A Deletion in the Simian Virus 40 Large T Antigen Impairs Lytic Replication in Monkey Cells In Vivo but Enhances DNA Replication In Vitro: New Complementation Function of T Antigen

CATHARINA MAULBECKER,^{1,2†} IAN MOHR,^{3‡} YAKOV GLUZMAN,^{3§}
JAMES BARTHOLOMEW,¹ AND MICHAEL BOTCHAN^{2*}

Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory,¹ and Department of Molecular and Cellular Biology,² University of California, Berkeley, California 94720, and Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724³

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We describe a new complementation function within the simian virus 40 (SV40) A gene. This function is required for viral DNA replication and virus production in vivo but, surprisingly, does not affect any of the intrinsic enzymatic functions of T antigen directly required for in vitro DNA replication. Other well-characterized SV40 T-antigen mutants, whether expressed stably from integrated genomes or in cotransfection experiments, complement these mutants for in vivo DNA replication and plaque formation. These new SV40 mutants were isolated and cloned from human cells which stably carry the viral DNA. The alteration in the large-T-antigen gene was shown by marker rescue and nucleotide sequence analysis to be a deletion of 322 bp spanning the splice-donor site of the first exon, creating a 14-amino-acid deletion in the large T antigen. The mutant gene was expressed in H293 human cells from an adenovirus vector, and the protein was purified by immunoaffinity chromatography. The mutant protein directs greater levels of DNA replication in vitro than does the wild-type protein. Moreover, the mutant protein reduces the lag time for in vitro DNA synthesis and can be diluted to lower levels than wild-type T antigen and still promote good replication, which is in clear contrast to the in vivo situation. These biochemical features of the protein are independent of the source of the cellular replication factors (i.e., HeLa, H293, COS 7, or CV1 cells) and the cells from which the T antigens were purified. The mutant T antigen does not transform Rat-2 cells. Several different models which might reconcile the differences observed in vivo and in vitro are outlined. We propose that the function of T antigen affected prepares cells for SV40 replication by activation of a limiting cellular replication factor. Furthermore, a link between the induction of a cellular replication factor and transformation by SV40 is discussed.

Simian virus 40 (SV40) was first isolated from cultures of rhesus monkey kidney cells, where it was described as a "nondetectable" passenger. Indeed, it was originally believed that SV40 was an almost ubiquitous contaminant of all such primary or established lines and that no detectable cytopathic effects could be observed (61). The titers of virus produced were at best 2 to 3 orders of magnitude lower in the homologous rhesus system relative to virus production in the African green monkey heterologous system. Furthermore, in another isolation of the virus, it was reported that SV40 could persist for many months after infection of virus-free homologous or heterologous primate cells such as human cells without having any visible effect (27). These early studies indicated that the SV40 lytic replication response can be highly attenuated and by implication may be a regulated process. Little is understood of these attenuation processes, which in principle could be achieved by the production of large numbers of defective "interfering" viral templates or by modulation of limiting viral or host *trans*-acting replication factors. While some evidence in support of the former

possibility is available (46, 57), most speculation about this in vivo attenuation of the natural (often called semipermissive) SV40 replication process is based on the enormous amount of biochemical information concerning the lytic growth of SV40 in the permissive African green monkey kidney cells. These many points for regulation can be incorporated into putative scenarios for the regulation of virus production but give little evidence of the host cells' involvement in the regulation of the viruses' replicative functions that were evident from the early studies.

The key viral replication protein is the large T antigen. It autoregulates its own synthesis and is itself translated from different mRNAs which have intrinsically different translation efficiencies (3, 31). The rate or amount of synthesis of this site-specific DNA-binding protein, which is involved in the unwinding of the DNA at the viral origin of DNA replication (30), could be part of the modulation processes referred to above. Phosphorylation and dephosphorylation of large T antigen by cellular enzymes may be pivotal control points for regulation (43b). T antigen produced in *Escherichia coli* cells is weak in certain assays in its abilities to bind to the SV40 origin of DNA replication and to aid in initiating DNA replication in vitro (43a). After specific phosphorylation at threonine 124 by cellular maturation-promoting factor kinase activity, the T-antigen protein is efficient at DNA binding and shows robust activity in initiating DNA replication (42). Moreover, it has been shown that certain phosphorylation sites must negatively regulate the activity of T antigen (43b), as the cellular protein called

* Corresponding author.

† Present address: Department of Molecular Cell Biology, Max-Planck Institute for Biophysical Chemistry, 3400 Göttingen, Germany.

‡ Present address: Department of Molecular and Cellular Biology, University of California, Berkeley, CA 94720.

§ Present address: Lederle Laboratories, American Cyanamide Co., Pearl River, NY 10965.

replication factor C required in the early or lag period steps of *in vitro* SV40 DNA synthesis is a phosphatase (63). These findings suggest that *in vivo*, persistent SV40 infection relies on a virus-cell interaction more in tune within a normal cell cycle control than would necessarily be an *a priori* extrapolation from the rather immediate response well characterized in the lytic cycle.

Modulation of host cell factors by SV40 infection, the involvement of these factors in the control of viral DNA synthesis, and the link between these functions and cellular transformation form an area of intense interest. For example, the well-known and well-studied role of the virus in oncogenic transformation may be a direct consequence of the ability of the large T and small t antigens to prepare a resting cell for S phase by inducing cellular factors necessary for cellular DNA replication. These same cellular factors probably participate in viral DNA replication, as it is only in S phase that the viral DNA replicates efficiently (6, 51). Here, it may be interesting to point out that the large T antigen can bind to at least five cellular proteins. Among the cellular proteins that interact with the T antigen are the retinoblastoma (Rb) gene product (7), p118/120 (10), and the p53 protein (34). There is evidence that these three cellular proteins play some role in repressing cellular progression into the S phase (8), which in the case of the Rb protein was suggested to be mediated by differential phosphorylation throughout the cell cycle (2, 4, 8, 64). Thus, an interesting antagonism between the T antigen's innate ability to stimulate synthesis of DNA and these cellular protein's abilities to indirectly, or directly, in the case of p53 (14, 60, 65), inhibit DNA synthesis may play a further role in attenuating viral DNA synthesis. However, to date, mutations in T antigen which reduce or abolish Rb or p53 interactions have no measured effects on *in vivo* virus production (28, 34, 49, 69). It is, however, entirely possible that these functions are redundant for high-level viral replication in cell culture and that double mutants in the "Rb pocket" region of T and the p53-binding site would have a phenotype for virus production. Alternately, such mutants may become lytic in infections of rhesus cells.

In addition to the ability of T antigen to modulate the activity of cellular factors by direct binding to cellular proteins, both T antigens are known to activate the transcription of cellular genes by *trans* activation (29, 37, 44, 69). This *trans* activation may enhance or be necessary for transformation and DNA replication. A key focus, then, for the present studies of SV40 DNA replication is what cellular factors involved in viral replication are indeed modulated during the cell cycle and which of these activities are in turn affected by the well known *trans*-activation functions of large T antigen.

From the above perspective, we have been interested in the SV40 genomes carried by the human cell lines GM637 and XP12ROSV. Unlike most postcrisis SV40-transformed human cell lines, which contain replication-defective integrated SV40 genomes, both of these lines have been reported to carry persistent multicopy viral plasmids (33, 41, 52) capable of replication in human cells. These established SV40 cell lines originate from a viral infection of the host cells. Because the free viral DNA replicates extrachromosomally (41), the viral replicon *per se* must indeed be active. We had presupposed that if these cells remain permissive for viral DNA replication, either interesting new mutations in the viral replication system had occurred or the cells themselves had established some potent way of attenuating viral runaway DNA replication. Studies with both stably trans-

formed simian cells (19, 39) and human cells (16) show that mutations which inactivate the replication functions arise in response to this pressure and lead to defective integrated viral genomes incapable of spontaneous excision. Selection for such mutants is clearly provided by these systems, as viral replication leads to cell death. Some human cell lines maintain viral DNA extrachromosomally in a precrisis state (15, 70), and if such disabling mutations do not arise, the cell lines do not survive (16, 45).

