JOURNAL OF VIROLOGY, Apr. 1993, p. 1987–1995
0022-538X/93/041987-09$02.00/0

Vol. 67, No. 4

Simian Virus 40 (SV40) T-Antigen Mutations in Tumorigenic Transformation of SV40-Immortalized Human Uroepithelial Cells

CHINGHAI KAO,[1,2] PAUL HAUSER,[2,3] WILLIAM S. REZNIKOFF,[1] AND CATHERINE A. REZNIKOFF[2,3]*

Department of Biochemistry[1] and Department of Human Oncology,[3] Wisconsin Comprehensive Cancer Center,[2] University of Wisconsin, Madison, Wisconsin 53792

Received 25 August 1992/Accepted 22 December 1992

pSV2*Neo*, a plasmid that contains the wild-type simian virus 40 (SV40) origin of replication (ori), is widely used in mammalian cell transfection experiments. We observed that pSV2*Neo* transforms two nontumorigenic SV40-immortalized human uroepithelial cell lines (SV-HUC and CK/SV-HUC2) to G418 resistance (G418^r) at a frequency lower than that at which it transforms SV-HUC tumorigenic derivatives (T-SV-HUC). Transient expression studies with the chloramphenicol transferase assay showed that these differences could not be explained by differences in *Neo* gene expression. However, when we replaced the SV40 ori in pSV2*Neo* with a replication-defective ori to generate G13.1*Neo* and G13.1′*Neo*, the G418^r transformation frequency of the SV40-immortalized cell lines was elevated. Because SV40 T antigen stimulates replication at its ori, we tested plasmid replication in these transfected cell lines. The immortalized cell lines that showed low G418^r transformation frequencies after transfection with pSV2*Neo* showed high levels of plasmid replication, while the T-SV-HUC that showed high G418^r transformation frequencies failed to replicate pSV2*Neo*. To determine whether differences in the status of the T-antigen gene contributed to the phenomenon, we characterized the T-antigen gene in these cell lines. The results showed that the T-SV-HUC had sustained mutations in the T-antigen gene that would interfere with the ability of the T antigen to stimulate replication at its ori. Most T-SV-HUC contained a super-T-antigen replication-defective ori that apparently resulted from the partial duplication of SV40 early genes, but one T-SV-HUC had a point mutation in the ori DNA-binding domain of the T-antigen gene. These results correlate with the high G418^r transformation frequencies with pSV2*Neo* in T-SV-HUC compared with SV-HUC and CK/SV-HUC2. Furthermore, these results suggest that alterations in SV40 T antigen may be important in stabilizing human cells immortalized by SV40 genes that contain the wild-type SV40 ori, thus contributing to tumorigenic transformation. This is the first report of a super T antigen occurring in human SV40-transformed cells.

In vitro transformation systems provide important models with which to study complex biochemical and genetic changes that contribute to multistep tumorigenesis. Because of the limited life span of normal diploid cells in culture, a necessary first step in establishing such models is cell immortalization (5). Unlike in rodent cells, immortalization of human cells in culture does not occur spontaneously and has been difficult to achieve reproducibly with chemical carcinogens (12, 18, 53). However, human cell immortalization can be achieved routinely with small DNA tumor viruses or their oncoprotein genes (5). For example, immortalization of many human epithelial cell types has been reported with simian virus 40 (SV40) or the SV40 T antigen gene (6, 8, 20, 29, 40). In contrast to observations made with rodent cells, in which SV40 immortalization is usually accompanied by tumorigenic transformation, SV40-immortalized human epithelial cells usually have not undergone tumorigenic transformation (5, 19, 52). Therefore, SV40-immortalized human cell lines have been considered useful models for studying the ability of various chemicals, irradiation, and oncogenes to cause tumorigenic transformation or progression in different human cell types (44).

Our laboratory has developed two multistep in vitro-in vivo transformation systems with SV40 (wild-type virus)-infected and SV40 T antigen gene (plasmid)-transfected immortalized human uroepithelial cells (HUC), the SV-HUC (8) and CK/SV-HUC2 (21) transformation systems, respectively. Tumorigenic transformation and/or neoplastic progression of SV-HUC and CK/SV-HUC2 has been achieved after exposure of cells in vitro to chemical carcinogens (2, 42) or transfection with the EJ/*ras* human bladder cancer oncogene (7, 21, 39). Thus, we have generated two isogenic series of cell lines that represent different steps in multistep HUC transformation.

The presence and role of SV40 genes and their transcripts in these human transformation systems must be considered. SV40 is a small DNA tumor virus that has not been identified as an etiologic agent in human cancers. It contains a 5,243-bp double-stranded DNA genome that replicates several rounds within one host cell cycle starting from a single replication origin (ori). This ori also contains two promoters for the expression of the virally encoded genes. One of these virally encoded genes is the large-T-antigen gene. SV40 T antigen is a multifunctional protein (4, 32, 51) with DNA helicase (54), RNA helicase (45), and ATPase (3) activities. SV40 T antigen upregulates the expression of early virus genes and downregulates the expression of late virus genes (17, 23, 41). SV40 T antigen forms complexes with several cellular proteins, including DNA polymerase (49) and two tumor suppressor gene products, pRB (11) and p53 (27, 31, 37, 49). Interactions between T antigen and pRB and p53 in our

* Corresponding author.

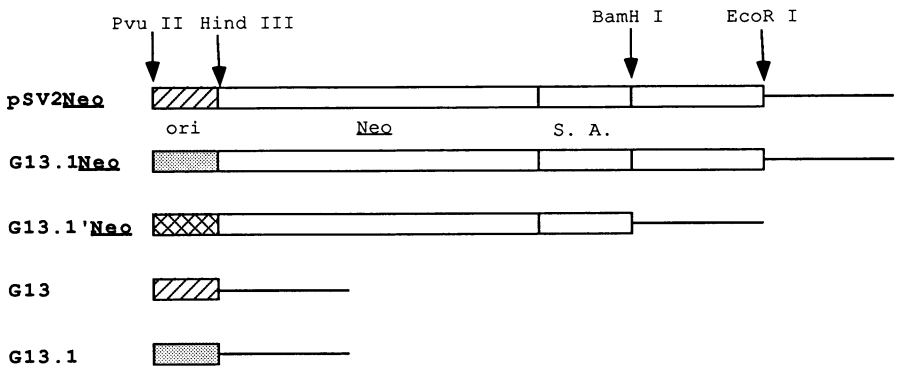

FIG. 1. Maps of the *Neo* gene expression plasmids (pSV2*Neo*, G13.1*Neo*, and G13.1'*Neo*) and of the two SV40 ori-containing plasmids (G13 and G13.1) used in this study. pSV2Neo and G13 contain the wild-type SV40 ori as a *Pvu*II-*Hin*dIII SV40 ori fragment. G13.1*Neo* and G13.1 contain a mutant SV40 ori from plasmid pX-8 as a *Pvu*II-*Hin*dIII mutant SV40 ori fragment. G13.1'*Neo* contains a mutant SV40 ori from plasmid p6-1 as a *Pvu*II-*Hin*dIII mutant SV40 ori fragment. Further details are given in Materials and Methods. S. A., a fragment containing both splicing and polyadenylation signals.

transformation systems have been considered as part of a separate study (20a). Most relevant to the present report is the observation that SV40 T antigen initiates DNA replication from its ori (10, 22, 47). If unchecked, virus replication leads to cell lysis and cell death in permissive cell types (52). Cells that are permissive or semipermissive for SV40 but survive infection, including monkey and human cells, must by definition generate mechanisms to limit virus replication. In monkey and human cells, these mechanisms have included selection for mutations in the T-antigen gene that inactivate its ability to initiate replication (14, 15, 48).

