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Mutational Analysis of Simian Virus 40 T Antigen: Stimulation of Cellular DNA Synthesis and Activation of rRNA Genes by Mutants with Deletions in the T-Antigen Gene

KENNETH J. SOPRANO,[1] NORBEL GALANTI,[1] GERALD J. JONAK,[1] SCOTT McKERCHER,[1] JAMES M. PIPAS,[2] KEITH W. C. PEDEN,[3] AND RENATO BASERGA[1]*

Department of Pathology and Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania 19140[1]; Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260[2]; and Howard Hughes Medical Institute Laboratory, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205[3]

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The biological activity of several deletion mutants of simian virus 40, cloned in pBR322, was determined. Three functions of the simian virus 40 *A* gene were studied: (i) the ability to express T antigen; (ii) the ability to induce cell DNA replication; and (iii) the ability to reactivate silent rRNA genes in hybrid cells. Recombinant plasmid DNA was introduced into cells by manual microinjection or by transfection. The results (together with previous reports) indicate that the critical sequences for these three functions are located separately on the simian virus 40 *A* gene, as follows: (i) the sequences necessary for the detection of the common antigenic determinant of T antigen extend from nucleotide 4147 to nucleotide 4001 (map units 0.45 to 0.42); (ii) the sequences critical for the stimulation of cell DNA synthesis extend from nucleotide 4327 to nucleotide 4001 (map units 0.49 to 0.42); and (iii) those critical for the reactivation of rRNA genes extend approximately from nucleotide 3827 to nucleotide 3526 (map units 0.39 to 0.33).

As summarized in the preceding paper (26), genetic and biochemical evidence indicates that the early region of the simian virus 40 (SV40) genome is required for many of the biological activities of the virus. This region is needed for viral DNA replication (35), autoregulation of early genes (1, 14, 29, 38), cell transformation (2, 3, 15, 16, 18, 23, 37), and stimulation of cellular DNA and RNA syntheses (4, 9, 12, 13, 19, 22, 27, 30, 31, 34, 39). Although the early region codes for two proteins, the large T antigen (containing 708 amino acids) and the small t antigen (containing 174 amino acids), most of these functions have been attributed to the large T antigen. As is true of many other proteins which exhibit multiple functions (for a review, see reference 17), some of the functions of the large T antigen can be localized to specific domains. These domains can be determined in some detail by studying the functions of modified, cloned SV40 DNAs introduced into the nuclei of cells by the manual microinjection technique of Graessmann and Graessmann (10). By microinjecting fragments of SV40 DNA, Galanti et al. (9) have previously been able to distinguish those sequences in the T-antigen gene that are critical for the stimulation of cell DNA replication from those that are critical for the activation of silent rRNA genes. In the present communication we report further experiments in which this technique was applied to the cloned deletion mutants of SV40 described in the preceding paper (26) to refine the map positions of T-antigen coding regions involved in these two activities.

MATERIALS AND METHODS

Growth and preparation of cells. *ts*13 cells, a G_1-specific temperature-sensitive mutant originally derived from BHK cells (20), were grown at 34°C on glass cover slips as previously described. Before microinjection, *ts*13 cells were made quiescent by allowing them to grow for 7 days in Dulbecco minimal essential medium supplemented with 1% calf serum. After microinjection, cells were incubated at 34°C for 24 h in the same medium supplemented with [^{3}H]thymidine (0.7 μCi/ml). All media were warmed to the temperature of incubation before use.

NIH 3T3 cells (a kind gift from Geoffrey M. Cooper, Sidney Farber Cancer Institute, Boston, Mass.) were made quiescent in 1% serum. After microinjection, they were incubated at 37°C without changing the medium. TC-7 monkey cells were grown in Dulbecco medium supplemented with 10% serum at 37°C. They were usually microinjected while exponentially growing (9).

Human-mouse hybrid cells (55–54 cells) were derived by Croce (6) from the fusion of HT-1080 human

fibrosarcoma cells with mouse peritoneal macrophages. Karyotype and isozyme analyses of this hybrid have shown that 55–54 cells contain all of the human chromosomes and 18 different mouse chromosomes, including 3 chromosomes where mouse rRNA genes are located (7). This cell line is routinely cultured in Dulbecco medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum. Its karyotype is periodically checked in stock cultures.