Thus, stably transformed lines such as GM637 and XP12ROSV carrying replication-competent SV40 genomes are interesting material for investigating attenuation of a replication-competent SV40 genome. We have cloned the free SV40 genomes from these human cell lines and found that they retain a limited replication efficiency. They are indeed mutant forms of the virus with dramatically reduced DNA replication effectiveness when introduced into the normally permissive monkey cells. Moreover, persistent plasmid DNA can be detected in transfected CV1 cells, though lysates of these cultures do not produce virus stock with a measurable titer. By a variety of genetic rescue and inactivation studies, we show that the defect for replication in monkey cells lies within the first exon of the A gene. Unexpectedly, the mutant T-antigen protein shows activity better than that of wild type for replicating SV40 DNA in cell-free *in vitro* assays. Therefore, this function of T antigen does not seem to involve roles of the protein normally addressed by present *in vitro* DNA replication assays, and we propose that the alleles of the gene define an inductive function of T antigen which can elevate or mobilize a basal cellular activity needed for DNA replication. As the mutant genomes are defective for transformation, it seems likely that this function links an important (and unknown) function necessary for both activities.

MATERIALS AND METHODS

Cell lines. The GM637-b cell line was obtained from the National Institute of General Medical Sciences (Human Genetic Mutant cell repository). These cells will be referred to here as GM637 cells. GM637 cells were produced by SV40 transformation of human skin fibroblasts and isolated as a clonal cell line (National Institute of General Medical Sciences catalog of human cell lines). XP12ROSV cells were obtained from J. Cleaver; they are a subclone of the cell line called XP12RO (52). The cell lines were derived by SV40 transformation of skin fibroblasts (XP12RO cells) from a patient carrying the autosomal recessive disease xeroderma pigmentosum (47, 52). CV1-P, COS 7, H293, and HeLa cells were obtained from the Cold Spring Harbor Cell Culture Facility. The GM638 cell line has been described previously (16). CV1-H cells were a gift from Melvin DePamphilis. All cells were passaged in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

GMSV40 and XPSV40 DNA refer to the extrachromosomal DNA isolated by Hirt extraction (26) of GM637 and XP12RO cells, respectively. COS 7 cells are SV40-transformed CV1 cells that contain replication-defective SV40 genomes but constitutively produce large amounts of T antigen (17). Human 293 (H293) cells are human embryonic kidney cells transformed by transfection with adenovirus 5 fragments (21). H293 cells express the early adenovirus transforming genes E1A and E1B. HeLa cells are cervical carcinoma cells that express human papillomavirus type 18 transforming proteins E6 and E7 (55).

Plasmids and constructs. The recombinant plasmids

pGMSV40 and pXPSV40 were constructed by cloning the extrachromosomal SV40 genomes isolated and gel purified from the Hirt supernatant of GM637 and XP12ROSV cells, respectively, into pUC19 (obtained from New England BioLabs) at the *Bam*HI site. pJYM is wild-type SV40 cloned into pML1 at the *Bam*HI sites (38). To create adenovirus-SV40 hybrid virus, a modified adenovirus vector was used (16a). pAC4C+pTGX1 is an adenovirus 5 derivative that lacks E1A and E1B coding regions (18, 59) but contains the T antigen under the control of the adenovirus major late promoter. To replace the wild-type T-antigen sequence with the GMSV40 T-antigen sequence, GMSV40 DNA was cleaved with *Stu*I, *Bam*HI, and *Nci*I. The DNA sequence extending from SV40 positions 5190 to 2534 was inserted into the vector. The vector was first hydrolyzed with *Hind*III, and then the ends were filled in with Klenow polymerase. Then the vector was cleaved with *Bam*HI, and the mutant fragment was ligated into the vector. For the in vitro replication studies, a wild-type SV40 plasmid called pwtSV40 (which contains the entire SV40 genome) and a mutant derivative (p8-4) of pwtSV40 (which contains a defect in the origin of replication) were used as templates (59).

Transfections. For DNA transfections, we used the calcium phosphate coprecipitation method (22) with modifications suggested by Weber and Schaffner (66). Cells were transferred 1 day prior to transfection to give a monolayer of cells 80% confluent on the day they were transfected. Plasmid DNA was digested to release and linearize the SV40 insert prior to transfection of the unligated DNA. In cotransfection experiments, 2 μ g each of the DNAs of interest was used, while in experiments to produce infectious virus (e.g., Table 1), 1 μ g was used. The DNA precipitate was exposed to the cells for 10 h. The cells were then shocked with 15% glycerol-Dulbecco modified Eagle medium for 1 to 3 min depending on the cell line used, rinsed with phosphate-buffered saline solution, and incubated with fresh medium. At 12 h postshock, the cells were split if necessary. The first time point is calculated from the time elapsed after the glycerol shock.

Extrachromosomal DNA was isolated from the Hirt supernatant and purified by RNase and proteinase digests and repeated phenol extractions following standard procedures. One quarter of the DNA was digested overnight with *Mbo*I (New England BioLabs) and analyzed by Southern analysis of 1% agarose gels using nick-translated 32 P-labeled SV40 DNA as a probe.

In transformation assays, Rat-2 cells (5×10^5 cells) were transfected with 1 μ g of SV40 DNA and stained with methylene blue for focus formation at 3 weeks posttransfection.

Purification of GMSV40 and wild-type SV40 T antigens. The T-antigen expression plasmid was cotransfected with a fragment of adenovirus DNA containing sequences from map units 4 to 100 into H293 cells by calcium phosphate coprecipitation. Virus plaques were picked from the H293 cell infected medium, and the lysate was assayed for T-antigen protein by radioimmunoassay with PAb 405 anti-T antibody (24, 25) and for SV40 DNA by dot blot hybridization with labeled SV40 DNA. The H293 lysate, which contained either Ad5SVRII2 (wild-type T-antigen recombinant) or Ad5SVRII2/GM (mutant T-antigen recombinant), was used to infect H293 cells for protein expression. The mutant virus stock was plaque purified on H293 cells. To extract T antigen from HeLa Spinner cells, the cells were coinfecting with the wild-type adenovirus helper and the

defective virus stock. In a control experiment, T antigen was expressed by transfection of pGMSV40 and pwtSV40 into CV1 cells. T-antigen localization and production of the mutant and wild-type proteins were thus measured side by side. The protein was purified by immunoaffinity chromatography as described previously (56, 59). Protein concentrations were measured by the Bradford assay (1a) with bovine serum albumin as a standard.

Preparation of in vitro replication extracts. Cytoplasmic replication extracts were prepared from HeLa and H293 cells by suspension in hypotonic buffer and Dounce homogenization (59). CV1-H cell replication extracts were prepared as described by Decker et al. (9) with the following modifications. Cells were preincubated for 5 min at 4°C in hypotonic buffer and then scraped free with a rubber policeman and lysed in a Dounce homogenizer with 15 strokes of pestle B. The hypotonic buffer contained acetate instead of chloride salts (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5], 5 mM potassium acetate, 1.5 mM magnesium acetate, 0.5 mM dithiothreitol). The cells were left on ice to incubate for 45 min and then centrifuged in a Sorvall HB4 rotor at 10,000 rpm for 10 min. The protein concentration (5 to 8 mg/ml) was determined as described above. The supernatant, which contains the cytoplasmic extract, was frozen on dry ice-ethanol and stored in liquid nitrogen. Prior to use in the in vitro replication reactions, the extracts were spun for 10 min at 12,000 rpm, and the supernatant was used for the replication assay.

In vitro replication reactions. Reactions were performed essentially as described by Stillman and Gluzman (59). Replication reaction mixture volumes of 50 μ l contained 40 mM HEPES (pH 7.5); 8 mM $MgCl_2$; 0.5 mM dithiothreitol; 100 μ M each dCTP, dGTP, and dTTP; 2.5 μ M dATP; 25 μ M [α - 32 P]dATP (1,000 cpm/pmol); 200 μ M CTP, GTP, and UTP; 40 mM creatine phosphate; and 1 μ g of creatine phosphokinase (Sigma) as an ATP-generating system. Typical reaction mixtures contained 300 ng of template DNA (pSV origin plus and pSV origin minus), 1 μ g of T antigen, and 150 μ g of cytoplasmic extracts. pSV origin-minus plasmid was used to measure the amount of repair synthesis and to correct the number of picomoles obtained from pSV origin-plus replication to obtain absolute replication values. The reaction mixtures were incubated at 37°C for various amounts of time, and reactions were terminated by the addition of EDTA to 10 mM and carrier DNA (50 μ g). The DNA was precipitated by the addition of ice-cold 8% trichloroacetic acid–1% sodium pyrophosphate onto Whatman GF-C glass fiber filters. The specific activity of the newly synthesized DNA was determined by scintillation counting and corrected for the amount of repair synthesis obtained with pSV origin-minus plasmid.