We report here that similar types of T-antigen mutations are generated in SV40-immortalized HUC that have undergone tumorigenic transformation. We transfected a pSV2 *Neo*-derived EJ/*ras* expression vector into SV-HUC and CK/SV-HUC2. The *Neo* gene in this construct is controlled by the SV40 early gene promoter and results in G418 resistance (G418^r) (50). We observed a very low efficiency of transformation to G418^r by pSV2*Neo* in SV-HUC and CK/SV-HUC2 compared with T-SV-HUC, the chemically transformed tumorigenic derivatives of SV-HUC, and also compared with control cell lines that do not contain SV40 genes.

We found that T antigen stimulates replication from the SV40 ori of pSV2*Neo*-transfected SV40-immortalized cell lines, resulting in a low G418^r transformation frequency with this vector. However, similar plasmid replication was not observed in T-SV-HUC, the isogenic tumorigenic derivatives of SV-HUC. We discovered that these T-SV-HUC had sustained mutations in the T-antigen gene, including those generating a super T antigen, that would interfere with their ability to stimulate replication from the SV40 ori.

MATERIALS AND METHODS

Cells. Two SV40-immortalized cell lines, SV-HUC and CK/SV-HUC2, were used in this study. The methods of establishment and the characteristics of these cell lines have been described (8, 21, 42). In brief, SV-HUC was established after infection of cultured normal ureteral HUC with wild-type SV40 (8). At passage 15 (p15), SV-HUC had one SV40 integration site, was near diploid karyotypically, expressed SV40 T antigen, and did not produce infectious SV40 (8). CK/SV-HUC2 was established from a similar HUC culture but after transfection with a plasmid carrying SV40 early genes with an ori-negative (ori$^-$) expression element (21). At p23, CK/SV-HUC2 also had one SV40 integration site and expressed SV40 T antigen, but this cell line showed losses of chromosomes 3p, 11p, and 13q (21). Neither SV-HUC (p15) nor CK/SV-HUC2 (p23) was tumorigenic when inoculated into athymic nude mice (8, 21).

However, exposure of SV-HUC to chemical carcinogens, including the polycyclic hydrocarbon carcinogen 3-methylcholanthrene, resulted in tumorigenic transformation, which allowed us to generate the series of SV-HUC isogenic tumorigenic derivatives, T1, T2, T7, T9, T10, and T16 (42), that were also used in this study. Cell lines derived from a subcutaneous tumor transplant of T1, TT1, and a lung tumor metastasis from a tail vein inoculation of T1, mTT1, were also used in this study. We used as controls in this study two carcinoma HUC lines, T24 and 647V, that were derived from clinical bladder cancers, and also the NIH 3T3 rodent fibroblast cell line. None of these control cell lines contains the SV40 genome. All cells were cultured in 100-mm plastic petri dishes (P100; Corning, Corning, N.Y.) at 37°C in an atmosphere of 5% CO_2. HUC were cultured in 1%FBS-F12+, a medium that contained fetal bovine serum (GIBCO BRL, Gaithersburg, Md.) and Ham's F12 medium (GIBCO BRL) and that was developed for this cell type (43). NIH 3T3, T24, and 647V cells were cultured in 10%CS-F12, a medium that contained calf serum (GIBCO BRL) and Ham's F12 medium.

Plasmids. As shown in Fig. 1, in plasmid pSV2*Neo*, the expression of the G418^r *Neo* gene is driven by the SV40 early gene promoter derived from a *Pvu*II-*Hin*dIII fragment of the SV40 ori (50). Three functions are encoded by this *Pvu*II-*Hin*dIII region, the early gene promoter, the late gene promoter, and the ori. This *Pvu*II-*Hin*dIII region also contains three SV40 T-antigen-binding sites (46). G13.1*Neo* and G13.1*Cat* were constructed by replacing the *Pvu*II-*Hin*dIII fragment of pSV2*Neo* and pSV2*Cat* (American Type Culture Collection, Rockville, Md.) with the corresponding *Pvu*II-*Hin*dIII fragment from pX-8, in which an 8-bp sequence including a *Bgl*I site at the SV40 ori was replaced with an 8-bp *Xho*I linker (21). For G13.1'*Neo*, the *Pvu*II-*Hin*dIII fragment was from p6-1, in which a 6-bp sequence including a *Bgl*I site was deleted and the *Bam*HI-*Eco*RI fragment of pSV2*Neo* was replaced with a *Bam*HI-*Eco*RI linker. G13 and G13.1 were constructed by replacing the *Neo* gene *Hin*dIII-*Eco*RI fragments of pSV2*Neo* and G13.1*Neo*, respectively, with *Hin*dIII-*Eco*RI linkers.

DNA transfection. Plasmid DNA was precipitated by the calcium phosphate transfection method exactly as previously described (21). The selection medium for *Neo* gene expression contained 200 U of G418 (GIBCO BRL) per ml and was added 48 h after transfection. G418^r colonies were stained with crystal violet approximately 2 weeks after transfection.

CAT assay. Cells were dispersed and collected 2 days after transfection. The cell pellet was resuspended in 200 μl of ice-cold hypotonic buffer (25 mM Tris-Cl [pH 7.5], 2 mM $MgCl_2$) and placed in a dry ice-ethanol bath for 5 min. The tube was transferred to a 37°C water bath for 5 min. The freeze-thaw regimen was repeated four more times. The cell debris was pelleted. The supernatant was quickly frozen in a dry ice-ethanol bath. The lysate was stored at −70°C. The chloramphenicol acetyltransferase (CAT) reaction (24) was performed at 37°C for 25 to 30 min with a cell extract containing 50 μg of protein in the presence of 0.05 μCi of [^{14}C]chloramphenicol (55 mCi/mmol; Amersham)–0.53 mM acetyl coenzyme A–0.22 M Tris-Cl (pH 7.5). The CAT reaction mixture was extracted once with ethyl acetate. The ethyl acetate layer was dried and resuspended in 15 μl of fresh ethyl acetate before being spotted on a thin-layer chromatography plate. The chromatography was allowed to run for 30 min in a chromatography tank containing 19:1 (vol/vol) chloroform-methanol. The thin-layer chromatography plate was air dried, and autoradiography was accomplished by exposing the plate to Kodak X-Omat AR film at −70°C.

SV40 ori replication assay. Cells were washed once with cold phosphate-buffered saline (PBS) and lysed with 1 ml of ice-cold lysis buffer (16.7 mM glucose, 8.3 mM Tris-Cl [pH 8.0], 3.3 mM EDTA [pH 8.0], 133 mM NaOH, 0.67% sodium dodecyl sulfate [SDS]) 1, 2, and 3 days after transfection. The cell lysate was collected with a rubber policeman and neutralized with 500 μl of ice-cold neutralization buffer (3 M potassium acetate, 2 M acetic acid). The cell debris was pelleted, and the supernatant was extracted twice with phenol-chloroform and once with chloroform. DNA was precipitated with 2 volumes of ethanol. The DNA pellet was dissolved in 80 μl of DNA buffer (10 mM NaCl, 10 mM Tris [pH 7.9], 0.1 mM EDTA). DNA (10 μl) was digested with restriction enzyme *Hin*dIII (5 U) or *Hin*dIII (5 U) plus *Dpn*I (3.5 U). DNA fragments were separated by electrophoresis through a 1% agarose gel and transferred to a Hybond-N membrane (Amersham, Arlington, Ill.). Southern blot hybridization was performed by use of the same protocol as that previously described (21).