Cloning and characterization of SV40 deletion mutants. The cloning, characterization by restriction enzyme analysis, and sequencing of the deletion mutants used in these studies have been described in detail in the accompanying paper (26).

SV40 nomenclature. For convenience, the map coordinates of the SV40 genome will be given in this paper (whenever feasible) both in map units and in nucleotide residue numbers. The map units and residue numbers are those given by Tooze (40). Map units and nucleotide numbers are given clockwise.

Microinjection of recombinant DNA. DNA fragments were delivered directly into nuclei of cells by the glass capillary microinjection technique described by Graessmann and Graessmann (10). Samples were dissolved in 10 mM Tris-hydrochloride (pH 7.4) at 25°C and routinely centrifuged for 15 min in an Eppendorf centrifuge before microinjection. At least 100 cells were microinjected with each DNA preparation. Unless otherwise stated, DNA solutions to be microinjected in these experiments were adjusted to a concentration of 0.1 mg/ml.

TABLE 1. Ability of SV40 deletion mutants to express T antigen in TC-7 monkey cells after microinjection[a]

Plasmid or deletion mutant[b]	Deletion limit	Coordinates	% T-positive cells
pSVB3			98
pSV2G	1782–2533	1.0–0.143	94
*dl*1135	5114–5082	0.636–0.629	100
*dl*1047	4007–3570	0.424–0.341	64
*dl*1151	3798–3472	0.385–0.322	68
*dl*1140	2792–2763	0.192–0.187	44
*dl*1136[c]	5067–4262	0.614–0.461	0
*dl*1137	4453–4423	0.509–0.504	7[d]
*dl*1138	4339–4311	0.488–0.482	7[d]
*dl*1046	4146–3867	0.451–0.398	0
*dl*1001	4001–3476	0.423–0.322	60
*dl*1055	3620–3566	0.351–0.340	32
*dl*1139	3557–3526	0.339–0.332	32
*dl*1058	3290–3148	0.288–0.260	42
*dl*1061	3048–2907	0.231–0.215	64
*dl*1066	2809–2730	0.196–0.181	88
*dl*1141	2648–2681	0.171–0.165	50

[a] All plasmids were microinjected manually into the nuclei of TC-7 cells. The cells were fixed and stained for T antigen after 24 h.

[b] pSVB3 is wild-type SV40 cloned into pBR322 (24); pSV2G is the 4,492-base-pair *Bam*HI-*Eco*RI fragment cloned into pBR322 (9); *dl*1135, *dl*1047, *dl*1151, and *dl*1140 are in-phase deletion mutants; the rest are out-of-phase deletion mutants (26).

[c] Missing both large T splice junctions.

[d] Only cytoplasmic immunofluorescence.

Transfection. For certain experiments it was necessary to introduce the DNA into cells by calcium phosphate-mediated transfection. A modification of the method described by Graham et al. (11) was used. 55–54 cells were plated at a concentration of 2×10^5 per 60-mm dish. After 24 h, the medium was changed to calcium-free, serum-less medium. After 3 h, this medium was removed and replaced with 5 ml of fresh calcium-free medium and 0.5 ml of transfection cocktail. This contained 1 μg of the cloned DNA of interest, 10 μg of 55–54 high-molecular-weight carrier DNA, 125 mM $CaCl_2$, 140 mM NaCl, and 0.75 mM Na_2HPO_4. After 4 h, the medium and cocktail were removed, the cells were washed thoroughly, and fresh complete medium containing [^{3}H]uridine (New England Nuclear Corp.; 2.5 Ci/mol, 1 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) was added. The cells were harvested 24 h later and analyzed for reactivation of the silent mouse rRNA genes (32). With this procedure, from 8 to 10% of the transfected cells became T positive in 48 h.

Detection of T antigen by immunofluorescence. Cells were fixed in cold methanol for 15 min at 4°C. SV40 T antigen was visualized by the indirect immunofluorescence technique (28), using hamster anti-T serum and, as a second antibody, fluorescein-conjugated goat anti-hamster gamma globulin.

Autoradiography. Cells were continuously labeled with [^{3}H]thymidine (New England Nuclear Corp.; 6.7 Ci/mol, 0.7 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) added immediately after microinjection or transfection, and autoradiographs were made and analyzed by standard procedures.