RESULTS

Replication properties of plasmid DNA. The free plasmid DNAs persisting in the human cell lines GM637 and XP12ROSV are homogeneous but different from each other. This was first indicated by the electrophoretic behavior of the DNAs in Southern blot experiments, in which only single species of supercoiled DNAs could be detected. We noticed that restriction fragments spanning the A gene were smaller than those of wild type. The *Hind*III-B fragment in particular was diagnostic for a deletion of approximately 320 bp. The *Hind*III-B fragments of both the GM637 and XP12ROSV genomes were distinct from each other in that the GM637 *Hind*III fragment had a mobility corresponding to that of an

TABLE 1. Plaque assays on CV1 cells^a

SV40 construct	No. of plaques/dish ^b
pA	0
pB	11,400
pC	0
pD	11,000
pGMSV40	0
pXPSV40	0
pwtSV40	12,000
pJYM	21,000
Medium from untransfected cells	0

^a Indicated plasmid DNAs (1 μg each) were hydrolyzed with *Bam*HI to release the SV40 DNA from the plasmid vector and transfected into CV1 cells as indicated in Materials and Methods. At 120 h posttransfection, the plates were harvested and a freeze-thaw lysate of virus stock was obtained. Plaque assays were performed on CV1 cells. In the case of pwtSV40, pJYM, pB, and pD constructs, a 100-fold dilution of the medium from the 120-h time point of the transient replication assay (Fig. 1) was used to infect CV1 cells. The number of plaques obtained was corrected for the 100-fold dilution. pGMSV40, pXPSV40, pA, and pC media were used undiluted.

^b From transfected cells.

846-bp fragment, while the XP12ROSV fragment electrophoresed as an 864-bp fragment. The wild-type fragment is 1,169 bp. The homogeneity of the Hirt-extracted plasmid DNAs persisted over continued cell passages and was confirmed by sequence analysis across the end points of the *Hind*III-B fragment. To analyze SV40 plasmid DNAs in the human cell lines GM637 and XP12ROSV, we cloned the DNAs extracted from Hirt supernatants after hydrolysis with *Bam*HI. We cloned the plasmid DNAs from independent Hirt extracts, and these results substantiated the homogeneity of the extrachromosomal DNA. We refer to these DNAs as GMSV40 from GM637 cells and XPSV40 from XP12ROSV cells.

To characterize the biological potential of these cloned genomes, we asked if the cloned DNAs could support plaque production in CV1 cells. The cloned viral DNAs were released from the plasmids and transfected into CV1 cells in an attempt to produce infectious stocks. Lysates from these cultures were then assayed for infectious virus by plaque assay (Table 1). While neither plasmid would form infectious virus as measured by plaque assay on CV1 cells, we found that the plasmids could establish themselves as a latent episome without apparent cell lysis for at least 10 serial passages of the transfected cultures (Fig. 1B [CVGM]). For this later experiment, we harvested the transfected CV1 cells and split them 1:3 each week for 10 weeks. Again, infectious virus were not detected in the medium from these cultures after this medium was used in plaque assays on CV1 cells.

We assayed the replication potential of the cloned GMSV40 and XPSV40 DNA in CV1 cells by using a transient replication assay. The input unreplicated DNA was distinguished from replicated DNA by digestion with the methylation-sensitive restriction enzyme *Mbo*I. The plasmid DNA replicated in the *E. coli* host cannot be hydrolyzed by *Mbo*I because of specific methylation. After replication in eucaryotic cells, the methylation pattern is altered and the DNA becomes susceptible to *Mbo*I digestion. Replication was monitored by the appearance of *Mbo*I restriction fragments on Southern blots that were probed with nick-translated SV40 DNA.

The autoradiograph in Fig. 1A shows transfections into CV1 cells of cloned wild-type SV40, SV80, and GMSV40 DNAs. pSV80 is a cloned DNA coding in part for a T antigen defective in origin binding. The clone was originally con-

FIG. 1. Transient replication assays in CV1 cells of GMSV40 and wild-type SV40 genomes and a cotransfection with the replication-incompetent SV80 genome. (A) Kinetics of DNA replication when 4 μg each of GMSV40, wild-type SV40, and SV80 form III was transfected into CV1 cells. The DNA isolated from the Hirt supernatants was digested with *Mbo*I. Two of the *Mbo*I cleavage sites present in wild-type SV40 are deleted in GMSV40 such that the two types of DNA can easily be distinguished from each other. The DNA was analyzed by gel electrophoresis on 1% agarose gels and Southern blot hybridization to ³²P-labeled SV40 DNA. Input *Mbo*I-insensitive form III DNA is visible in the lanes as the 5,243-bp band in the wild-type transfection and the 4,920-bp band in the GMSV40 transfection. Replicated SV40 DNA is cleaved by *Mbo*I. (B) Data for complementation by SV80 cotransfection. Cotransfections of GMSV40 or wild-type SV40 with SV80 contained 2 μg of each of the SV40 genomes per dish. Lane CVGM shows the Hirt-extracted DNA 10 weeks posttransfection by GMSV40 DNA alone.

structed from the integrated SV40 sequences rescued from the human cell line SV80 (16). At 12 h posttransfection, there was no SV40 replication in either wild-type SV80 or GMSV40-transfected CV1 cells. At 24 and 36 h, prior to virion formation, both wild type and GMSV40 began to replicate, but GMSV40 replication was significantly reduced compared with that of wild type, despite equal amounts of input DNA. The SV80 DNA fails to replicate as previously reported (16). In multiple experiments of this sort, we found that wild-type SV40 was at least 20-fold better than the mutant in its ability to replicate in CV1 cells. Similar deficiencies were found for the XPSV40 mutant genome (see Fig. 8). In all these experiments, it was important to measure

FIG. 2. Construction of hybrid clones from pGMSV40 and pJY. Identical constructs were made with XPSV40 genomes. The results were identical for both pXPSV40 and pGMSV40, and for simplicity, we show only the results for pGMSV40. Both plasmids were digested with *PflmI*, and the two backbone vector fragments were isolated together with the 4,558- to 1,007-bp *PflmI* SV40 fragment from low-melting-point agarose. The *PflmI* fragment of pGMSV40 was inserted into the pJY vector backbone (pC subclone), and the wild-type pJY-*PflmI* fragment was inserted into the pGMSV40 backbone (pD subclone) by cutting the appropriate bands from the gel, mixing them, and ligating them in low-melting-point agarose. pC and pD were distinguishable from their parent plasmid backbone by *PvuII* digests, since the vector in pGMSV40 is pUC whereas the pJY vector is pBR322, while the *PflmI* inserts differ because of the deletion in the A gene and the creation of a new *PvuII* site in the promoter-enhancer region of GMSV40 (68a). The pC and pJY plasmids were digested with *PflmI*, and the isolated *PflmI* fragments were digested with *HindIII*. pA was constructed by ligating the pJY backbone fragment with the 4,558- to 5,172-bp *PflmI*-*HindIII* fragment of pC and the 5,172- to 1,007-bp *HindIII*-*PflmI* fragment of pJY. pB was constructed in a similar manner, except that the source of the *PflmI*-*HindIII* fragment was pJY, while the *HindIII*-*PflmI* fragment came from pC. *PvuII* digestion was used to confirm the identity of the fragments. The GMSV40 sequences in the figure are represented as thick lines, and wild-type sequences are represented by thinner lines. Numbering is with respect to the SV40 genome. The *Bam*HI sites at which wild-type and GMSV40 were cloned into pUC (pGMSV40) and pBR322 (pwtSV40) are indicated. The restriction enzymes indicated in the figure simply denote the borders of fragments used in the swaps. The autoradiogram shows the transient replication assays of subclones of pGMSV40 and pwtSV40 in CV1 cells. pA, pB, pC, pD, pGMSV40, pXPSV40, pJY, and pwtSV40 were digested with *Bam*HI to release the SV40 insert and transfected by calcium phosphate coprecipitation in CV1 cells. SV40 genomes were transfected (2 μ g/60-mm-diameter dish), and the replicated DNA was analyzed by *Mbo*I digestion.

replication rates prior to virion assembly and reinfection. Therefore, we emphasize the comparisons between mutants and wild type at the 24- and 36-h points. By 120 h, reinfection and a second round of replication could be detected for the wild type (Fig. 1; see Fig. 8). It is clear from Fig. 1 (see also Fig. 8) that the GM mutant is defective for replication, but the data do not preclude a secondary defect in virion formation. Capsid protein can be detected in CVGM lines, and virions are produced (40). However, these virions are not infectious at low multiplicity, presumably because of the T-antigen defect characterized below. We note that lack of titratable infectious virus can be the result of a replication-defective T antigen or a role for T antigen in virion assembly. The data presented here indicate that the DNA is defective for replication.

