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A Functional Simian Virus 40 Origin of Replication Is Required for the Generation of a Super T Antigen with a Molecular Weight of 100,000 in Transformed Mouse Cells

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We used two recombinant plasmids, one containing wild-type simian virus 40 DNA (pSVR1) and the other containing a simian virus 40 genome with a defective origin of replication (pSVR1-origin-minus) to transfect NIH3T3 cells. Quantitation of T-antigen synthesis by indirect immunofluorescence at 48 h after transfection with either DNA revealed the same percentage of T-positive nuclei. The transformation frequencies observed were also similar with both plasmids. Immunoprecipitation of [^{35}S]methionine-labeled cell extracts showed the expected 94,000-dalton (94K) T and 17K t antigens in all clones examined. In pSVR1-generated transformants, a 100K super T antigen was also detected. Transformants isolated from pSVR1-origin-minus transfection, however, never expressed this 100K super T antigen, and some of these clones originally also showed greatly reduced levels of 94K T antigen. However, after growth in culture for several generations, the levels of 94K T antigen synthesis in these underproducer clones were dramatically increased. A direct correlation between the amounts of T antigen synthesized and the ability to grow independently of anchorage was observed. The mechanism which brings about increasing levels of T-antigen synthesis in some of the clones is not clear, but it appears not to be due to changes in either the copy number or the methylation pattern of the integrated simian virus 40 DNA.

Simian virus 40 (SV40) encodes two early proteins, large T and small t antigens, with molecular weights of 94,000 (94K) and 17K, respectively (34). Both proteins are derived from a single transcript whose 5′ end lies near the viral origin of replication.

The large T antigen has been shown to be involved in transformation, in some cases including maintenance of certain transformed phenotypes in nonpermissive cells (34). T antigen also participates directly in many biochemical events, including initiation of viral DNA replication in lytically infected permissive cells (30), down-regulation of early RNA synthesis (22, 31), activation of host ribosomal genes (28), stimulation of cellular DNA synthesis (13), provision of adenovirus helper function (6), high-affinity binding to specific DNA sequences at the SV40 origin of replication (32), in vitro ATPase activity (33), and formation of a stable complex with a cellular 54K phosphoprotein (18).

In comparison the functions of small t antigen have not been clearly defined. Studies with deletion mutants that do not produce t antigen, but only T antigen, reveal that t is necessary for the full expression of some of the above functions of T. It has also been suggested that t antigen may have a role in transformation, perhaps as a hormone (25). Recently, Rubin et al. (23) showed that a recombinant plasmid that makes only t was unable to function in abortive transformation assays with a variety of cell lines. However, mixed infection with a T-producing, t-deletion mutant led to permanent transformation, again suggesting that t itself has an adjuvant or promoting role in transformation by SV40 T.

Variant-sized SV40 large T antigens have been helpful in distinguishing the molecular requirements for the different functions of T in lytic growth and in transformation. For example, transformed mouse cells sometimes contain a variant 100K T antigen in addition to the T and t antigens (2, 27). The presence of this variant 100K T antigen is well correlated with anchorage-independent growth in SV40-transformed mouse cells (2). Recently we and others (24; S. Chen, G. Blanck, and R. Pollack, submitted for publication) have shown that in SVT2, an

SV40-transformed BALB/c 3T3 cell line contains only an off-size early region of SV40 about 4.4 kilobases (kb). The only detectable virus-specific protein is 100K. Upon subcloning or passaging in culture, a normal-sized early region of 2.7 kb and lytic size 94K T and 17K t are detected (Chen et al., in preparation).

To further clarify the mechanism of T in maintaining the transformed state, we and others have used SV40 mutants constructed by directed mutagenesis. For example, Gluzman et al. (9) have shown that the lytic and transforming functions of large T can be genetically separated. More recently, temperature-sensitive mutants of SV40 and a nonconditional mutant of SV40 have been described, which also retain the ability to transform rat cells, but are unable to replicate SV40 DNA (7, 21, 29).

Most recently, mutations have also been constructed which eliminate the SV40 replication origin function. For example, an SV40 mutant that lacks six nucleotides at the *BglI* site in the SV40 replication origin is unable to replicate its DNA (10). This mutant is able to express its early genes and to transform permissive monkey cells (8) and semipermissive human fibroblasts (26). This origin-defective SV40 has an enhanced transformation ability in human fibroblasts compared with that of wild-type virus (26).