Immunoprecipitation and Western blot (immunoblot) analysis. Cells were collected from four P100 dishes and washed with ice-cold PBS as described for the CAT assay. Cells were collected and lysed by the addition of 1 ml of lysis buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 100 U of aprotinin per ml, 10 μg of leupeptin per ml). The cell debris was pelleted in a microcentrifuge, and the supernatant was used for immunoprecipitation with anti-T-antigen Ab-2 (recognizes the C-terminal domain of T antigen; Oncogene Science, Manhasset, N.Y.) and protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.). The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis through a 7.5% polyacrylamide gel and then transferred to an Immobilon-P membrane (Millipore, Bedford, Mass.). The immunoblotting procedure was done by use of the Immun-Blot kit manufacturer's protocol (Bio-Rad Laboratories, Richmond, Calif.) with anti-T-antigen Ab-1 (recognizes the N-terminal domain of T antigen; Oncogene Science) and Ab-2 (see above).

RNA PCR. Total RNA was prepared by the guanidine isothiocyanate method as described previously (21). RNA polymerase chain reaction (PCR) amplification was done by use of the protocol accompanying the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.). Two sets of primers encompassing the whole large-T and small-t antigen coding region were used to amplify SV40 early transcripts. The first set of primers consisted of an upstream primer, 5′-TTGGAGGCCTAGGCTTTTGC-3′, and a downstream primer, 5′-TCCTTTAAACAGCCAGTATC-3′, encompassing nucleotides 3540 to 5199. The second set of primers consisted of an upstream primer, 5′-GGATTCAGTGGTG TATGACT-3′, and a downstream primer, 5′-GCTGCAATA AACAAGTAAC-3′, encompassing nucleotides 2652 to 3615.

Cloning of PCR products. PCR products were cloned into a T-Vector by use of the protocol accompanying the pT7Blue T-Vector kit (Novagen, Inc., Madison, Wis.). Plasmid DNA was prepared from white colonies and checked for content of the correct insert by restriction enzyme digestion and agarose gel electrophoresis.

DNA sequencing. Plasmids containing the desired PCR product were sequenced by use of a Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, Ohio) with nine primers spanning the whole SV40 T-antigen coding region. These primers were 5′-TTGGAGGCCTAG GCTTTTGC-3′, 5′-TTCTTGTATAGCAGTGCAGC-3′, 5′-GTTTCCTCTGCTTCTTCTGG-3′, 5′-CAGCCTGTTGGCA TATGGTT-3′, 5′-GGATTCAGTGGTGTATGACT-3′, 5′-TCCTTTAAACAGCCAGTATC-3′, 5′-CAGGGAATTAAT AACCTGGA-3′, 5′-GCATTGCTTTGCTTCTTATG-3′, and 5′-GCTGCAATAAACAAGTTAAC-3′. Plasmid DNA was purified from 3 ml of an overnight bacterial culture by the alkaline lysis method (34) and dissolved in 60 μl of TE buffer. DNA (8 μl) was treated with RNase for 30 min at 37°C, denatured with alkali, neutralized, precipitated, and dried. The DNA pellet was redissolved in 10 μl of a reaction mixture including 20 ng of primer. The reaction was carried out by use of the manufacturer's suggested conditions. Samples (3.5-μl volume) were loaded on a gel (6% polyacrylamide, 8 M urea) after being heat denatured at 80°C for 5 min. After 2 or 5 h at 75 W, the gel was immersed in fixing solution (10% acetic acid, 12% methanol), dried, and exposed to X-ray film.

RESULTS

pSV2*Neo*-mediated G418^r transformation is inefficient in SV-HUC compared with T-SV-HUC. Vector pSV2*Neo*, which contains the *Neo* gene under the control of the SV40 early gene promoter (Fig. 1), is commonly used in gene transfection studies as a selectable marker because *Neo* expression results in G418^r. In four independent experiments, pSV2*Neo* was transfected into two independently derived SV40-immortalized HUC lines, SV-HUC and CK/SV-HUC2, and also into NIH 3T3 cells and two human bladder carcinoma cell lines, T24 and 647V, which do not contain the SV40 genome. Results showed a significantly lower yield of G418^r colonies in both of the cell lines, SV-HUC and CK/SV-HUC2, that contain the T-antigen gene (Table 1).

pSV2*Neo* was also transfected into six independently derived tumorigenic derivatives of SV-HUC, T-SV-HUC (42). Although there was some variation in the transformation frequency among these T-SV-HUC, the overall effi-

TABLE 1. G418^r transformation with *Neo* constructs with or without the SV40 genome[a]

Cell line	No. of G418^r colonies in the presence of:		
	pSV2*Neo*	G13.1*Neo*	G13.1′*Neo*
SV40 immortalized			
SV-HUC	0.25 ± 0.43	125 ± 10	107 ± 20
CK/SV-HUC2	4 ± 2	70 ± 16	78 ± 20
Control			
T24	210 ± 50	188 ± 48	180 ± 58
647V	71 ± 5	71 ± 5	60 ± 16
NIH 3T3	>400	>400	ND
SV40 tumorigenic[b]			
mTT1	154 ± 9	129 ± 42	
T2	254 ± 21	249 ± 21	
T7	412 ± 29	432 ± 60	
T9	167 ± 12	158 ± 14	
T10	139 ± 7	138 ± 24	
T16	59 ± 5	53 ± 3	

[a] Cells were transfected with 10 μg of plasmid DNA as indicated. Two days posttransfection, 200 mg of G418 per ml was added to the culture medium. G418^r colonies were counted after 2 weeks. Each determination represents the average ± standard deviation number of G418^r colonies from four separate P100 dishes representing either two or four independent experiments. Control cell lines do not contain the SV40 genome. ND, not determined.

[b] For comparison, in this experiment, there were 0 and 120 ± 28 G418^r colonies in the presence of pSV2*Neo* and G13.1*Neo*, respectively, for SV-HUC.

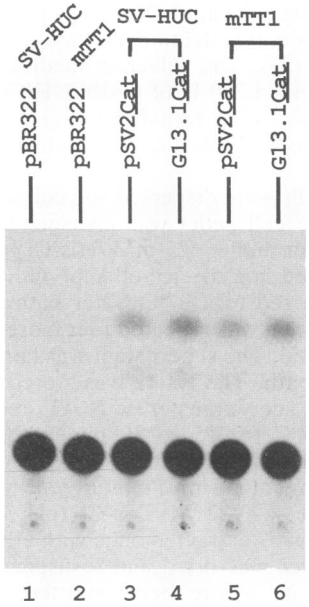

FIG. 2. Results of the CAT assay that was used to determine whether there are differences in CAT expression between wild-type (pSV2*Cat*) and mutant (G13.1*Cat*) SV40 promoters in two cell lines, SV-HUC and mTT1. There were no differences in expression levels between the cell lines (compare lanes 3 to 6). pBR322 (with no *Cat* gene) was used as a negative control (lanes 1 and 2).

ciency of transformation to G418^r was comparable to that seen in the three cell lines that do not contain the SV40 genome (Table 1). Therefore, whatever property prevented efficient G418^r transformation by pSV2*Neo* in CK/SV-HUC2 and SV-HUC was altered in the tumorigenic derivatives, T-SV-HUC, and was not present in the control cell lines, which do not contain SV40 T antigen.

Efficient transient *Neo* gene expression from pSV2*Neo* in SV-HUC. We addressed the possibility that SV40 T antigen (or some other cellular factor) downregulates the expression of the *Neo* gene from the SV40 early promoter in pSV2*Neo* in SV-HUC but not in the tumorigenic derivatives. We compared gene expression from pSV2*Cat* (a vector in which the *Cat* gene was substituted for the *Neo* gene) in SV-HUC with that in a representative isogenic tumorigenic derivative of SV-HUC, mTT1. Transient gene expression studies showed no difference in CAT expression between the cell line that was efficiently transformed by pSV2*Neo* and the cell line that was inefficiently transformed (Fig. 2). These results are inconsistent with the hypothesis that the failure to recover G418^r colonies after transfection of SV-HUC with pSV2*Neo* results from poor *Neo* gene expression in this cell line.