Mapping the lesion for DNA replication in CV1 cells. To genetically map the lesions in the cloned DNAs, a variety of recombinant SV40 molecules were made. The experiments whose results are given in Fig. 2 and Table 1 map the lesions for DNA replication and production of infectious virus to the same region, which spans nucleotides 4558 to 5172 of SV40. We note that the replication assay here is sensitive to virion formation, as the time points are extended compared with those in Fig. 1. This result substantiates the plaque assay data of Table 1. Interestingly, both the GMSV40 and XPSV40 DNAs had disabling mutations in this region (data not shown). Thus, when a *PflmI*-*HindIII* restriction fragment which spans nucleotides 4558 to 5172 was placed into the wild-type SV40 genome (construct pA), 20-fold-reduced

replication levels and no plaque formation were detected. Moreover, when the wild-type fragments spanning this region were inserted into the mutant backbone, wild-type replication was detected (construct pD), as was plaque formation (Table 1).

In order to identify the precise nature of the mutations, the deleted forms of the mutant *HindIII*-B fragment were subcloned into M13mp19 vectors, and the SV40 sequences were determined. This study showed that nucleotides, including nucleotides 4965 to 4644, are deleted in both GMSV40 and XPSV40. Thus, both DNAs contain a deletion of 322 nucleotides that deletes part of the coding sequences for large T antigen and all the sequences that are unique for small t antigen (Fig. 3). The deletion destroys the large-T-antigen splice donor but stops short of the small-t-antigen donor site. The deletion also places the small-t-antigen stop codon out of frame with respect to the coding sequence, and thus, a modified large T antigen can be produced. New mRNA species predicted by the deletion have indeed been verified by S1 analysis (data not shown). The sequence shows that small t antigen cannot be produced and that large T antigen contains a deletion of 16 amino acids (residues 67 to 82) from the region encoded by the end of the first exon and an addition of 2 new amino acids (Tyr-Lys) from sequences that would normally be in the large-T-antigen intron. The net effect is a reduction of 14 amino acids in the large T antigen. Interestingly, the nucleotides of the large-T-antigen gene that are deleted at the 5' border of the deletion would code for a Lys-Tyr sequence, and they are replaced by nucleo-

FIG. 3. Genotypes of GMSV40 and XPSV40 in comparison with wild-type SV40 small- and large-T-antigen-coding sequences. The map shows the early region of SV40 with the nucleotide numbers referring to positions in the wild-type genome. Letters inside boxes refer to amino acids and indicate both the amino acids lost from the wild type by the deletion in GMSV40 and XPSV40 and the amino acid changes at the C-terminal end of the first exon. The nucleotides between nucleotides 4966 and 4643 are deleted, and two codons from the C-terminal end of the small-t-antigen region just before its donor site are placed in frame with the large-T-antigen-coding sequences in both genomes. The deletion of 322 bp, the point mutation at 4,642 bp that changes an A to a C, and the 18-bp duplication at positions 4461 to 4479 in XPSV40 are indicated. The exons and introns of the wild-type small and large T antigens are given for comparison, and exon 2 domains discussed in the text are indicated. IVS, intervening sequence.

tides which encode for the amino acids Tyr-Lys in the mutant protein. This tyrosine codon is constructed by a cytosine-to-adenosine transversion, as shown in Fig. 3. Although the XPSV40 genome carries the same deletion as the GMSV40 genome, there is a duplication of 6 amino acid codons within the second exon, resulting in a net reduction of 8 amino acids for the XPSV40 T antigen relative to that of wild type. This duplication does not appear to play a role in the mutant phenotype, as the marker rescue fragment which defined the phenotype was the *Pf*mI-to-*Hind*III fragment (nucleotides 4558 to 5172) stopping just 5' of the duplication.

To corroborate the predictions of the sequence data, we transfected the cloned GMSV40 DNA into CV1 cells in side-by-side experiments with wild-type SV40 and analyzed the T antigen produced by both alleles. Both the wild-type and mutant T antigens accumulated in the nucleus, and no differences in T-antigen levels could be detected by immunofluorescence. However, as shown in Fig. 4, the mutant T antigen which was purified by immunoaffinity chromatography from CV1 cells (see Materials and Methods) was smaller than the wild-type protein by about 2,000 Da. Furthermore, immunoprecipitation using PAb 108 antibody, which is directed at the amino-terminal end of T antigen (23), showed that neither GMSV40 nor XPSV40 produces small t antigen (data not shown).

Small t antigen is known to be a transactivator of gene expression (37). Given that the deletions in the GMSV40 and

XPSV40 DNAs eliminate the production of small t antigen, we were interested to know if expression of small t antigen *in trans* could rescue wild-type replication of the two DNAs. Cotransfection with small-t-antigen expression plasmid pW2-t/cDNA (37) did not rescue or increase the replication activity of the mutants. In these experiments, small-t-antigen expression was positive when monitored with PAb 108 in parallel transfections in which only pW2-t/cDNA was used (data not shown). We conclude that the primary defect of these SV40 genomes lies in the large T antigen and not in a lack of small-t-antigen production.

In vitro DNA replication directed by the GMSV40 T antigen. Several studies have shown that mutations in the first exon of large T antigen result in markedly reduced replication rates for the mutant genomes (49, 53, 58). The only known function encoded in the first exon related to DNA replication is a binding domain for α -polymerase, and it is not known whether this interaction is critical for replication (30a). As all of the known biochemical activities of the protein in SV40 DNA replication (e.g., ATP hydrolysis, helicase activity, and site-specific DNA binding) map to the second exon, we were interested to explore the *in vitro* properties of the mutant T antigen. To this end, we created a defective adenovirus-SV40 hybrid virus which could produce the mutant GMSV40 allele of large T antigen in human cells (see Materials and Methods). *In vivo* labeling studies

FIG. 4. GMSV40 T antigen (lane GM) and wild-type T antigen (lane wt) isolated from monkey cells. Each lane on the 5% sodium dodecyl sulfate-polyacrylamide gel, which was stained with Coomassie blue, contains 1 μ g of T antigen that was produced through transfection of CV1-H cells. The wild-type-T-antigen protein migrates slightly faster than the 97-kDa molecular size marker, in agreement with a predicted size of 96 kDa. GMSV40 runs at about 94 kDa, presumably because of the deletion of amino acids 67 to 82 (2 new amino acids are added also, which results in a net loss of 14 amino acids in GMSV40 T antigen). The molecular sizes of the commercial Bio-Rad markers (lanes MW) are given in kilodaltons at the right.

with H293 cells infected with the mutant and wild-type hybrid viruses showed no difference in apparent stability between the mutant and wild-type T antigens. As the apparent mutation mapped for both the GMSV40 and the XPSV40 genomes were identical, we chose to focus on the GMSV40 T antigen.

The ability of GMSV40 and wild-type SV40 T antigens to direct DNA synthesis in H293 cytoplasmic extracts was examined (Fig. 5). In this experiment, we determined the kinetics of SV40 DNA replication at different concentrations of T antigen. As seen from this data, 0.25 μ g of T antigen represents a suboptimal protein concentration for stimulating in vitro replication by wild-type T antigen. The rate of GMSV40 T-antigen-directed in vitro replication at this concentration is approximately 25-fold greater than wild-type-T-antigen-directed replication. Increasing the amount of T antigen to 0.5 μ g per reaction enabled wild-type T antigen to support replication. However, GMSV40 T antigen still supported replication fivefold better than wild-type T antigen after 60 min of incubation. At optimal wild-type-T-antigen concentrations of 1 μ g, the difference in replication rates between GMSV40 T antigen- and wild-type-T-antigen-directed DNA synthesis was only 2.5-fold at 60 min. GMSV40 T antigen purified from HeLa cells thus is up to 25-fold more

FIG. 5. T-antigen concentration-dependent in vitro DNA replication. The numbers of picomoles used for this graph were corrected for the amount of repair synthesis as described in Materials and Methods. Values below 1 pmol are lower than the value for repair synthesis and represent background. Symbols: $\square$, GMSV40-directed replication; $\blacksquare$, wild-type SV40-directed replication.

efficient in directing SV40 DNA replication in vitro at low T-antigen concentrations.