In the present study we have examined the transformation activity in mouse cells of a newly constructed origin-minus SV40, by using plasmids containing either the wild-type SV40 or origin-minus SV40. The expression of T, t, and the variant T proteins in the resulting transformants was analyzed by immunoprecipitation with hamster anti-SV40 tumor serum and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The growth properties of the isolated transformants were also studied.

MATERIALS AND METHODS

Cells. NIH3T3 cells were kindly provided by M. Wigler. All cells were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum at 37°C in an atmosphere of 10% CO_2. The cells were routinely checked for pleuropneumonia-like organisms by the Hoechst staining method.

Construction of bacterial plasmids. The plasmid carrying an SV40 with a defective origin of replication (pSVR1-origin-minus) used in these experiments was constructed from recombinant plasmids which contain various portions of the SV40 genome that had been cloned separately in pBR322 for other reasons. One, pSVBgl-Bam, contains SV40 sequences extending clockwise from the *Bam*HI site at 0.14 map units (m.u.) to the *BglI* site at 0.66 m.u. (approximately 2,800 base pairs). The SV40 sequences in the other, pSVBgl-RI, extend from the *BglI* site to the *Eco*RI site at 1.0 m.u. (approximately 1,750 base pairs). These plasmids were constructed by first digesting SV40 form 1 DNA with *BglI*, rendering the linear molecules blunt ended by treatment with S1 nuclease (100 U/μg of DNA, incubated at 30°C for 1 h) and attaching *Bam*HI linkers (CCGGATCCGG; Collaborative Research, Inc.) by standard techniques (17). The DNA was digested with *Bam*HI and *Eco*RI, and samples were mixed with pBR322 that in one case had been digested with *Bam*HI and in another had been digested with *Bam*HI plus *Eco*RI. After ligation and transformation of *Escherichia coli* HB101, the correct clones were identified by restriction endonuclease digestion of small-scale plasmid preparations. The origin-defective mutant of SV40 was constructed from these two recombinant plasmids plus the plasmid pSVR1, which contains the entire SV40 genome inserted in the *Eco*RI site of pBR322, as follows. First, the SV40 insert from pSVBgl-Bam was inserted into pSVBgl-RI at the *Bam*HI site. This was done by liberating the SV40 sequences from pSVBgl-Bam by digestion with *Bam*HI, and inserting this fragment into the *Bam*HI site of pSVBgl-RI, to generate pSVBam-RI-origin-minus. This procedure resulted in a plasmid that contained all of the SV40 genome except for the region between 0 and 0.14 m.u. To complete the construction, pSVR1 was first digested with *Eco*RI and *Bam*HI, and the fragment from 0 to 0.14 m.u. (730 base pairs) was purified on a 0.6% agarose gel. pSVBam-RI-origin-minus was digested to completion with the restriction endonucleases *Fnu*dII and *Eco*RI and partially with *Bam*HI. This allowed for the purification of the SV40 sequences from 0.14 to 1.0 m.u. Equimolar amounts of each gel-purified fragment were incubated with T4 DNA ligase overnight, heated to 70°C to inactivate the ligase, and digested with *Eco*RI. This DNA was then mixed with pBR322 that had been digested with *Eco*RI and treated with calf intestinal phosphatase, ligated, and transfected into *E. coli* HB101. Ampicillin-resistant colonies were screened for plasmids containing an SV40 insert by the hybridization method of Grunstein and Hogness (11). Colonies that were positive were further screened by restriction endonuclease digestion of small-scale plasmid preparations. The resultant plasmid (pSVR1-origin-minus) has a one base transition and a net insertion of 4 base pairs. The structure of the origin of replication region of this plasmid is shown in Fig. 1. The sequence was determined by the Maxam-Gilbert technique (19).

Transfection of NIH3T3 or CV1 cells with plasmid DNA. The transfection procedure used for NIH3T3 cells was essentially as described previously (36). For CV1 cells, the diethylaminoethyl-dextran method was used (20). For both methods, the cells were plated out 24 h before transfection at 10^6 cells per 100-mm petri dish. Fresh medium was given 4 h before the addition of the DNA. For NIH3T3 cells, calf thymus DNA (final concentration, 5 μg/ml) was used as carrier DNA, the calcium phosphate precipitate was left on the cells for 4 h, and then fresh medium was added. For CV1 cells a sample of diethylaminoethyl-dextran-DNA was left on the cells for 2 h. After 24 h, NIH3T3 cells were plated out at 10^5 per 60-mm petri dish for low serum selection of dense foci. Three weeks later several clones were picked with steel cloning rings for further analysis. Some of the cells were also plated out at 10^5 per 60-mm petri dish in 0.33% agarose on top of plates previously coated with 0.5% agarose. Several