Efficient G418^r transformation of SV-HUC by ori$^-$ *Neo* constructs. SV40 T antigen interacts with its ori, affecting DNA replication as well as gene transcription (17, 22, 23, 41). To address the possibility that differences between the nontumorigenic and tumorigenic SV40-immortalized cells in this interaction could explain their different G418^r transformation efficiencies with pSV2*Neo*, we constructed two new *Neo* gene vectors, G13.1*Neo* and G13.1′*Neo*, each with a different mutation in its SV40 ori (Fig. 1). The replication-defective mutation in G13.1*Cat* (the same as that in G13.1*Neo*) apparently did not interfere with transcription promotion activity, as determined by a transient expression assay (Fig. 2). With these vectors, there was no difference in G418^r transformation frequencies between pSV2*Neo* and the three control cell lines, which do not contain the SV40 genome (Table 1). Therefore, G418^r transformation with our ori$^-$ constructs was equivalent to that with the pSV2*Neo* construct. There was also no difference in the ability to cause G418^r transformation of T-SV-HUC between the ori$^-$ constructs and pSV2*Neo* (Table 1). In contrast, the G418^r transformation efficiencies for both SV-HUC and CK/SV-HUC2 were significantly increased when the ori$^-$ constructs were used to introduce the *Neo* gene (Table 1). Thus, the phenomenon of inefficient G418^r transformation of SV-HUC and CK/SV-HUC2 by pSV2*Neo* that we had observed requires the presence of a wild-type SV40 ori.

Inhibition in *trans* of efficient G13.1*Neo*-mediated G418^r transformation of SV-HUC by a wild-type ori-containing plasmid. To further confirm that a wild-type SV40 ori was a required factor in the poor transformation frequencies for SV-HUC and CK/SV-HUC2, we performed a mixed transfection experiment in which we tested the ability of the wild-type SV40 ori to interfere with efficient G418^r transformation of SV-HUC by ori$^-$ constructs. In these experiments, the frequencies of G418^r transformation of SV-HUC and mTT1 by G13.1*Neo* were compared with the frequencies obtained when a vector containing a wild-type SV40 ori was added to the transfection mixture. The G418^r transformation frequencies for both SV-HUC and CK/SV-HUC2 decreased in a dose-dependent manner with increasing concentrations of such a vector, e.g., pSV2*Cat* (Fig. 3A). The deleterious effect of the wild-type SV40 ori on the G418^r transformation of SV-HUC by G13.1*Neo* did not depend on the gene encoded in the plasmid vector, because it was observed in experiments with constructs that did not encode any gene (Fig. 3B). Importantly, a wild-type SV40 ori had no effect in *trans* on G418^r transformation by G13.1*Neo* of the tumorigenic SV-HUC derivative mTT1 (Fig. 3).

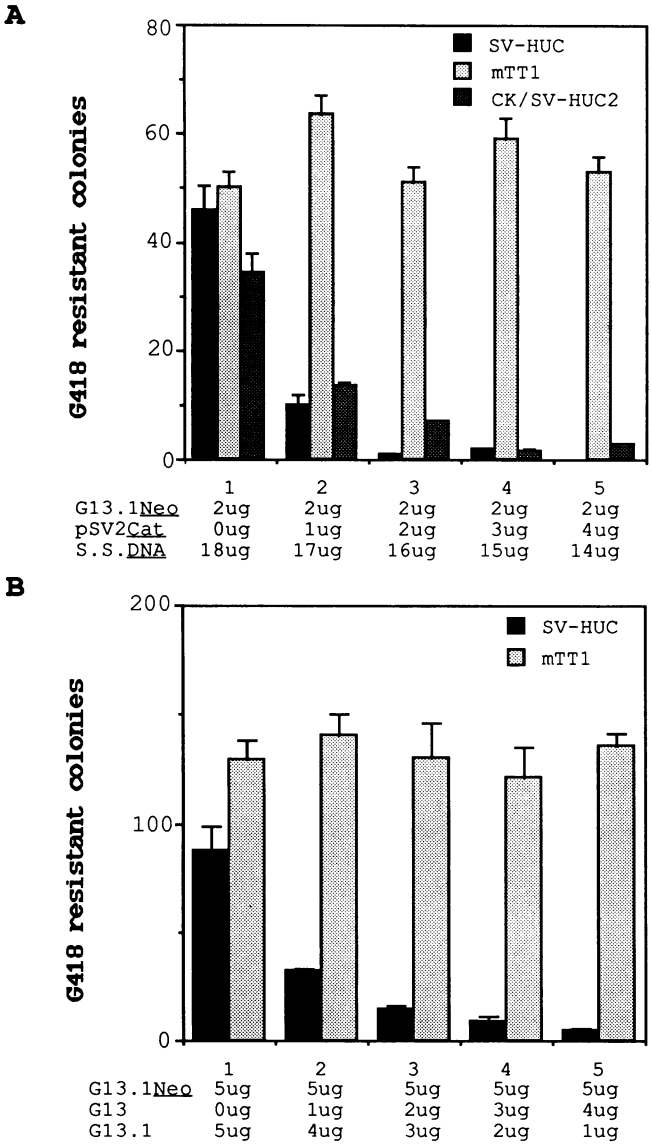

FIG. 3. Determination of interference of a wild-type SV40 ori with the recovery of G418^r colonies in SV-HUC or mTT1 after transfection with ori$^-$ G13.1*Neo*. (A) The addition of pSV2*Cat* decreased the G418^r transformation of SV-HUC and CK/SV-HUC but not mTT1 by G13.1*Neo* in a dose-dependent manner. This graph shows the average of four experiments. In this experiment, salmon sperm (S.S.) DNA was used to standardize the DNA concentration. (B) The phenomenon was again reproduced with SV-HUC by use of an SV40 expression element that does not encode a gene. The numbers of G418^r colonies in mTT1 were unaffected by SV40 ori in these two experiments, each of which was reproduced four times.

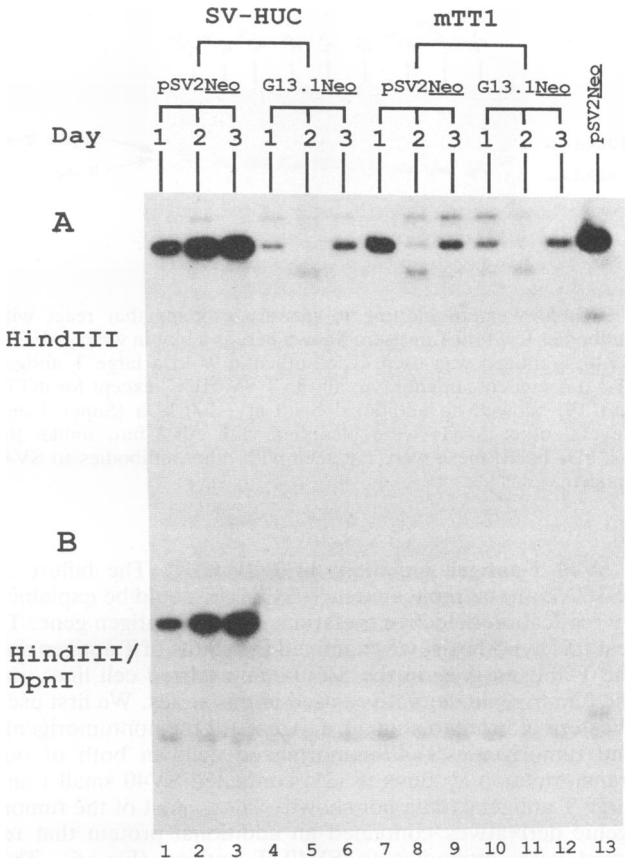

FIG. 4. Southern blot analysis to determine whether pSV2*Neo* is replicated in either SV-HUC or mTT1 1, 2, or 3 days after transfection of cells with 10 μg of pSV2*Neo* or G13.1*Neo*. Cellularly replicated plasmid DNAs were distinguished from transfected plasmid DNAs (A) by their resistance to *Hin*dIII and *Dpn*I (B). The results showed *Hin*dIII- and *Dpn*I-resistant bands in only SV-HUC and not mTT1 and only after transfection with pSV2*Neo* and not G13.1*Neo*. pSV2*Neo* was used as a probe.