A lag of 15 to 25 min prior to the onset of DNA synthesis was observed with 1 μ g of wild-type T antigen per reaction

FIG. 6. In vitro replication in CV1, COS 7, H293, and HeLa cell cytoplasmic extracts supported by either GMSV40 or wild-type SV40 T antigens. Reaction mixes contained 200 μ g of cytoplasmic extracts, 300 ng of DNA template, and 1, 2, or 3 μ g of T antigen and were incubated for 2 h at 37°C. The picomol values for replication were corrected for repair synthesis. The open squares represent GMSV40 T-antigen-directed in vitro replication, and the solid squares represent wild-type-T-antigen-directed replication.

(Fig. 5), a result in agreement with those of published reports (59, 67, 68). Only after 30 min at any concentration does wild-type T antigen promote measurable amounts of DNA synthesis. In contrast, the lag period in DNA replication observed for synthesis initiated by GMSV40 T antigen was considerably shortened (Fig. 5). Thus, in vitro, GMSV40 T antigen seems to be more efficient than wild-type T antigen for DNA replication when compared on the basis of concentration dependence and effect on presynthesis lag time.

These results were surprising, given the striking defects of GMSV40 DNA in vivo in monkey cells. To test whether the replication defects might be specific to simian cells, we prepared replication extracts from a variety of cells: CV1 and COS 7 (simian cells) and H293 and HeLa (human cells). In vitro replication was measured at different T-antigen concentrations, and the results obtained with the different cellular replication extracts are shown in Fig. 6. Standard protocols suggest that optimal replication rates in monkey cells require 2 to 4 μ g of T antigen per reaction (35). Human cells reach levels of optimal DNA synthesis at 0.5 to 1 μ g of T antigen per reaction (59, 67). The monkey cell extracts CV1 and COS 7 contained 0.5, 1, 2, and 3 μ g of T antigen. The human cell extracts contained 0.25, 0.5, 1, and 2 μ g of T antigen per reaction. The difference in replication efficiencies between GMSV40 and wild-type SV40 T antigens is greatest at suboptimal protein concentrations. We conclude that at all T-antigen concentrations employed in both monkey and human cell extracts, the GMSV40 T antigen was more active in promoting in vitro DNA replication than the wild-type T antigen, although the differences were greatest with the simian extracts (Fig. 7).

Although the cell extracts used in the above experiment were prepared from different cell types, the T antigens were prepared only from human cells. A possible way to explain the behavior of the mutant GMSV40 T antigen in vivo is to invoke specific modifications of the T antigen when it is produced in a specific host cell. Thus, modifications found specifically in CV1 cells might cripple the GMSV40 T antigen. Alternatively, specific modifications occurring in the infected HeLa cells might activate the T antigen, which would otherwise be mutant if translated and produced in simian cells. To explore this point, the adenovirus vector was used to infect both human and simian cells, and the T antigens from these sources were purified and assayed as before. As shown in Fig. 7, the T antigen from GMSV40 was still more active than

wild-type T antigen, regardless of the source of the protein. To test the influence of adenovirus-induced T-antigen modifications, we prepared mutant and wild-type T antigens by transfection of the respective pUC cloned genomes into CV1 cells. Again, the mutant T antigen was more effective than the wild type at initiating in vitro replication.

First exon deletion defines a genetically distinct function in the large T antigen. Although the large-T-antigen protein is a multifunctional polypeptide with discrete domain activities, intracistronic complementation by different mutants for functions required for DNA replication has not been reported. These results may indicate that although the active T-antigen complex at the replication origin functions as an oligomer, most activities (e.g., ATP hydrolysis, ATP binding, helicase activity, and DNA binding) must be present on a single polypeptide chain. In fact, some mutations in the second exon of the T antigen give a *trans*-dominant negative effect, down modulating replication promoted by wild-type T antigen (11). There was a hint that this might not occur with the new mutants characterized here. We reasoned that the replication effect defined by the GMSV40 and XPSV40 genomes might affect an indirect function of T antigen required in vivo. This was suggested by the unique position of the deletion in the first exon of the large-T-antigen gene. For example, it is conceivable that a unique or specific function of the first exon of the large-T-antigen gene is carried by those amino acids which are shared by the small-t-antigen gene, which is not directly involved in the mechanics of DNA replication. This notion would then be compatible with the existence of a mutant large-T-antigen protein that is indeed more active than the wild-type protein with respect to all direct functions required for DNA replication. We were thus interested in exploring the question of separate activities within the T-antigen gene, as other indirect replication functions of T antigen could be supplied by separate polypeptides incapable of direct replication functions. A precedence for multiple functional complementation groups in the A gene was established by Tornow et al. (62). However, the function they mapped was at the carboxyl terminus of the protein, is important for capsid protein synthesis, and thus affects a postreplication function. To determine whether the mutants described here define a different activity, two different assays were done.

First, we transfected the GMSV40 and XPSV40 genomes into SV40-transformed permissive cells harboring well-de-

FIG. 7. Comparison of DNA replication in H293 and CV1 cell extracts promoted by either GMSV40 (GM T) or wild-type T (wt T) antigens produced in HeLa or CV1 cells. (A and C) DNA replication in H293 cell extracts. Graph A compares replication with GMSV40 and wild-type SV40 T antigens produced in HeLa cells using H293 extracts *in vitro*. Graph C shows replication in H293 extracts promoted by both T-antigen proteins produced in CV1 cells. (B and D) replication rates of the HeLa cell-produced T antigens in CV1 extracts (B) and the CV1 cell-produced T antigens in CV1 cell extracts (D). Open squares represent GMSV40 T antigen-directed replication, and solid squares represent wild-type-T-antigen-directed replication rates.

finer T-antigen mutants. C2 cells are simian cells transformed by a replication-incompetent SV40 genome (39), as are the human cell lines GM638 and SV80 (16). The SV80 line carries a T antigen with a single amino acid substitution in the DNA-binding domain of the protein, while the C2 and GM638 proteins carry single but different changes in the ATP-binding-helicase domains of the protein. These cell lines will support wild-type SV40 replication but will not provide a T-antigen source which can complement exon 2 lesions in DNA replication functions (for example, see Fig. 2 in reference 16). As can be seen in Fig. 8, these cell lines supported the replication of the mutant XPSV40 and GMSV40 genomes to levels at least as great as if not greater than what is found for wild-type SV40. While recombination between circular DNA and the integrated genomes can occur, this recombination is too rare to account for the immediate and wild-type levels of replication detected in these assays. Furthermore, we note that the pattern of amplification detected is solely that of the mutant genomes. For the transfected C2 cells, visible lysis and cell death with

classical SV40-induced cytopathic effect occurred 1 week after transfection. The harvested virus would not form plaques on CV1 cells.

Next, we asked whether cotransfection of the GMSV40 DNA would be complemented by the replication defect in the cloned mutant SV80 allele of T antigen in a direct transfection of CV1 cells. As can be seen in the bottom section of Fig. 1, the SV80 plasmid and the GMSV40 plasmid effectively complement each other. Multiple rounds of reinfection by virions and replication occurred during the course of these cotransfection experiments, and the entire monolayer of CV1 cells lysed at 200 h posttransfection. We cannot effectively compare the relative replication efficiencies of the two genomes, given the resolution of the autoradiogram in Fig. 1B. However, DNA sequencing of the viral origin of both viral DNAs showed no deviations in the core *ori* region and immediately flanking sequences for either genome, although some differences in the 72-bp enhancer were detected (data not shown). These particular results were substantiated in separate experiments wherein the GMSV40

FIG. 8. Transfection of GMSV40, XP, and wild-type SV40 into recipient C2, CV1, GM638, and SV80 cells. GMSV40, XP, or wild-type SV40 DNA (2 μ g) was transfected into C2 or CV1 (monkey) or SV80 or GM638 (human) cells. DNA was collected by Hirt extraction at 12, 24, and 120 h after the glycerol shock. One-fourth of the DNA at each time point was digested with *Mbo*I. C2 transfections of both GMSV40 and wild-type SV40 DNAs resulted in lysis after 120 h of incubation. SV80 cells showed slower cell death, which after 1 week resulted in complete destruction of the monolayer, a response that is more characteristic of crisis in human cells. CV1 cells showed cell lysis and cytopathic effects only with wild-type SV40 DNA.

genomes were cotransfected with the small-t-antigen vector described above. This vector contains an effective SV40 origin of DNA replication and is intact across the region of deletion in the GMSV40 genome. Complementation of these replication defects of GMSV40 genome was not detected, nor did cell lysis occur. In a parallel transfection with the SV80 genome plus the GMSV40 genome, cell lysis occurred as described above. This comparison implies that recombination was not playing a role in these assays.