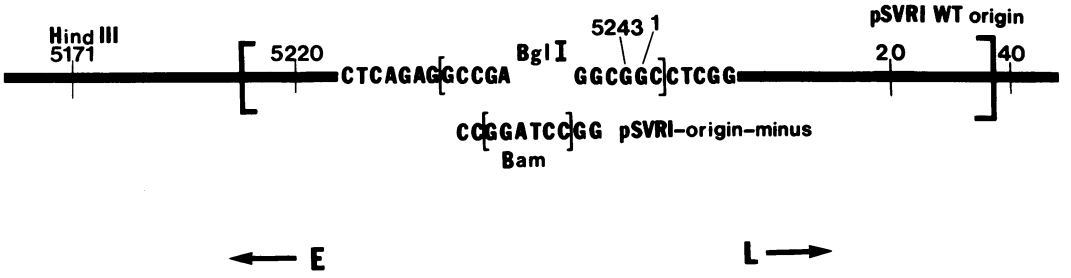

FIG. 1. The *Bgl*I site is bracketed in pSVR1. The *Bam*HI site is bracketed in pSVR1-origin-minus. The entire 10-mer *Bam* linker was inserted into the SV40 origin of replication in place of the CCGAGG of the *Bgl*I site. The pSVR1 sequence is broken to show the homology between pSVR1-origin-minus and pSVR1 sequences.

anchorage-independent colonies were picked from these dishes for further studies. At 48 h after transfection cover slips with either NIH3T3 or CV1 cells were scored for T antigen-positive cells by nuclear immunofluorescence.

Replication and production of SV40 by the plasmid DNA. To check for SV40 DNA replication, permissive monkey cells were infected with either the wild-type (pSVR1) or mutant SV40 plasmid (pSVR1-origin-minus). Low-molecular-weight DNA was collected by the Hirt method (14) 72 h posttransfection, separated on 1.5% agarose gel, transferred to nitrocellulose paper, and hybridized to ^{32}P-labeled pSVR1 probe as described elsewhere (1a).

To examine virus production, the cell lysate was collected 10 days after transfection of plasmid DNA into CV1 cells, freeze-thawed three times, and used to infect fresh plates of CV1 cells. After 48 h the infected cells were labeled with [^{35}S]methionine for 2 h, and cell extracts were prepared, immunoprecipitated with hamster anti-SV40 tumor serum, and analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (2).

Analysis of the integrated SV40 DNA in NIH3T3 cells. Genomic digests and Southern blotting were carried out as described previously (1a, 35).

RESULTS

Origin-minus SV40 mutant is unable to replicate or yield virus. Six nucleotides have been deleted at the *Bgl*I site. (0.67 m.u.), and 10 bp of *Bam* linker inserted at this site. This substitution resulted in the replacement of the *Bgl*I site with a *Bam*HI site flanked by two new *Hpa*II sites (Fig. 1). To test the ability of this SV40 mutant to replicate in permissive monkey cells, the following experiments were carried out.

The plasmid was linearized by *Eco*RI digestion and transfected into CV1 cells by the diethylaminoethyl-dextran method (20). At 72 h posttransfection, low-molecular-weight DNA was extracted from the transfected cells by the procedure of Hirt (14). The DNA was separated on agarose gel and then transferred to nitrocellulose paper and hybridized with ^{32}P-labeled pSVR1 probe. Both form I and form II DNA were detected in cells transfected with pSVR1. In cells transfected with pSVR1-origin-minus, only a trace amount of input plasmid DNA remained, whereas neither form I nor form II DNA was present, indicating that this plasmid cannot replicate in permissive monkey cells (data not shown). The inability of pSVR1-origin-minus mutant plasmid to replicate in monkey cells was due to the defective origin. Cotransfection with a frameshift mutant, pSVR1-H, was used to show this. The frameshift mutant is unable to replicate in monkey cells due to lack of a functional SV40 T antigen (D. Lewis et al., Proc. Natl. Acad. Sci. U.S.A., in press). However, cotransfection of pSVR1-H and pSVR1-origin-minus plasmid DNAs into monkey cells resulted in replication of SV40 DNA as detected by Southern blot analysis (data not shown). Therefore, the pSVR1-origin-minus plasmid must make a SV40 T antigen with full lytic function.