Replication of pSV2*Neo* in SV-HUC but not T-SV-HUC. In the presence of a wild-type SV40 ori, T antigen stimulates DNA replication (10, 22, 47). We tested the possibility that pSV2*Neo* plasmid replication occurred in SV-HUC but not T-SV-HUC. To distinguish bacterial plasmid DNAs that were transfected into the cells from plasmid DNAs that were replicated by the host mammalian cells, we made use of the sensitivity of bacterially replicated plasmid DNAs to the restriction enzyme *Dpn*I, which results from *dam* methylation of GATC sequences by bacteria (13, 25). SV-HUC and a representative T-SV-HUC, mTT1, were transfected with pSV2*Neo* or G13.1*Neo* and examined by the Southern blot technique at intervals posttransfection for *Dpn*I-resistant plasmid sequences. The overall quantity of pSV2*Neo* DNA increased in SV-HUC from days 1 to 3, and this DNA was resistant to *Dpn*I digestion (Fig. 4, lanes 1 to 3). On the other hand, the quantity of pSV2*Neo* DNA did not increase in mTT1 in 3 days following transfection, and this DNA was sensitive to *Dpn*I digestion (Fig. 4, lanes 7 to 9). The quantity of plasmid DNA did not increase in either cell line when the ori$^-$ construct G13.1*Neo* was used (Fig. 4, lanes 4 to 7 and 11 to 13). This experiment was also done with CK/SV-HUC2 and all the other T-SV-HUC (Table 1) used in this study. The replication of pSV2*Neo* occurred in CK/SV-HUC2 but not in the other T-SV-HUC, with the exception of weak replication in T2 (data not shown). Thus, there was an almost perfect correlation between poor G418^r transformation by pSV2*Neo* in SV40-immortalized cells and the ability of this plasmid to be replicated in cells after transfection. We hypothesized that plasmid replication was stimulated by the presence of wild-type T antigen in SV-HUC and CK/SV-HUC2 and that this effect was deleterious to the cells. However, this hypothesis left unanswered the question of why T-SV-HUC do not replicate pSV2*Neo*.

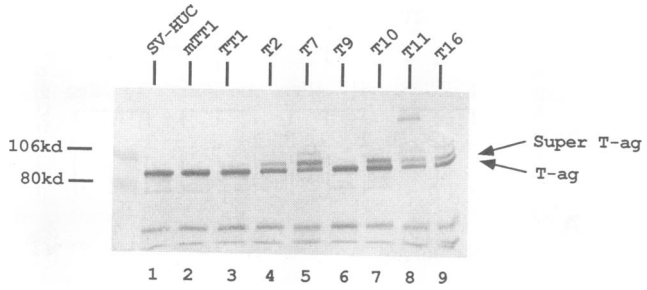

FIG. 5. Western blotting to identify proteins that react with antibodies to SV40 T antigen. Shown here is a blot in which Ab-2 to SV40 T antigen was used. The authentic 94-kDa large T antigen (T-ag) is evident. In addition, all the T-SV-HUC, except for mTT1 and T9, showed an additional band at ~100 kDa (Super T-ag). Several other bands were identified with Ab-2 but, unlike the 100-kDa band, these were not seen with other antibodies to SV40 antigen.

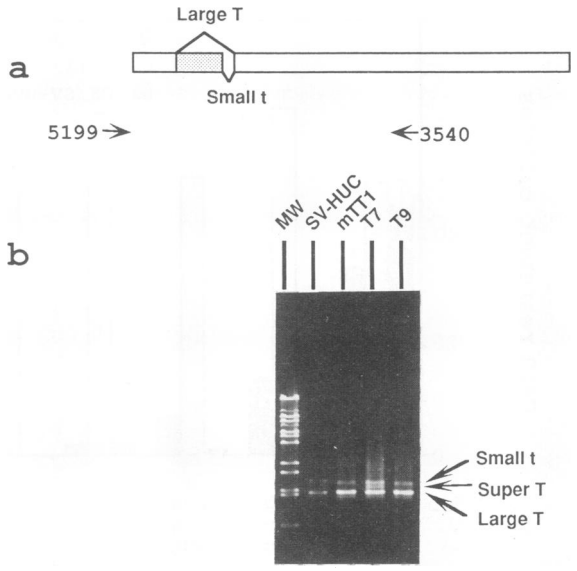

FIG. 6. PCR amplification of SV40 early transcripts. SV40 large T and small t antigens were encoded by the same SV40 early transcripts but with different splicing events (a). For examination of these transcripts in our cell lines, PCR amplification of SV40 early transcripts was done with an upstream primer, 5199 (20-mer, from nucleotides 5180 to 5199), and a downstream primer, 3540 (20-mer, from nucleotides 3540 to 3559). The wild-type viral genes yielded two products, one for SV40 large T antigen and one for small t antigen, in all cell lines examined (b). Both of these bands were present in all cell lines tested. In addition, the T7 cell line contained an additional band between the two bands for authentic large T and small t antigens, corresponding to super T antigen. The molecular weight marker (MW) was λ DNA digested with the restriction enzyme *Bst*EII.

SV40 T-antigen mutations in T-SV-HUC. The failure of pSV2*Neo* to be replicated in T-SV-HUC could be explained by replication-defective mutations in the T-antigen gene. To test this hypothesis, we examined the status of T antigen and the T-antigen gene in the SV40-immortalized cell lines and the tumorigenic derivatives used in this series. We first used Western blotting to study T antigen. All the nontumorigenic and tumorigenic SV40-immortalized cells in both of our transformation systems (8, 21) contained SV40 small t and large T antigens (data not shown). Also, most of the tumorigenic derivatives contained an additional protein that reacted with antibodies to SV40 T antigen (Fig. 5). This additional band could be detected with two different antibodies recognizing different epitopes of T antigen (see Materials and Methods). It was approximately 100 kDa in size (authentic large T antigen is 94 kDa in size) and was therefore similar in size to the super T antigen described for SV40-transformed mouse cells (30). This super T antigen was observed in all T-SV-HUC, except for mTT1 and T9. T7, T11, and T16 also contained other, larger proteins that reacted with only one antibody to SV40 T antigen. However, in this study, we characterized only the 100-kDa super T antigen. The correlation between these altered T antigens in the T-SV-HUC and the observed failure to replicate pSV2*Neo* was good, except for mTT1 and T9 (Fig. 5, lanes 2 and 6).

We therefore sequenced the T antigens of mTT1 and T9 to determine whether the T-antigen genes in these cell lines had sustained other types of mutations that would alter DNA replication. A PCR technique was used to amplify the SV40 T-antigen coding region from SV-HUC, mTT1, and T9 with two set of primers (see Materials and Methods). The first set of primers encompassed the 5′ part of SV40 early transcripts from nucleotides 3540 to 5199 (Fig. 6a). PCR amplification with this set of primers resulted in two products that were separated into two bands by 1% agarose gel electrophoresis. These two PCR products were derived from the authentic SV40 early transcripts for the large T and small t antigens. SV-HUC, mTT1, and T9 contained two authentic SV40 early transcripts, as shown by these two bands (Fig. 6b). The second set of primers encompassed the 3′ part of SV40 early transcripts from nucleotides 2652 to 3615. PCR amplification with this set of primers revealed only one PCR product derived from the RNAs of SV-HUC, mTT1, and T9 (data not shown). The PCR products were cloned into a V vector and sequenced (see Materials and Methods). As shown in Fig. 7, the SV40 T antigen in mTT1 contained one point mutation at codon 221, from TTA (Leu) to TTT (Phe), which is located in the SV40 ori DNA-binding domain (38). T9 contains four copies of the SV40 genome (as estimated by Southern blot analysis; data not shown). To date, we have sequenced four clones of PCR products from each set of primers described above and have not yet identified a T-antigen mutation in T9. In summary, T-antigen mutations that inactivate SV40 ori have been identified in all but one T-SV-HUC.

