the instability of the mutants in the Cys clusters is not known but can be reproduced in vitro by heat inactivation experiments in the presence and absence of reducing agents (R. Goswami and K. Rundell, unpublished observations). Instability might be accounted for by the existence of intramolecular disulfide bonds which stabilize the protein. Although the existence of intracellular disulfide bonds has been questioned in the past because of the reducing atmosphere in the cell, recent studies have indicated that the average cellular environment is adequate to permit disulfide bond formation where the conformation of the polypeptide brings the two cysteine residues into close proximity (5).

It is also possible that small-t antigen is stabilized by the binding of a metal ion. Although small-t has not yet been shown to bind any metal ions, similar cysteine motifs in other proteins such as the human papillomavirus E7 or bovine papillomavirus E6 proteins (7, 24) have been shown to be involved in zinc binding.

The instability of several of the mutant small-t antigens made it impossible to correlate other biologic effects with the

failure of these to enhance transformation of rat F111 cells, because inability of the small-t antigens to promote transactivation or theophylline resistance might simply reflect the rapid turnover of the mutant proteins.

Three of the point mutants studied produced stable small-t antigens and were defective in transformation. These were mutants Val-134, Cys-161, and the double mutant, Met-120 Glu-123. With these mutants and the deletion mutant *dl*890, it has been possible to dissociate transformation of rat F111 cells from ability of the mutant proteins to transactivate the EII promoter and render CV1 cells theophylline resistant.

It is more difficult to determine the significance of the binding of the two cellular proteins to small-t antigen. Clearly, none of the mutants were totally defective in this function. However, the levels of the cellular proteins bound may be important if activation or inhibition of any enzymatic functions of these proteins occurs as a consequence of small-t binding. It has been shown previously (19) that at least in permissive infection, sufficient quantities of small-t antigen are present to interact with all the cellular proteins present. If complete interaction is necessary for maximal effects, reduced interaction of mutant small-t antigens with these cellular proteins may explain, in part, the mutant phenotype. Similarly, binding alone may not be sufficient for activation or inhibition of functions of these proteins.

Two of the mutants which affect transformation have alterations in residues of a spacer region which separates the two CysXCysXXCys clusters. However, the WT behavior of a mutant at Val-136 showed that not all mutations in this region will affect small-t function. Hydropathy plots have shown that this is the most hydrophilic portion of the unique domain of small-t antigen and separates two highly hydrophobic domains in which the Cys clusters lie. Mutations in this region may influence protein conformation, without leading to the pronounced instability noted with the Cys mutations. Alternatively, residues in this region may be required for some specific function as yet undetermined. It will be of interest to generate additional point mutations in this region, in addition to insertions and deletions that alter the spacing between the two CysXCysXXCys clusters to determine their effect on properties of the small-t antigen.

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