To further test the mutant plasmid's capacity to replicate viral DNA and to generate virions, a more sensitive assay was carried out. Both wild-type and mutant plasmid DNAs were transfected into CV1 cells. Ten days later the lysate was collected and used to infect in fresh CV1 cells. After 48 h, the cells were labeled with [^{35}S]methionine, extracted, and immunoprecipitated with hamster anti-SV40 tumor serum or normal hamster serum and analyzed on polyacrylamide gel electrophoresis (Fig. 2). Lane A shows mock infection of CV1 cells. Lane B shows CV1 infected with wild-type SV40 virus 777. Lane C shows CV1 infected with lysate from pSV-3 transfection. pSV-3, provided by P. Berg, is a plasmid with wild-type SV40 cloned at the *Bam*HI site of pBR322. Lane D is same as lane C, except the plasmid used was pSVR1, a wild-type SV40 cloned at the *Eco*RI site of pBR322. Lane E shows CV1 infected with pSVR1-origin-minus as described above, and lane F shows CV1 infected with another origin-defective SV40 mutant prepared by Gluzman (10). Lanes B, C, and D show the expected large T and small t antigens and also a breakdown product of large T. Neither lane E nor lane F shows any produc-

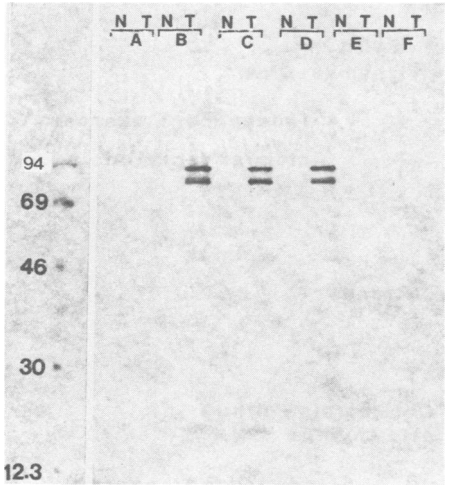

FIG. 2. CV1 cells were transfected with plasmid DNA, and 10 days later the lysates were collected and used to infect freshly plated CV1 cells. At 48 h postinfection the cells were labeled with [^{35}S]methionine, and cell extracts were prepared and immunoprecipitated with either normal hamster serum (N) or hamster antitumor serum (T) and analyzed on 10 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: (A) mock infection, (B) wild-type SV40 virus strain 777, (C) pSV-3 (wild-type SV40 plasmid from P. Berg), (D) pSVR1, (E) pSVR1-origin-minus, and (F) pOri$^-$ (another origin-defective plasmid from Y. Gluzman).

tion of the proteins encoded by SV40. Therefore, permissive cells transfected with either of the two origin-defective SV40 plasmids are unable to produce mature virions from an otherwise undetectable low level of replication.

Transformation of NIH3T3 cell by wild-type SV40(pSVR1) or origin-minus SV40(pSVR1-origin-minus) plasmids. Transformation was carried out with SV40 DNA cloned in plasmid pBR322. The plasmid DNA contained either the wild-type SV40 cloned at the *Eco*RI site of the plasmid (pSVR1) or the origin-minus SV40(pSVR1-origin-minus) as described above. NIH3T3 cells were transfected with either type of the *Eco*RI-linearized plasmid by the calcium phosphate method with calf thymus DNA as the carrier (36). Immunofluorescence of T antigen at 48 h posttransfection with either type of plasmid DNA yielded 0.2 ± 0.05% of T antigen-positive cells per μg of input DNA. This indicates that the SV40 early promoter had not been negatively affected by the manipulation used to construct this plasmid. Similar results have been obtained after transfection into more readily transfected HeLa cells (D. Lewis and J. Manley, unpublished data).

Three weeks later transformants were counted or cloned either as dense foci in the low-serum assay or as colonies in the anchorage independence assay. Some of the transformed colonies isolated from the pSVR1-origin-minus transfection in the agarose assay were bigger than those from pSVR1 transfection. However, no difference in anchorage transformation frequency was detected in NIH3T3 cells transfected with the two different plasmid DNAs (6.5 ± 3.0 agarose colonies per 10^5 cells for pSVR1 and 6.0 ± 2.0 agarose colonies per 10^5 cells for pSVR1-origin-minus).

The pSVR1-origin-minus mutant appears to transform NIH3T3 cells about threefold more efficiently than does the origin-defective SV40 mutant described by Gluzman (10; N. Nicholson, unpublished observation). The basis for this difference is unclear. All dense foci arising in low serum were isolated and tested for nuclear T antigen by immunofluorescence. Since mock-infected NIH3T3 cells sometimes give rise to false dense foci, the number of dense foci generated by the two plasmids could not be quantitated with these cells.