SV40 super T antigen results from a 147-bp repeat in the 5′ end of T antigen. To confirm and characterize the altered super SV40 T-antigen transcript identified (above) in most of the tumorigenic cell lines, we first analyzed the 5′ end of SV40 early transcripts from a representative T-SV-HUC in this series, T7, by using a PCR technique. This PCR analysis revealed a third SV40 early transcript in addition to the two authentic transcripts (Fig. 6b). We hypothesized that this additional SV40 early transcript might encode super T antigen. Cloning and sequencing of this altered SV40 early transcript revealed a 147-bp (49-amino-acid) repeat extending from codons 83 to 132. The remaining 3′ end of the T7 SV40 early transcript was also sequenced by use of the same technique and shown to contain no mutations.

DISCUSSION

While using the pSV2*Neo* vector as a vehicle for the selectable marker gene *Neo* in oncogene transfection exper-

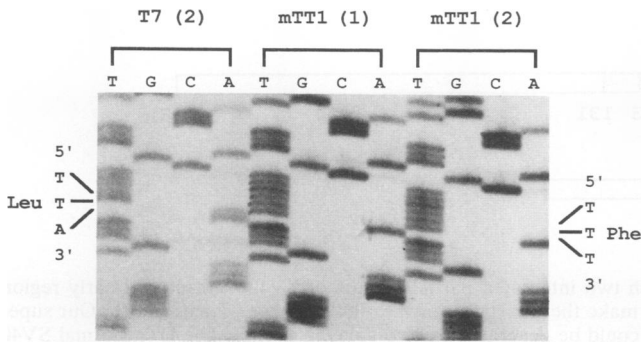

FIG. 7. Sequencing of mTT1 reveals a new mutation. Codon 221 of the wild-type SV40 T antigen is TTA, which encodes leucine (Leu). The SV40 T antigen in mTT1 contained a A → T transversion mutation at codon 221 that resulted in an amino acid change from leucine to phenylalanine (Phe). T7 was used to show the wild-type T-antigen sequence. Two clones of mTT1 [(1) and (2)] showed the same mutation. T7 clone 2 [(2)] showed a wild-type sequence.

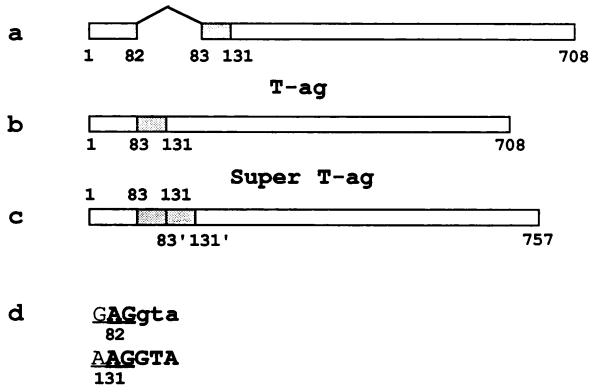

FIG. 8. mRNA structure for super T antigen. (a) The coding sequence for SV40 T antigen encodes 708 amino acids and contains an intron between codons 82 and 83. (b) Splicing for SV40 T antigen connects codon 82 to codon 83. (c) Super T antigen contains an additional 49 amino acids (repeating codons 83 to 131) besides authentic SV40 T antigen. Super T antigen therefore contains 757 amino acids. (d) The sequence near codon 131 is similar to the sequence near codon 82, which contains an authentic splicing donor site for SV40 T antigen. The numbers indicated are codon numbers for SV40 T antigen.

iments, we fortuitously observed a highly significant difference in G418^r transformation frequencies between SV40-immortalized HUC (SV-HUC) and their chemically induced tumorigenic derivatives (T-SV-HUC) (Table 1). We systematically pursued a mechanistic explanation for this difference, as reported here.

First, the observation that pSV2*Neo* is inefficient in G418^r transformation of SV-HUC led us to the discoveries that plasmid vectors, such as pSV2*Neo*, containing SV40 ori are replicated extensively when transfected into cells that contain a wild-type T antigen (Fig. 4) and that this replication is associated with poor transformation frequencies (Table 1 and Fig. 3). This finding has practical significance in that it demonstrates that expression elements containing SV40 ori, such as pSV2*Neo*, may not be suitable for some SV40-immortalized cells. A simple replacement of the wild-type ori with a mutant ori, however, corrects the problem (Table 1 and Fig. 3).

Second, the observation that pSV2*Neo* is not replicated in tumorigenic derivatives of SV-HUC, T-SV-HUC, but does efficiently transform these cells to G418^r (Table 1 and Fig. 4) led us to the discovery that T-SV-HUC sustained mutations in their T-antigen genes that would affect their abilities to initiate DNA replication from SV40 ori. The T-antigen mutations that we characterized were of two types. Western blot analysis revealed several T antigens of different sizes (Fig. 5) similar to those observed in rodent cells, including 100-, 115-, and 145-kDa altered T antigens (30, 33, 35). These altered T antigens are not only defective for but also interfere with wild-type T-antigen ori function (9, 16, 36). One of the altered T antigens found in our studies, the 100-kDa T antigen, was present in most T-SV-HUC. This altered T antigen was similar in size to the super T antigen observed by Levitt et al. in SV40-transformed mouse cells (30). We cloned and sequenced this candidate super T antigen from a representative T-SV-HUC, T7 (Fig. 6 and 8). This first example of a human SV40 super T antigen contains a duplication of 49 amino acids from codons 83 through 132 in the T-antigen gene (Fig. 8). It is interesting that codons 132 and 133 contain the same sequence as the splicing donor region of SV40 T antigen: AGGTA (Fig. 8). Codon 132 is just before the splicing donor site, and codon 83 is just after the splicing acceptor site, so the connection between codons 132 and 83 could be explained by a splicing event. Levitt et al. reported a coding sequence for a 100-kDa super T antigen that contained two integrated partial repeats of the SV40 genome (Fig. 9) (30). It is possible that a similar partial repeat of the SV40 genome exists in most of our tumor cell lines, and this rearrangement produces super T antigen by two splicing events, as proposed in Fig. 9. Super T antigen has been identified in several cell types that are nonpermissive or semipermissive for SV40 and has been implicated to interfere with the replicative function of T antigen (9, 16, 36). Our data suggest that super T antigen may play a similar role in our SV-HUC transformation system (8). In this respect, we note that most of the tumor cell lines have multiple copies of the T-antigen gene (20a) and also contain an apparently normally sized T antigen. Thus, the proposed role of SV40 super T antigen in our system would necessarily be of a transdominant nature.

Super T antigen was not detected by Western blotting in two tumor cell lines, mTT1 and T9 (Fig. 5), that showed efficient pSV2*Neo* G418^r transformation. We examined the T-antigen gene in these two cell lines for other types of T-antigen mutations. mTT1 contains only one copy of the SV40 genome (unpublished Southern blot data). Sequencing showed that this single copy of the SV40 genome in mTT1 has sustained an A → T transversion mutation (Fig. 7) that falls within the SV40 ori DNA-binding domain. Such a mutation could in theory inactivate the DNA replication competence of T antigen (38). This latter hypothesis is consistent with our results showing that pSV2*Neo* is not replicated in mTT1 cells. To date, we have not identified SV40 T-antigen mutations in any of four clones sequenced from the T9 tumor cell line. However, T9 contains at least four copies of the T-antigen gene, and further cloning and more extensive sequencing will be required to exclude this possibility.