RESULTS

Expression of T antigen in TC-7 cells microinjected with DNA from deletion mutants of SV40. DNAs of various SV40 mutants containing deletions of T-antigen coding sequences, as described by Pipas et al. (26), were first microinjected into the nuclei of TC-7 cells to determine the ability of each recombinant clone to express T antigen in this cell line. In these experiments the SV40 DNA was microinjected as pBR322 recombinants. At 24 h after microinjection, the cells were fixed and stained for T antigen, and the percentage of T-positive cells was determined. Those DNAs with in-phase deletions (*dl*1135, *dl*1047, *dl*1151, and *dl*1140) gave a high percentage of T-positive cells, as did those with out-of-phase deletions distal to (toward the codons of the carboxyl end of T antigen) map coordinate 0.42 (*dl*1001, *dl*1055, *dl*1139, *dl*1058, *dl*1061, and *dl*1066) (Table 1). Based on the nucleotide sequence data, the T antigens of this set of out-of-phase deletion mutants would contain a normal T-antigen sequence varying in length from 272 to 670 amino acids from the amino terminus. In each case the intensity and location of fluorescing material were typical of cells injected with wild-type SV40 DNA. Micro-

injection of pSV *dl*1137, *dl*1138, and *dl*1046, all of which contain out-of-phase deletions mapping proximal to nucleotide 4192 (i.e., code for T antigens of less than 223 amino acids) resulted in either very weak or no immunofluorescence, even after repeated microinjection. (pSV *dl*1136, which is missing large T splice junctions, also failed to produce detectable T antigen.) In the case of the two weakly positive DNAs (*dl*1137 and *dl*1138), which code for T antigens of 121 and 160 amino acids, respectively, the detectable immunofluorescence was cytoplasmic. We conclude that the four mutants with in-phase deletions and those with out-of-phase deletions that permit the synthesis of a T-antigen polypeptide containing at least 272 amino acids (*dl*1001) of the wild-type sequence direct the synthesis of T antigen sufficiently stable to be readily detected by immunofluorescence. These T-related proteins accumulate in the cell nucleus. Putative T-antigen polypeptides of 121 or 160 amino acids (*dl*1137 and *dl*1138) derived from mutants with somewhat more proximal out-of-phase deletions are either unstable or lack many antigenic determinants reactive with the anti-T serum used. These polypeptides appear to remain largely cytoplasmic. Finally, a mutant DNA that codes for an amino-terminal T polypeptide of 32 amino acids (*dl*1136) fails to produce detectable antigen.

Stimulation of cellular DNA synthesis. One of the biological activities of the large T antigen in the infected host cells is stimulation of cell division (see reference 40 for a review). This mitogenic activity is accompanied by stimulation of cellular DNA and rRNA syntheses (4, 9, 12, 13, 30, 31, 34, 39).

To study the induction of DNA synthesis by the various cloned deletion mutants of SV40, quiescent NIH 3T3 and *ts*13 cells were microinjected with recombinant plasmids. Control cells were injected with either pBR322, pSV2G (a clone containing the entire T-antigen gene of SV40), or pSVB3 (a clone containing the entire SV40 genome). NIH 3T3 cells were incubated at 37°C and *ts*13 cells were incubated at 34°C in conditioned Dulbecco medium supplemented with 1% calf serum in the presence of [^{3}H]thymidine. At 24 h after microinjection, the cells were fixed, stained for T antigen, and processed for autoradiography.

The results obtained after microinjection of the deletion mutants (Table 2) show that deletion of any sequences distal to nucleotide 4001 does not affect the ability of the microinjected DNA to stimulate cell DNA synthesis in either *ts*13 or NIH 3T3 cells. Deletions which lead to presumptive termination at a site proximal to nucleotide 4311 (*dl*1138, *dl*1137, and *dl*1046) are unable to induce cell DNA synthesis.

TABLE 2. Stimulation of cell DNA synthesis and activation of rRNA genes by SV40 deletion mutants

DNA microinjected	% of cells in DNA synthesis		Reactivation of rRNA genes
	3T3	*ts*13	
None	3	6	–
pBR322	4	5	–
pSVB3	95	97	+
pSV2G	90	90	+
*dl*1135	90	ND[a]	+
*dl*1047	56	64	–
*dl*1151	29	50	–
*dl*1140	74	66	+
*dl*1136	5	ND	–
*dl*1137	4	ND	–
*dl*1138	3	ND	–
*dl*1046	7	ND	–
*dl*1001	52	80	–
*dl*1055	40	40	–
*dl*1139	22	19	–
*dl*1058	ND	50	+
*dl*1061	40	40	+
*dl*1066	72	98	+
*dl*1141	74	80	+

[a] ND, Not done.