Reduced anchorage growth in early passage pSVR1-origin-minus transformants. Transformed foci that arose in the low-serum and the anchorage assays were isolated for further analysis. The geneology of these clones and subclones is shown in Fig. 3. Three clones isolated as dense foci in the low-serum assay were analyzed further for their ability to form colonies in high and low sera and in agarose (Table 1). Colonies isolated from either pSVR1 or pSVR1-origin-minus transfection had comparable colony-forming ability in either high or low serum. In the anchorage assay, the three clones isolated from pSVR1 gave a similar number of colonies (Table 1). However, plating efficiencies in agarose of three of the five pSVR1-origin-minus transformants were reduced. Only two of the clones isolated from pSVR1-origin-minus mutant transfection showed plating efficiencies similar to those of the wild-type clones, whereas the other three clones showed a 2- to 10-fold decrease in agarose growth (Table 1). The difference in anchorage-independent growth of the clones was found to be related to their expression of large T antigen.

94K T and 100K super T are produced by wild-type pSVR1-transformed mouse cells. The original clones isolated from transfection with the wild-type pSVR1 plasmid were analyzed by immunoprecipitation to detect virus-specific proteins. The cells were labeled with [^{35}S]methionine and immunoprecipitated with either normal hamster serum or hamster antitumor serum and analyzed on 10 to 20% gradient polyacrylamide gels (Fig. 4). All clones examined showed the expected large T and small t antigens and also the nonviral 54K protein, which binds tightly to

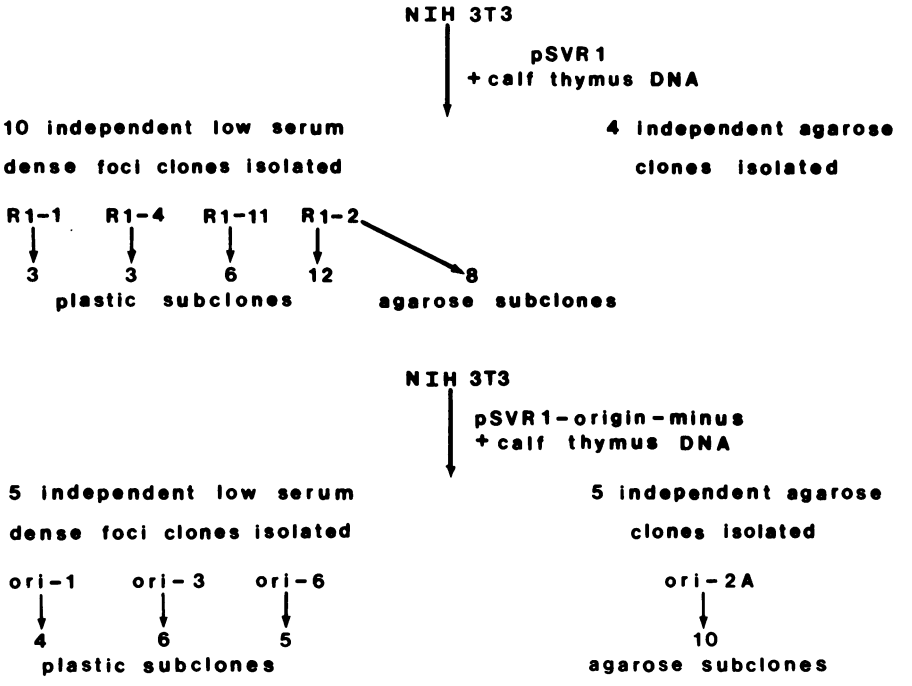

FIG. 3. Geneology of primary clones and subclones of transformed NIH3T3 cells derived from either transfection with pSVR1 or pSVR1-origin-minus DNA. Plastic subclones were picked from 10^2 plates with a steel cloning ring. Agarose colonies were isolated from 10^4 plates after 3 weeks.

T antigen (18). Seven of the 10 primary clones examined soon after isolation also expressed the 100K super T antigen. The three remaining clones showed the 100K T after 7 to 10 passages in culture (data not shown).

NIH3T3 cells transformed by SV40 virus strain 777 (Fig. 4, lanes A) showed both the 94K T and the 100K variant T. Plasmid transformant clone R1-1 (Fig. 4, lanes B) showed mostly the 94K T antigen and a trace amount of the 100K T. Clones R1-4 (Fig. 4, lanes C) produced only the 94K T. Clone R1-11 (Fig. 4, lanes D) contained both 94K and 100K T antigens. These three clones, R1-1, R1-4, and R1-11, were carried in culture for 3 months (about 15 passages), and [^{35}S]methionine-labeled cell extracts were then immunoprecipitated with hamster antitumor serum. All three clones now express 17K, 94K, and 100K proteins (Fig. 4, lanes E, F, and G, respectively).