The demonstration that most tumorigenic derivatives of SV-HUC are unable to initiate DNA synthesis from SV40 ori and show T-antigen mutations suggests a role for such mutations in our transformation system. An obvious mechanism by which mutant T antigens could play a role in our

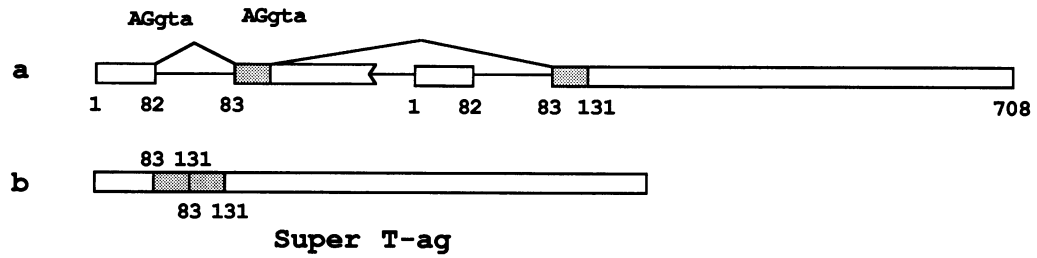

FIG. 9. (a) Model for the formation of super T antigen (30), in which two integrated partial repeats of SV40, a truncated early region followed by a complete early region in the same orientation, combine to make the transcript for the 100-kDa super T antigen. (b) Our super T antigen also contains a duplication (from codons 83 through 131) and could be generated by two splicing events. The first, normal SV40 splicing event connects codon 82 to codon 83 in the 5′ end. The second splicing event connects codon 131 in the 5′ part of the repeat to codon 83 in the 3′ part of the repeat.

system is by interfering with SV40 T-antigen-activated SV40 DNA replication and resultant cell damage and death. However, this interpretation does not explain why such mutations were only present in tumorigenic cells and not in immortal cells. One possibility is that the generation of super T antigen occurred in response to the carcinogen treatment that was part of the transformation protocol that generated T-SV-HUC (42). Extensive data demonstrate that carcinogens stimulate unscheduled DNA synthesis of integrated viral DNA sequences as well as cellular DNA sequences. Autoreplication of SV40 sequences during unscheduled DNA synthesis could lead to amplification of viral DNA sequences (26, 28). Such amplification could be deleterious to cell survival. The generation of mutations, such as super T antigen, that damper SV40 DNA synthesis during carcinogen-induced unscheduled DNA synthesis would give such cells a selective survival advantage. Alternatively, the mutant T antigens identified were more oncogenic than the wild-type T antigen. It would be possible to address this question by transfecting the mutant T antigens into SV-HUC and testing the tumorigenicity of the transformants.

The T-SV-HUC carcinogen-induced tumorigenic transformants used in this system show highly significant nonrandom genetic losses similar to those observed in clinical bladder cancers, including losses of genes on chromosomes 3p, 11p, 13q, and 18q (55), as well as activation of Ha-*ras* by a point mutation (20a). Our studies have showed that the chemicals that are transforming in this system are also mutagenic in the cells that they transform (1, 2). Therefore, we have hypothesized that a mechanism by which chemical carcinogens contribute to SV-HUC tumorigenic transformation involves causing activating mutations in oncogenes or inactivating mutations in suppressor genes (2). The present data do not contradict this hypothesis. However, they indicate that the SV40 T-antigen gene may also be an important target for mutagenesis in tumorigenic transformation of cells containing SV40. These data also provide the first example of an SV40 super T antigen in human cells and the first example of T-antigen mutations in a HUC transformation system.

ACKNOWLEDGMENTS

We gratefully acknowledge M.-H. Jeng for technical assistance with the CAT assay and comments on the manuscript.

This work was supported by National Cancer Institute grants RO1-CA29525-11 and PO1-CA51987-01 and by funds from the Haertle Foundation and the Evelyn Mercer Professorship awarded to W. S. Reznikoff. C. Kao was supported by a W. H. Peterson Fellowship and a Haertle Foundation Predoctoral Fellowship.

REFERENCES

1. **Bookland, E. A., C. A. Reznikoff, M. Lindstrom, and S. Swaminathan.** 1992. Induction of thioguanine-resistant mutations in human uroepithelial cells by 4-aminobiphenyl and its N-hydroxy derivatives. Cancer Res. **52:**1615–1621.
2. **Bookland, E. A., S. Swaminathan, R. Oyasu, K. W. Gilchrist, M. Lindstrom, and C. A. Reznikoff.** 1992. Tumorigenic transformation and neoplastic progression of human uroepithelial cells after exposure in vitro to 4-aminobiphenyl or its metabolites. Cancer Res. **52:**1606–1614.
3. **Bradley, M. K., T. F. Smith, R. H. Lasthrop, D. M. Livingston, and T. A. Webster.** 1987. Consensus topography in the ATP binding site of the simian virus 40 and polyomavirus large tumor antigens. Proc. Natl. Acad. Sci. USA **84:**4026–4030.
4. **Butel, J. S., and D. L. Jarvis.** 1986. The plasma-membrane-associated form of SV40 large tumor antigen: biochemical and biological properties. Biochim. Biophys. Acta **865:**171–195.
5. **Chang, S. E.** 1986. In vitro transformation of human epithelial cells. Biochim. Biophys. Acta **823:**161–194.
6. **Chang, S. E., J. Keen, E. B. Lane, and J. Taylor-Papadimitriou.** 1982. Establishment and characterization of SV40-transformed human breast epithelial cell lines. Cancer Res. **42:**2040–2053.
7. **Christian, B. J., C. Kao, S.-Q. Wu, L. F. Meisner, and C. A. Reznikoff.** 1990. EJ/*ras* neoplastic transformation of simian virus 40-immortalized human uroepithelial cells: a rare event. Cancer Res. **50:**4779–4786.
8. **Christian, B. J., L. J. Loretz, T. D. Oberley, and C. A. Reznikoff.** 1987. Characterization of human uroepithelial cells immortalized in vitro by simian virus 40. Cancer Res. **47:**6066–6073.
9. **Clayton, C. E., M. Lovett, and P. W. Rigby.** 1982. Functional analysis of a simian virus 40 super T-antigen. J. Virol. **44:**974–982.
10. **Conrad, S. E., C.-P. Liu, and M. R. Botchan.** 1982. Fragment spanning the SV40 replication origin is the only DNA sequence required in cis for virus excision. Science **218:**1223–1225.
11. **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.
12. **Dipaolo, J. A.** 1983. Relative difficulties in transforming human and animal cells in vitro. JNCI **70:**3–7.
13. **Geier, G. E., and P. Modrich.** 1979. Recognition sequence of the dam methylase of Escherichia coli K12 and mode of cleavage of Dpn I endonuclease. J. Biol. Chem. **254:**1408–1413.
14. **Gish, W. R., and M. R. Botchan.** 1987. Simian virus 40-transformed human cells that express large T antigens defective for viral DNA replication. J. Virol. **61:**2864–2876.
15. **Gluzman, Y., J. Davison, M. Oren, and E. Winocour.** 1977. Properties of permissive monkey cells transformed by UV-irradiated simian virus 40. J. Virol. **22:**256–266.
16. **Gurney, T., Jr., and E. G. Gurney.** 1990. An explanation of the origin and persistence of SV40 "super-T" antigens, p. 210. *In*