Reactivation of silent rRNA genes. Soprano et al. (33) have previously shown that microinjection of the T-antigen gene is capable of reactivating silent mouse rRNA genes in the human-mouse 55–54 hybrid cells. These cells have a full complement of human chromosomes plus 18 mouse chromosomes, including 3 chromosomes on which the mouse rRNA genes are located. Under standard growth conditions, these cells continuously express human rRNA. The mouse rRNA genes are repressed but can be activated by infection with SV40 (32), a variety of adenovirus-SV40 hybrid viruses (33, 34), and microinjection with a variety of SV40 DNA fragments (9, 33).

We have continued these studies on identifying the T-antigen gene sequences that are critical for this function by using the cloned deletion mutants described above. Exponentially growing 55–54 cells were microinjected or transfected with deletion mutant recombinant plasmids. Microinjected and non-microinjected cells were labeled for 24 h with [^{3}H]uridine and [^{14}C]uridine, respectively. The rRNA was then isolated and analyzed by electrophoresis in 0.6% agarose–2.4% polyacrylamide gels to score for the appearance of the human and mouse 28S species. DNAs with in-phase deletions either near the beginning (*dl*1135) or near the end (*dl*1140) of the T-antigen coding sequence activated mouse rRNA genes (Table 2). However, DNAs with in-phase deletions corresponding to amino acid residues 270 to 417 (*dl*1047) or 340 to 450

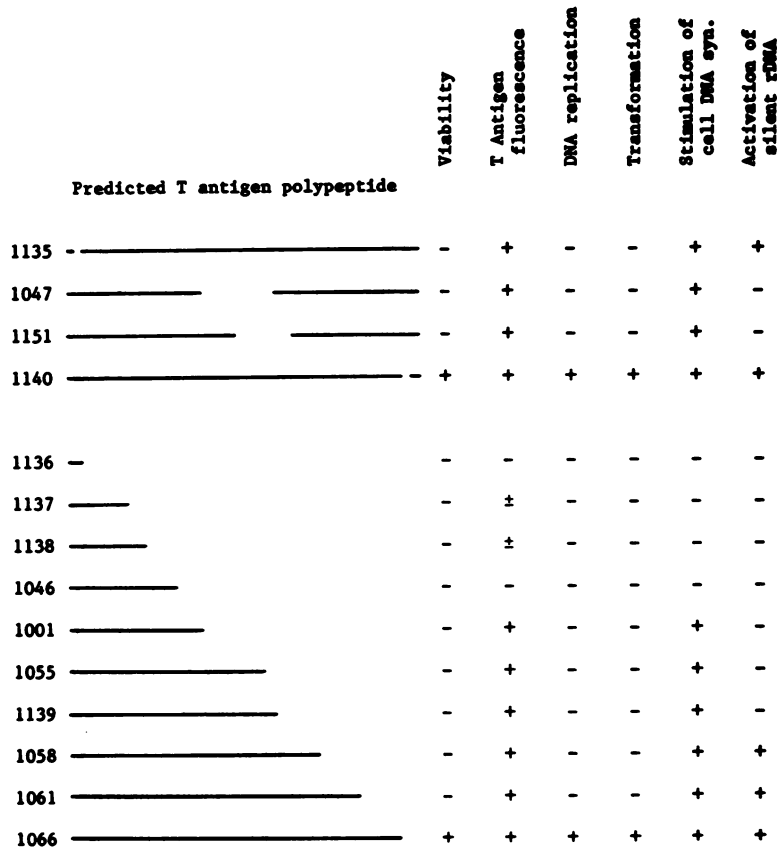

FIG. 1. Summary of the findings of the biological functions of SV40 deletion mutants presented in this and the accompanying paper (26).

(*dl*1151) were inactive, as were out-of-phase deletions that left fewer than 420 translatable codons of the T-antigen gene (*dl*1001, *dl*1055, and *dl*1139). Out-of-phase mutants which retained more than 509 translatable codons (*dl*1058, *dl*1061, and *dl*1066) were active. Of particular note is the observations that *dl*1151 DNA stimulates DNA synthesis, but does not activate rRNA genes.