Subclones were also isolated from four primary clones (R1-1, R1-2, R1-4, and R1-11) (Fig. 3). R1-2 and R1-4 did not show the 100K protein initially, but their subclones all express the 100K super T. A total of 27 subclones have been examined, and all express the 100K protein in addition to 17K and 94K t antigens, as judged by immunoprecipitation and sodium dodecyl sulfate-gel electrophoresis (data not shown). Therefore either by passaging in culture or by subcloning, all clones and subclones originating from pSVR1-transfected cells eventually gave rise to the 100K T.

Four primary clones were also isolated from the anchorage assay (Fig. 3). Soon after isolation these clones were labeled with [^{35}S]methionine, and cell extracts were prepared and immunoprecipitated with hamster antitumor serum. All four clones expressed 100K, 94K, and 17K t antigens. Eight subclones were isolated from agarose, and all of these also expressed the 100K variant T antigen in addition to the 94K and 17K t antigens (data not shown).

Since these transformants were generated by a plasmid containing wild-type SV40 DNA, the sequences encoding the 100K variant protein that we and others have described earlier (2, 27) cannot have arisen from preexistent variant sequences in virus stocks, but must be a consequence of a frequent event occurring soon, but not necessarily immediately, after the infection of mouse cells by wild-type SV40 DNA.

94K T, but not 100K super T, is produced in mouse cells transformed with pSVR1-origin-minus mutant plasmid. NIH3T3 cells were transfected with DNA from the pSVR1-origin-minus mutant plasmid. Five primary dense foci were picked from a low-serum assay and analyzed for

TABLE 1. Growth properties of mouse transformants isolated from transfection with pSVR1 or pSVR1-origin-minus plasmids[a]

Cells	Avg no. of colonies per 10^2 cells ± SE		Avg no. of colonies >0.2 mm per 10^4 cells ± SE
	10% FCS	1% FCS	
R1-1	25.7 ± 3.7	3.5 ± 0.4	13.0 ± 0.6
R1-4	13.7 ± 0.1	4.0 ± 1.2	11.0 ± 1.7
R1-11	13.3 ± 0.9	3.5 ± 1.2	15.3 ± 2.0
Ori-1	26.0 ± 3.1	3.0 ± 0.8	20.7 ± 2.9
Ori-3	12.7 ± 1.5	2.5 ± 0.4	1.3 ± 0.2
Ori-4	15.3 ± 3.5	3.5 ± 0.7	2.7 ± 0.6
Ori-6	20.0 ± 4.2	2.9 ± 0.9	8.7 ± 0.9
Ori-7	18.8 ± 2.4	3.9 ± 0.9	21.7 ± 2.7

[a] Three clones isolated from pSVR1 transfection and five clones isolated from pSVR1-origin-minus transfection as dense foci in the low-serum assay were analyzed further for their growth properties in 1% and 10% fetal calf serum (FCS) and in 0.33% agarose. For colony formation in 1% or 10% serum, the plates were fixed and stained with crystal violet at the end of 10 days. For the agarose assay, the plates were scored under a dissecting scope 3 weeks later for colonies >0.2 mm in diameter. All cells were fed twice a week.

virus-specific protein shortly after isolation. Immunoprecipitation was done on [^{35}S]methionine-labeled cell extracts, and proteins were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All five clones expressed the 94K and 17K t antigens and host 54K protein, but none of the clones showed any trace of the 100K protein detected in clones transformed by the wild-type plasmid.

pSVR1-origin-minus transformants showed an unexpected dispersion in 94K T antigen production. Two of the five clones, Ori-1 and Ori-7 (Fig. 5, lanes A and E), showed considerably more 94K T than did the other three clones, Ori-3, Ori-4, and Ori-6 (Fig. 5, lanes B, C, and D). Equal amounts of trichloroacetic acid-precipitable protein were used in the immunoprecipitation assay, so a difference in 94K T expression implies that different amounts of this protein are being synthesized, or that the 94K T in one clone is more stable than that in another. This clonal variation in 94K T antigen expression has been reproducibly observed in many experiments.