GMSV40 and XPSV40 genomes are defective for transformation of rodent cells. The results described above were consistent with the hypothesis that the SV40 T antigens encoded by the mutant genomes were defective in functions needed for lytic SV40 DNA replication in an indirect manner or in replication functions not measured by the *in vitro* replication system. We thus looked for known functions of T antigen which might have an indirect impact on *in vivo* replication. From immunoprecipitation data, it was clear that the mutant T antigens bind to the cellular retinoblastoma (p107) protein and to p53, as does the wild-type protein (9a). We also asked if the GMSV40 T antigen could transactivate

TABLE 2. Transformation assay

Source of DNA	No. of foci in exp ^a :			
	1	2	3	4
pSV40	118	72	63	56
pGMSV40	0	0	0	0
pXPSV40	0	4	0	3
pBR322	0	0	ND	ND

^a Foci were counted on a Rat-2 cell monolayer at 3 weeks posttransfection of 5×10^5 cells with 1 μ g of DNA. ND, no datum. Experiments were performed at different times. Recipient cells in experiments 3 and 4 were rapidly proliferating.

the SV40 late promoter. To do this, we asked if pGMSV40 could activate the plasmid pL2N-CAT (29) in a cotransfection of CV1 cells. pL2NCAT contains the chloramphenicol transacetylase gene reporter linked to the SV40 late promoter (with a small deletion at the origin of SV40 replication). In side-by-side assays with the plasmid 6-1 (20) (which provides a wild-type T antigen), no differences in transactivation were detected (data not shown). In these experiments, an internal β -galactosidase reporter plasmid was used to monitor transfection efficiencies. These experiments thus do not provide evidence that these *in vivo* functions of the mutant T antigen are affected. However, as T-antigen activation of the late SV40 promoter is complex, involving multiple pathways, some of which require SV40 DNA binding and others of which do not, this experiment is difficult to interpret, as one pathway could be affected and the other not. The recent work of Zhu et al. (69) is certainly consistent with the concept that multiple *trans*-activating domains of T antigen are required for full function. Those results are thus consistent with the notions that T antigen can stimulate transcription through multiple pathways and that critical cellular promoters may respond differently from the SV40 late promoter to T antigen.

The mutant SV40 genomes rescued from these human cells are clearly defective for transformation of rodent cells. These data are shown in Table 2. In multiple independent parallel assays, the mutant genomes were at least 20- to 100-fold reduced in their abilities to morphologically transform the established Rat-2 fibroblast indicator line. This reduction was observed when the DNAs were transfected into confluent or growing Rat-2 cells. In any case, these particular mutants of T antigen may be more dependent on small-t-antigen functions for transformation than a wild-type large T antigen would be. We would like to know if the mutant T antigens retain their abilities to immortalize or transform other indicator cell lines, particularly because the genomes were rescued from transformed human cells. Nevertheless, the results clearly show that the mutant T antigens have lost some unknown activity that manifests in the pleiotropic SV40 transformation phenotype. These results reinforce a point recently made by Montano et al. (44). Those workers showed that the large T antigen and small t antigen have a common transformation domain, at least one of which is required for SV40 function in transformation. The mutant genomes described here may lack this function.

DISCUSSION

The transforming and replication functions of the SV40 early proteins have been widely studied, yet no clear insights into the relationships between these activities have emerged. Specifically, it has long been assumed that the transforming

functions serve the virus either positively or negatively to regulate its vegetative propagation in permissive environments. Transformation can thus be seen as an abortive infection in a nonpermissive cell. The large T antigen is a complex protein, and it has been clearly shown that at least two distinct transforming functions are encoded in the protein (see reference 58 and the references therein). One of these activities is believed to be manifested as a result of large T antigen's ability to interact with the cellular Rb or p107 proteins. Yet mutations in SV40 large T antigen which cripple the virus for transformation and incapacitate large T antigen's abilities to bind these cellular proteins are apparently wild type for DNA replication and virion production. Large T antigen can also associate with the cellular p53 protein through the auspices of a second large-T-antigen protein domain. As cellular p53 can act as an antioncogene in some assays, an attractive hypothesis would be that this association between large T antigen and p53 suppresses viral replication (60, 65). The deletions present in GMSV40 and XPSV40 may delete a third distinct domain of T antigen required for transformation. However, as pointed out by Montano et al. (44), the first exon of T antigen may influence its wild-type interaction with Rb or p53.

The deletion mutants described in this study are interesting in that they provide support for the notion that an indirect function of large T antigen is required both for lytic replication and for transformation. The deletion of amino acids 67 through 82 of the first exon of the large-T-antigen gene results in a protein that is defective for viral replication *in vivo*, yet the purified protein itself seems to be a very active T antigen for replication *in vitro*. These results argue that the affected function is distinct from the known replication activities of large T antigen. In addition, the deletion mutant can be complemented for replication by other known SV40 A-gene mutants. This is consistent with the putative requirement for the protein to act at some site other than the replication fork. Other SV40 mutants which directly affect replication functions show *trans*-negative effects on coinfection with wild-type DNA. We suggest that the GMSV40 T antigen is actually a highly active T antigen for the replication process itself *in vivo* but is defective in some early step which prepares the cells for replication. The complementing viral genomes (SV80 or C2) are defective for known replication functions (43), and we suggest that they can provide the early pre-DNA synthetic function. Possibly, T antigen functions as a monomer for this early function and is thus not poisoned by mutant proteins. That this deletion inactivates SV40 transformation argues directly that this particular T-antigen allele is defective in its interactions within the cell. Moreover, to date, mutations that disable direct replication functions such as DNA binding or helicase or ATPase activities are wild type for transformation. This last statement is true with the proviso that the mutation does not also affect nuclear localization or stability; the mutant T antigens from GMSV40 and XPSV40 are wild type for both of these aspects.

If the deletion mutants described here encode for a T antigen more active than that of wild type in *in vitro* DNA replication, what could account for its defect for replication *in vivo*? We suggest that the function affected concerns SV40's known ability to *trans* activate gene expression, which could elevate either directly or indirectly the activity of a limiting cellular replication factor. This factor may be sequestered in the cytoplasm and mobilized to a higher nuclear concentration as a result of viral gene action or may be modified to increase its specific activity. For example, Krauss et al. (32) have shown that phosphorylation of DNA

polymerase α increases its catalytic properties. In either case, the *in vitro* DNA replication systems may not be sensitive to these effects, since the cellular extracts are adjusted for optimal activity. Furthermore, it seems reasonable that a *trans*-activation function would be important for viral transformation as well as for DNA replication. An implication of this hypothesis would be that neither Rb binding nor p53 binding is sufficient for transformation without this *trans*-activation function (69) because the GMSV40 mutant is defective for transformation even though it binds Rb and p53 proteins as wild type (data not shown).

It is known that a *trans*-activation function maps to the first exon of large T antigen (69). The small t antigen also encodes for a *trans*-activation function (37), and the sequences deleted in the defective genomes described here overlap the common region shared by large T antigen and small t antigen. For this reason, we asked whether ectopic expression of small t antigen could complement the GMSV40 genome for *in vivo* replication. That it could not suggests that this domain carried by the large T antigen may be influenced by the presence of the exon 2-encoded protein and may be more readily assayed in our cell system. It will be interesting to know whether the small-t-antigen gene can effectively help the deletion mutant to oncogenically transform established rodent cells. We make a simple suggestion to link the *trans*-activation functions of large and small t antigens to the transformation helper effects and a replication requirement for a first exon domain. It remains to be shown where in the first exon these functions reside genetically (69). Linking the known *trans*-activation and transformation functions of the first exon to the replication defect shown by the GM (XP) mutants is of course speculation. One could alternatively explain our data by suggesting that the separate complementation function provided by the first exon entails a process not assayed by *in vitro* replication yet directly involved in replication *in vivo*. For example, T antigen may need to interact with chromatin-associated DNA in a way not mimicked by the *in vitro* system. Or, even though the mutant proteins show apparently normal nuclear localization and *in vivo* stabilities (data not shown), a special nuclear replication compartment which cannot be found by the mutants may be used. We favor our hypothesis mainly because it fits nicely with known activities of T antigen (5, 12, 13). Perhaps our unpublished observations concerning the phosphorylation state of the GM T antigen will provide a key for understanding the mutated functions of T antigen (see below).