DISCUSSION

An underlying assumption in the interpretation of our results with T-antigen mutants is that the altered activities of mutant DNAs are due to changes in the T-antigen protein. This assumption seems warranted, since it has been shown that the effects of microinjected SV40 DNA on cellular DNA synthesis are specifically inhibited by coinjected anti-T serum (8). A second assumption is that in those instances in which T antigen is readily detectable by immunofluorescence, the polypeptide predicted from the DNA sequence is present in the cell. In some instances this has been determined directly by immunoprecipitation and electrophoresis of the antigenic protein (25). However, with those mutant DNAs that produce little or no detectable immunofluorescence, we cannot distinguish between the presence of a nonreactive or poorly reactive polypeptide and unstable or very low levels of polypeptide. A third assumption is that the defectiveness of mutants with sequence changes distal to the small-t-antigen coding sequence is not due to changes in the rate of synthesis of the small t antigen. With these assumptions in mind, we will go on to describe our results and those of Pipas et al. (26) in terms of the predicted structures of mutant T antigens, as summarized in Fig. 1.

We discuss first the results presented in this paper and compare them with related experiments reported previously. Stimulation of cellular DNA synthesis is found with all T-antigen polypeptides extending from the amino terminus to amino acid 272 (*dl*1001) or further. T antigens shorter than 160 amino acids (*dl*1138) were inactive. (We ignore possible effects of extra carboxyl-terminal amino acids due to a shift in the

reading frame.) Therefore, the transition between inactive and active T antigen for cellular DNA synthesis is between amino acid 160 and 272; furthermore, amino acid residues 17 to 27 (*dl*1135) are not required. These results extend our prior conclusion (9) that the transition between active and inactive T antigen is between amino acid 160 and 272. Since it is not possible to separate T positivity from stimulation of cellular DNA synthesis, one could argue that the defect in cellular DNA activation may be due not to a lack of certain sequences but to defects in protein stability or its transport to the nucleus. For this reason, we suggest that one should look at these results as defining sequences that are not required rather than sequences that are necessary.

In regard to the activation of silent rRNA genes in 55-54 hybrid cells, T-antigen polypeptides extending from the amino terminus to amino acid 509 (*dl*1058) or farther are all active, whereas those shorter than 420 amino acids (*dl*1139) are inactive. Moreover, amino acids 17 to 27 (*dl*1135) are not required. The results with in-phase deletion mutants *dl*1047 are consistent with those conclusions, as are previous results with SV40 DNA fragments that had indicated a transition between active and inactive fragments between nucleotides 3736 and 3509 (9, 33). Since several mutants stimulate cellular DNA synthesis without activating rRNA genes, these two activities of T antigen are distinguishable, a conclusion reached earlier by Galanti et al. (9). Either these activities reflect different functions of the protein or activation of rRNA genes requires an additional activity. Reactivation of rRNA genes, as previously reported (9, 32–34), was an all-or-none phenomenon. A detectable peak of mouse 28S rRNA was either clearly present (+ in Table 2) or totally absent (− in Table 2).

If we compare the T-antigen structure required for the above cellular effects with that required for viral DNA replication (Fig. 1 and reference 26), it is clear, as concluded previously (26), that viral DNA replication is much more sensitive to a loss of amino acid sequences at the carboxyl terminus (e.g., *dl*1061 and *dl*1058) and within the body of the protein (*dl*1135 and *dl*1151). Stimulation of cellular DNA synthesis by T antigen therefore is probably not simply analogous to the initiation of viral DNA replication by T antigen. Similarly, by comparing the requirements for transformation and stimulation of cellular DNA synthesis or activation of rRNA genes, it is clear that the latter activities are insufficient for cell transformation. Whether they are at all related to the cell transforming function is still unknown. Finally, the concordance of viral DNA replication and transformation activities noted in Fig. 1 need not indicate that these activities are functionally related. The recent isolation of DNA-negative, transformation-positive mutants of SV40 (Stringer [35], Y. Gluzman [personal communication], and S. Pearson-White and D. Nathans [unpublished data]) indicates that these functions of T antigen are actually distinguishable also.

We are thus left with the notion, originally suggested by Weil et al. (42), that T antigen is a multifunctional protein. At the biological level, the study of mutants has identified the following distinct activities: viral DNA replication, cellular DNA synthesis, activation of rRNA genes, cell transformation, helper function for adenoviruses, and possibly autoregulation. It remains to be seen whether there is any commonality among these activities at the biochemical level.

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