94K T production increased in pSVR1-origin-minus transformants upon subculture. Three primary clones, Ori-1, Ori-3, and Ori-6, were carried in culture for 3 months (about 15 passages). Immunoprecipitation of these [^{35}S]methionine-labeled cell extracts indicated that Ori-1, which previously had shown high levels of 94K protein, still produced large amounts of 94K T. Ori-6, which originally showed an intermediate level of 94K antigen, now produced more of this protein. Ori-3, the clone which originally produced only low levels of the 94K protein, now also synthesized high levels of 94K T antigen (Table 2).

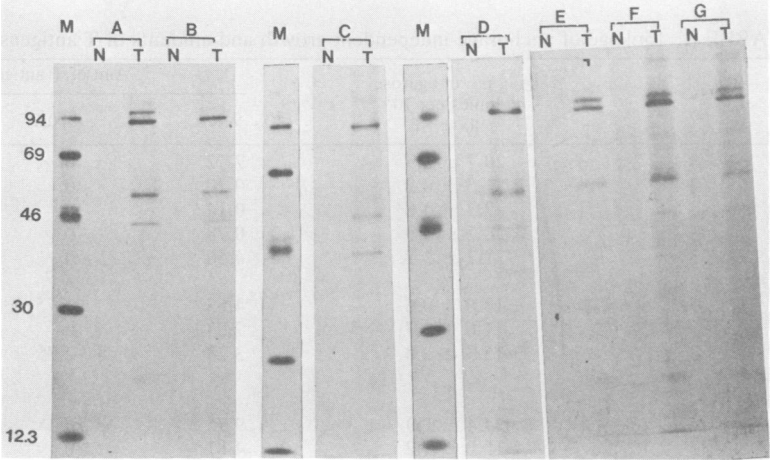

FIG. 4. [^{35}S]methionine-labeled cell extracts from NIH3T3 cells transformed with wild-type virus strain 777 or plasmid pSVR1. The labeled cell extracts were immunoprecipitated with either normal hamster serum (N) or hamster antitumor serum (T) and analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All immunoprecipitations were done with equal numbers of trichloroacetic acid-precipitable counts. Lane A contains NIH3T3 cells transformed by SV40 virus strain 777 and shows 17K t, 54K nonviral host protein, and 94K and 100K T antigens. Lanes B, C, and D contain R1-1, R1-4, and R1-11, respectively, analyzed shortly after isolation. R1-1 showed a trace amount of 100K T, none was detected in R1-4, and R1-11 showed more of the 100k T. Lanes E, F, and G contain R1-1, R1-4, and R1-11, respectively, after being in culture for 3 months (about 15 passages). All cells expressed the 100K variant T antigen.

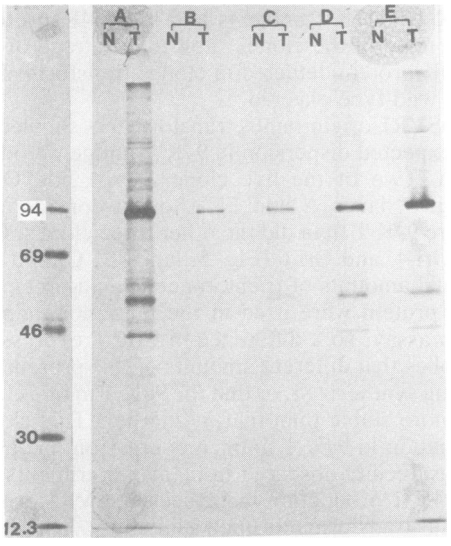

FIG. 5. Transformed NIH3T3 cells from transfection with the mutant plasmid pSVR1-origin-minus. The cells were labeled with [^{35}S]methionine, and cell extracts were prepared, immunoprecipitated with either normal hamster serum (N) or hamster anti-tumor serum (T), and analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Equal numbers of trichloroacetic acid-precipitable counts were used in the immunoprecipitation. Lanes: (A) Ori-1, overproduction of 94K protein; (B) Ori-3; (C) Ori-4, no overproduction of 94K; (D) Ori-6, moderate amount of 94K T antigen; and (E) Ori-7, similar to Ori-1 clone, abundant amounts of 94K.

The amounts of 94K and 100K T antigens on the autoradiograms were quantitated on a spectrophotometer equipped with a gel scanner (2). The relative amounts of the 94K T antigen present in each of the clones either shortly after isolation (3rd passage) or after 3 months in culture (15th passage) are presented in Table 2. Shortly after isolation of these clones there was almost a 40-fold difference between Ori-1 and Ori-3 clones (Fig. 5, lanes A and B; Table 2). After the cells have been in culture for 3 months, the differences are reduced to only twofold (Table 2). The difference in 94K T antigen expression between Ori-1 and Ori-6 was about 10-fold shortly after isolation of the clone, but at the end of the 3-month period there was almost no difference between the two clones (Table 2). Ori-4 and Ori-7 were also examined at early passage; Ori-7 showed high levels of 94K T antigen, whereas Ori-4 showed very low amounts of 94K protein, similar to those or Ori-3 (Table 2). These two clones were not further carried in culture.