M. Botchan, T. Grodzicker, and D. Livingston (ed.), SV40, polyoma, and adenoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Hansen, U., D. G. Tenen, D. M. Livingston, and P. A. Sharp.** 1981. T antigen repression of SV40 early transcription from two promoters. Cell **27**:603–612.
18. **Harris, C. C.** 1987. Human tissues and cells in carcinogenesis research. Cancer Res. **47**:1–10.
19. **Kahn, P., W. C. Topp, and S. I. Shin.** 1983. Tumorigenicity of SV40-transformed human and monkey cells in immunodeficient mice. Virology **126**:348–360.
20. **Kaighn, M. E., R. R. Reddel, J. F. Lechner, D. M. Peehl, R. F. Camalier, D. E. Brash, U. Saffiotti, and C. C. Harris.** 1989. Transformation of human neonatal prostate epithelial cells by strontium phosphate transfection with a plasmid containing SV40 early region genes. Cancer Res. **49**:3050–3056.
20a. **Kao, C.** Unpublished data.
21. **Kao, C., S.-Q. Wu, M. Bhattacharya, L. F. Meisner, and C. A. Reznikoff.** 1992. Losses of 3p, 11p, and 13q in EJ/*ras*-transformable simian virus 40-immortalized human uroepithelial cells. Genes Chromosomes Cancer **4**:158–168.
22. **Kelly, T. J.** 1988. SV40 DNA replication. J. Biol. Chem. **263**:17889–17892.
23. **Khoury, G., and E. May.** 1977. Regulation of early and late simian virus 40 transcription: overproduction of early virus RNA in the absence of a functional T-antigen. J. Virol. **23**:167–176.
24. **Kingston, R. E.** 1992. Introduction of DNA into mammalian cells, p. 9.0.1–9.9.6. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York.
25. **Lacks, S., and B. Greenberg.** 1975. A deoxyribonuclease of Diplococcus pneumoniae specific for methylated DNA. J. Biol. Chem. **250**:4060–4066.
26. **Lambert, M. E., S. Pellegrini, S. Gattoni-Celli, and I. B. Weinstein.** 1986. Carcinogen induced asynchronous replication of polyoma DNA is mediated by a *trans*-activating factor. Carcinogenesis **7**:1011–1017.
27. **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.
28. **Lavi, S.** 1981. Carcinogen-mediated amplification of viral DNA sequences in simian virus 40-transformed Chinese hamster embryo cells. Proc. Natl. Acad. Sci. USA **78**:6144–6148.
29. **Lemoine, N. R., E. S. Mayall, T. Jones, D. Sheer, S. McDermid, P. Kendall-Taylor, and D. Wynford-Thomas.** 1989. Characterization of human thyroid epithelial cells immortalized in vitro by simian virus 40 DNA transfection. Br. J. Cancer **60**:897–903.
30. **Levitt, A., S. Chen, G. Blanck, D. George, and R. E. Pollack.** 1985. Two integrated partial repeats of simian virus 40 together code for a super-T antigen. Mol. Cell. Biol. **5**:742–750.
31. **Linzer, D. I. H., and A. J. Levine.** 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell **17**:43.
32. **Livingston, D. M., and M. K. Bradley.** 1987. The simian virus 40 large T antigen. A lot packed into a little. Mol. Biol. Med. **4**:63–80.
33. **Lovett, M., C. E. Clayton, D. Murphy, P. W. J. Rigby, A. E. Smith, and F. Chaudry.** 1982. Structure and synthesis of a simian virus 40 super T-antigen. J. Virol. **44**:963–973.
34. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **May, E., J.-M. Jeltsch, and F. Gannon.** 1981. Characterization of a gene encoding a 115 K super T antigen expressed by a SV40-transformed rat cell line. Nucleic Acids Res. **9**:4111–4128.
36. **May, E., C. Lasne, C. Prives, J. Borde, and P. May.** 1983. Study of the functional activities concomitantly retained by the 115,000 M_r super T antigen, an evolutionary variant of simian virus 40 large T antigen expressed in transformed rat cells. J. Virol. **45**:901–913.
37. **Melero, J. A., D. T. Switt, W. F. Mangel, and R. B. Carroll.** 1979. Identification of new polypeptide species (48-55K) immunoprecipitable by antiserum to purified large T-antigen and present in SV40-infected and -transformed cells. Virology **93**:466.
38. **Paucha, E., D. Kalderon, R. W. Harvey, and A. E. Smith.** 1986. Simian virus 40 origin DNA-binding domain on large T antigen. J. Virol. **57**:50–64.
39. **Pratt, C. I., C. Kao, S.-Q. Wu, K. W. Gilchrist, R. Oyasu, and C. A. Reznikoff.** 1992. Neoplastic progression by EJ/ras at different steps of transformation in vitro of human uroepithelial cells. Cancer Res. **52**:688–695.
40. **Reddel, R. R., Y. Ke, B. I. Gerwin, M. G. McMenamin, J. F. Lechner, R. T. Su, D. E. Brash, J.-B. Park, J. S. Rhim, and C. C. Harris.** 1988. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res. **48**:1904–1909.
41. **Reed, S. I., G. R. Stark, and J. C. Alwine.** 1976. Autoregulation of simian virus 40 gene A by T antigen. Proc. Natl. Acad. Sci. USA **73**:3083–3087.
42. **Reznikoff, C. A., L. J. Loretz, B. J. Christian, S.-Q. Wu, and L. F. Meisner.** 1988. Neoplastic transformation of SV40-immortalized human urinary tract epithelial cells by in vitro exposure to 3-methylcholanthrene. Carcinogenesis **9**:1427–1436.
43. **Reznikoff, C. A., L. J. Loretz, D. M. Pesciotta, T. D. Oberley, and M. M. Ignjatovic.** 1987. Growth kinetics and differentiation in vitro of normal human uroepithelial cells on collagen gel substrates in defined medium. J. Cell. Physiol. **131**:285–301.
44. **Rhim, J. S.** 1989. Neoplastic transformation of human epithelial cells in vitro. Anticancer Res. **9**:1345–1366.
45. **Scheffner, M., R. Knippers, and H. Stahl.** 1989. RNA unwinding activity of SV40 large T antigen. Cell **57**:955–963.
46. **Schirmbeck, R., and W. Deppert.** 1988. Analysis of mechanisms controlling the interactions of SV40 large T antigen with the SV40 ori region. Virology **165**:527–538.
47. **Shalloway, D., T. Kleinberger, and D. M. Livingston.** 1980. Mapping of SV40 DNA replication origin region binding sites for the SV40 T antigen by protection against exonuclease III digestion. Cell **20**:411–422.
48. **Shiroki, K., and H. Shimojo.** 1971. Transformation of green monkey kidney cell lines and the replication of human adenovirus and SV40 in transformed cells. Virology **45**:163–171.
49. **Smale, S. T., and R. Tjian.** 1986. T-antigen–DNA polymerase α complex implicated in simian virus 40 DNA replication. Mol. Cell. Biol. **6**:4077–4087.
50. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
51. **Stahl, H., and R. Knippers.** 1987. The simian virus 40 large tumor antigen. Biochim. Biophys. Acta **910**:1–10.
52. **Tooze, J.** 1981. DNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
53. **Van Brunt, J.** 1989. Non-transformed cell lines: the problem of being human. Bio/Technology **7**:561–563.
54. **Wiekowski, M., M. W. Schwarz, and H. Stahl.** 1988. Simian virus 40 large T antigen DNA helicase. J. Biol. Chem. **263**:436–442.
55. **Wu, S.-Q., B. E. Storer, E. A. Bookland, A. J. Klingelhutz, K. W. Gilchrist, L. F. Meisner, R. Oyasu, and C. A. Reznikoff.** 1991. Nonrandom chromosome losses in stepwise neoplastic transformation in vitro of human uroepithelial cells. Cancer Res. **51**:3323–3326.