Another interesting and perhaps paradoxical finding reported here is that these T-antigen mutants, though deficient *in vivo*, are better than wild type in all of our *in vitro* assays. In fact, the mutant protein shows a phenotype *in vitro* similar to that of a dephosphorylated T antigen. The mutant T antigen shows a shortened lag in its kinetics of initiation and can function at a reduced T-antigen concentration. These properties of an underphosphorylated T antigen (43b, 63) are substantiated by our findings that the adenovirus vector produced GM T-antigen labels less effectively per mass with phosphate than does wild-type T antigen (1). Consistent with these observations, Jim Roberts (Seattle, Wash.) has reported (50) that the purified GMSV40 large T antigen cannot be further activated by replication factor C, which is the catalytic subunit of phosphatase IIa (63). These observations could explain the increased activity *in vitro* but do not readily explain decreased replication *in vivo*. It is tempting to link these observations with the recent discovery that SV40 small t antigen binds to phosphatase IIa and inhibits its activity (54). It is conceivable that with the wild-type T

antigen, the serine-phosphorylated state is necessary for *trans* activation and that the phosphatase IIa converts the protein from one that *trans* activates to one that is active in DNA replication. A deletion of this domain (and of small t antigen entirely) might lead to a protein incapable of *trans* activation but acting as an apparent "super" T antigen in replication. Reciprocally, specific phosphorylation may be required for a *trans*-activation function for which the GMSV40 allele may be defective. A switch between a *trans*-activator T antigen which functions early in the lytic cycle and a replication T antigen which functions later may thus be governed by such cycles of phosphorylation. This hypothesis, which can explain all of the data, can of course be directly tested.

Finally, we come back to a point addressed in the Introduction. The GMSV40-XPSV40 A-gene mutants described here do not completely inactivate the *in vivo* replication potential of the virus. In a sense, then, the deletion described creates an attenuated form of the viral replicon in that the DNA can persist without obvious lytic effects in the transformed cells. This point is consistent with previous findings which show that permissive or semipermissive cells cannot tolerate stable transformation by wild-type SV40. Furthermore, though the deletion cripples the SV40 for transformation in focus assays, it is possible that other oncogenic domains (Rb binding and p53 binding) may contribute to immortalization activities which may have played a role in the initial isolation of the human cells from which they were cloned. We assume that the viral variants preexisted in the wild-type stocks of SV40 used to establish the human cell lines GM637 and XP12ROSV, because both mutants (which result from an apparently illegitimate recombination) harbor precisely the same deletion. In any case, these mutants may provide a fortuitous tool for studying the cellular components involved in the control of transformation and viral replication *in vivo*.

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REFERENCES

1. Bartholomew, J., and M. Botchan. Unpublished data.
- 1a. Bradford, M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
2. Buchkovich, K., L. A. Duffy, and E. Harlow. 1989. The retinoblastoma protein is phosphorylated during the specific phases of the cell cycle. *Cell* **58**:1097-1105.
3. Buchman, A. R., M. Fromm, and P. Berg. 1984. Complex regulation of simian virus 40 early-region transcription from different overlapping promoters. *Mol. Cell. Biol.* **4**:1900-1914.
4. Chen, P.-L., P. Scully, J.-Y. Shew, J. Y. J. Wang, and W.-H. Lee. 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* **58**:1193-1198.
5. Cherington, V., M. Brown, E. Paucha, J. St. Louis, B. Spiegelman, and T. Roberts. 1988. Separation of simian virus 40 large T antigen transforming and origin-binding functions from the ability to block differentiation. *Mol. Cell. Biol.* **8**:1380-1384.
6. Chou, J., and R. Martin. 1975. DNA infectivity and the induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. *J. Virol.* **15**:145-150.
7. DeCaprio, J. A., J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**:275-283.
8. DeCaprio, J. A., J. W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica-Worms, C.-M. Huang, and D. M. Livingston. 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. *Cell* **58**:1085-1095.
9. Decker, S., M. Yamaguchi, R. Possenti, and M. DePamphilis. 1986. Initiation of simian virus 40 DNA replication *in vitro*: aphidocolin causes accumulation of early-replicating intermediates and allows determination of the initial direction of DNA synthesis. *Mol. Cell. Biol.* **6**:3815-3825.
- 9a. Dyson, N., and C. Maulbecker. Unpublished data.
10. Ewen, M. E., J. W. Ludlow, E. Marsilio, J. A. DeCaprio, R. C. Millikan, S. H. Cheng, E. Paucha, and D. M. Livingston. 1989. An N-terminal transformation-governing sequence of SV40 large T antigen contributes to the binding of both p110th and a second cellular protein, p120. *Cell* **58**:257-267.
11. Farber, J., K. Peden, and D. Nathans. 1987. *trans*-Dominant defective mutants of simian virus 40 T antigen. *J. Virol.* **61**:436-445.
12. Fried, M., and C. Prives. 1986. The biology of simian virus 40 and polyoma virus, p. 1-16. In M. Botchan, T. Grodzicker, and P. Sharp (ed.), *DNA tumor viruses*, vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. Gallo, G. J., G. Gilinger, and J. C. Alwine. 1988. Simian virus 40 T antigen alters the binding characteristics of specific DNA-binding factors. *Mol. Cell. Biol.* **8**:1648-1656.
14. Gannon, J. V., and D. P. Lane. 1987. p53 and DNA polymerase α complete for binding to SV40 T antigen. *Nature (London)* **329**:456-458.
15. Gimbrome, M., and G. Fareed. 1976. Transformation of cultured human vascular endothelium by SV40 DNA. *Cell* **9**:685-691.
16. Gish, W., and M. Botchan. 1987. Simian virus 40-transformed human cells that express large T antigens defective for viral DNA replication. *J. Virol.* **61**:2864-2876.
- 16a. Gluzman, Y. Unpublished data.
17. Gluzman, Y. 1981. SV40 transformed simian cells support the replication of early SV40 mutants. *Cell* **23**:175-182.
18. Gluzman, Y. (ed.). 1982. *Eukaryotic viral vectors*, p. 187-189. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. Gluzman, Y., and B. Ahrens. 1982. SV40 early mutants that are defective for viral DNA synthesis but competent for transformation of cultured rat and simian cells. *Virology* **22**:78-92.
20. Gluzman, Y., J. Frisque, and J. Sambrook. 1979. Origin defective mutants of SV40. Cold Spring Harbor Symp. Quant. Biol. **44**:293-300.
21. Graham, F., J. Smiley, W. Russel, and R. Nairu. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**:59-77.
22. Graham, F., and A. Van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-467.
23. Gurney, E. G., S. Tamowski, and W. Deppert. 1986. Antigenic binding sites of monoclonal antibodies specific for simian virus 40 large T antigen. *J. Virol.* **57**:1168-1171.
24. Harlow, E., L. Crawford, D. Pim, and N. William. 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. *J. Virol.* **39**:861-869.
25. Harlow, E., and D. Lane. 1988. *Antibodies. A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cultures. *J. Mol. Biol.* **26**:365-369.
27. Hsiung, G. D., and W. D. Gaylord, Jr. 1961. The vacuolating virus of monkeys. I. Isolation, growth characteristics, and inclusion body formation. *J. Exp. Med.* **114**:975-986.
28. Kalderon, D., and A. Smith. 1984. *In vitro* mutagenesis of a putative DNA binding domain of SV40 large T antigen. *Virology* **139**:109-137.
29. Keller, J. M., and J. C. Alwine. 1985. Analysis of an activatable promoter: sequences in the simian virus 40 late promoter

- required for T-antigen-mediated *trans* activation. *Mol. Cell. Biol.* 5:1859-1869.
30. Kelly, T. J. 1988. SV40 DNA replication. *J. Biol. Chem.* 263:17889-17892.
 - 30a. Kelly, T. J., et al. Cold Spring Harbor Symp. Quant. Biol., in press.
 31. Khali, K., J. Brady, and G. Khoury. 1987. Translational regulation of SV40 early mRNA defines a new viral protein. *Cell* 48:639-645.
 32. Krauss, S. W., D. Mochly-Rosen, D. E. Koshland, Jr., and S. Linn. 1987. Exposure of Hela DNA polymerase α to protein kinase C affects its catalytic properties. *J. Biol. Chem.* 262:3432-3435.
 33. Kuchterlapati, R., S. P. Hwang, N. Shimizu, J. K. McDougall, and M. R. Botchan. 1978. Another chromosomal assignment for a simian virus 40 integration site in human cells. *Proc. Natl. Acad. Sci. USA* 75:4460-4464.
 34. Lane, D. P., and L. V. Crawford. 1979. T antigen is bound to a host protein in SV40 transformed cells. *Nature (London)* 278:261-263.
 35. Li, J., and T. Kelly. 1984. Simian virus 40 DNA replication in vitro. *Proc. Natl. Acad. Sci. USA* 81:6973-6977.
 36. Lin, J.-Y., and D. T. Simmons. 1991. Stable T-p53 complexes are not required for replication of simian virus 40 in culture or for enhanced phosphorylation of T antigen and p53. *J. Virol.* 65:2066-2072.
 37. Loeken, M., I. Bikel, D. M. Livingston, and J. Brady. 1988. Trans-activation of RNA polymerase II and III promoters by SV40 small t antigen. *Cell* 55:1171-1177.
 38. Lusky, M., and M. Botchan. 1981. Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. *Nature (London)* 293:79-81.
 39. Manos, M. M., and Y. Gluzman. 1985. Genetic and biochemical analysis of transformation-competent, replication-defective simian virus 40 large T antigen mutants. *J. Virol.* 53:120-127.
 40. Maulbecker, C. 1989. Ph.D. thesis. University of California, Berkeley.
 41. Maulbecker, C., et al. Unpublished data.
 42. McVey, D., L. Brizuela, I. Mohr, D. Marshak, Y. Gluzman, and D. Beach. 1989. Phosphorylation of large tumour antigen by cdc2 stimulates SV40 DNA replication. *Nature (London)* 341:503-507.
 43. Mohr, I. J., M. P. Fairman, B. Stillman, and Y. Gluzman. 1989. Large T-antigen mutants define multiple steps in the initiation of simian virus 40 DNA replication. *J. Virol.* 63:4181-4188.
 - 43a. Mohr, I. J., Y. Gluzman, M. P. Fairman, M. Strauss, D. McVey, B. Stillman, and R. D. Gerard. 1989. Production of simian virus 40 large tumor antigen in bacteria: altered DNA-binding specificity and DNA replication activity of underphosphorylated large tumor antigen. *Proc. Natl. Acad. Sci. USA* 86:6479-6483.
 - 43b. Mohr, I. J., B. Stillman, and Y. Gluzman. 1987. Regulation of SV40 DNA replication by phosphorylation of T antigen. *EMBO J.* 6:153-160.
 44. Montano, X., R. Millikan, J. M. Milhaven, D. A. Newsome, J. W. Ludlow, A. K. Arthur, E. Fanning, I. Bikel, and D. M. Livingston. 1990. Simian virus 40 small tumor antigen and an amino-terminal domain of large tumor antigen share a common transforming function. *Proc. Natl. Acad. Sci. USA* 87:7448-7452.
 45. Neufeld, D., S. Ripley, A. Henderson, and H. Ozer. 1987. immortalization of human fibroblasts by origin-defective simian virus 40. *Mol. Cell. Biol.* 7:2794-2802.
 46. O'Neill, F. J., and D. Carroll. 1983. Role of defective SV40 genomes in establishment and maintenance of persistently infected primate cell lines. *Intervirology* 19:181-194.
 47. Park, S. D., and J. E. Cleaver. 1979. Postreplication repair: questions of its definition and possible alteration in xeroderma pigmentosum cell strains. *Proc. Natl. Acad. Sci. USA* 76:3927-3931.
 48. Paucha, E., D. Kalderon, R. W. Harvey, and A. E. Smith. 1986. Simian virus 40 DNA-origin-binding domain on large T antigens. *J. Virol.* 57:50-64.
 49. Pipas, J. M., K. W. C. Peden, and D. Nathans. 1983. Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T-antigen gene. *Mol. Cell. Biol.* 3:203-213.
 50. Roberts, J. Personal communication.
 51. Roberts, J., and G. D'Urso. 1988. An origin unwinding activity regulates initiation of DNA replication during mammalian cell cycle. *Science* 241:1486-1489.
 52. Royer-Pokora, B., W. D. Peterson, Jr., and W. A. Haseltine. 1984. Biological and biochemical characterization of an SV40-transformed xeroderma pigmentosum cell line. *Exp. Cell Res.* 151:408-420.
 53. Rutla, J. E., J. B. Christensen, and M. J. Imperiale. 1989. Viral growth, origin binding and p53 binding properties of simian virus 40 large T antigen transformation and replication mutants. *Oncogene Res.* 4:303-310.
 54. Scheidtmann, K. H., M. C. Mumby, K. Rundell, and G. Walter. 1991. Dephosphorylation of simian virus 40 large-T antigen and p53 protein by protein phosphatase 2A: inhibition by small-t antigen. *Mol. Cell. Biol.* 11:1996-2003.
 55. Seedorf, K., T. Oltersdorf, G. Krammer, and K. Rowekamp. 1987. Identification of early proteins of human papillomavirus type 16 (HPV 16) and type 18 (HPV 18) in cervical carcinoma cell lines. *EMBO J.* 6:139-144.
 56. Simanis, V., and D. Lane. 1985. An immunoaffinity purification procedure for SV40 large T antigen. *Virology* 114:88-100.
 57. Small, M. B., Y. Gluzman, and H. L. Ozer. 1982. Enhanced transformation of human fibroblasts by origin-defective simian virus 40. *Nature (London)* 296:671-672.
 58. Srinivasan, A., K. W. C. Peden, and J. M. Pipas. 1989. The large tumor antigen of simian virus 40 encodes at least two distinct transforming functions. *J. Virol.* 63:5459-5463.
 59. Stillman, B., and Y. Gluzman. 1985. Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. *Mol. Cell. Biol.* 5:2051-2060.
 60. Stürzbecher, H.-W., R. Brain, T. Maimets, C. Addison, K. Rudge, and J. R. Jenkins. 1988. Mouse p53 blocks SV40 DNA replication in vitro and downregulates T antigen DNA helicase activity. *Oncogene* 3:405-414.
 61. Sweet, B. H., and M. R. Hilleman. 1960. The vacuolating virus, SV40. *Proc. Soc. Exp. Biol. Med.* 105:420-427.
 62. Tornow, J., M. Polrino-Dodnar, G. Santangelo, and C. N. Cole. 1985. Two separate functional domains of simian virus 40 large T antigen: carboxyl-terminal region of simian virus 40 large T antigen is required for efficient capsid protein synthesis. *J. Virol.* 53:415-424.
 63. Virshup, D., and T. Kelly. 1989. Purification of replication protein C, a cellular protein involved in the initial stages of simian virus 40 DNA replication *in vitro*. *Proc. Natl. Acad. Sci. USA* 86:3584-3588.
 64. Wagner, S., and M. R. Green. 1991. A transcriptional tryst. *Nature (London)* 352:189-190.
 65. Wang, E. H., P. N. Friedman, and C. Prives. 1989. The murine p53 protein blocks replication of SV40 DNA in vitro by inhibiting the initiation functions of SV40 large T antigen. *Cell* 57:379-392.
 66. Weber, F., and W. Schaffner. 1984. Simian virus 40 enhancer increases RNA polymerase density within the linked gene. *Nature (London)* 315:75-77.
 67. Wobbe, R., F. Dean, Y. Murakami, L. Weissbach, and J. Hurwitz. 1986. Simian virus 40 DNA replication in vitro: study of events preceding elongation of chains. *Proc. Natl. Acad. Sci. USA* 83:4612-4616.
 68. Wold, M. S., J. J. Li, and T. J. Kelly. 1987. Initiation of simian virus 40 DNA replication *in vitro*: large-tumor antigen- and origin-dependent unwinding of the template. *Proc. Natl. Acad. Sci. USA* 84:3643-3647.
 - 68a. Yokota, P., and J. Bartholomew. Personal communication.
 69. Zhu, J., P. W. Rice, M. Chamberlain, and C. N. Cole. 1991. Mapping the transcriptional transactivation function of simian virus 40 large T antigen. *J. Virol.* 65:2778-2790.
 70. Zouias, D., K. Jha, C. Mulder, C. Basilico, and H. Ozer. 1980. Human fibroblasts transformed by the early region of SV40 DNA: analysis of "free" viral DNA sequences. *Virology* 104:439-453.