The ability of pSVR1-origin-minus clones to form colonies in agarose is related to the amount of the 94K T antigen (Fig. 6). As described above, only two of the clones had plating efficiencies similar to those of the clones isolated from wild-type plasmid transfection (Table 1). The two clones are Ori-1 and Ori-7, which contained considerably more 94K T antigen than Ori-3, Ori-4, and Ori-6 (Fig. 5, Table 2). The

TABLE 2. Linkage of anchorage-independent growth and amounts of T antigens[a]

Cells	Passage no.	Avg no. of agarose colonies per 10^4 cells ± SE	Amt of T antigens		
			94K	100K	Total
Ori-1	3	20.7 ± 2.9	7.72	0	7.72
Ori-3	3	1.3 ± 0.2	0.20	0	0.20
Ori-4	3	2.7 ± 0.6	0.14	0	0.14
Ori-6	3	8.7 ± 0.9	0.78	0	0.78
Ori-7	3	21.7 ± 2.7	4.56	0	4.56
R1-1	3	13.0 ± 0.6	4.63	1.75	6.38
R1-4	3	11.0 ± 1.7	7.03	0	7.03
R1-11	3	15.3 ± 2.0	3.28	2.75	6.03
Ori-1	15	24.4 ± 1.0	9.75	0	9.75
Ori-3	15	15.0 ± 1.2	4.81	0	4.81
Ori-6	15	21.6 ± 1.2	8.73	0	8.73
R1-1	15	17.3 ± 1.2	4.55	1.66	6.21
R1-4	15	15.1 ± 0.6	5.25	1.13	6.38
R1-11	15	18.1 ± 1.8	3.50	2.75	6.25

[a] For colony formation the cells were treated as described in footnote *a* Table 1. For quantitation of 94K T and the 100K variant T antigens, the autoradiograms were scanned on a Gilford 250 spectrophotometer equipped with a Gilford 2520 gel scanner. The area under the peak was then determined. The third passage of the cells was shortly after isolation of the clones. Some of the clones were carried in culture for 3 months (about 15 passages).

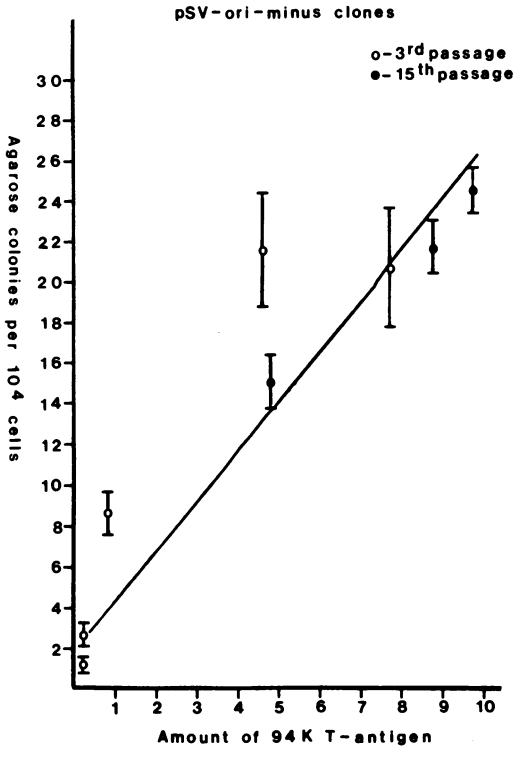

FIG. 6. Linkage of 94K T antigen and plating efficiency in agarose of Ori clones. Amounts of T antigens were determined as described in footnote *a* of Table 2. The slope of the line was determined by a least-squares linear regression.

amount of 94K T in Ori-1 and Ori-7 is statistically indistinguishable from the total amounts of 94K and 100K T in pSVR1 clones ($P > 0.20$) (Table 2).

Ori-1, Ori-3, and Ori-6 were tested for their ability to grow in agarose after being in culture for 3 months. Ori-1 showed a plating efficiency of $24.4 \pm 1.0/10^4$ cells plated, which is similar to the frequency obtained at earlier passage (Table 2). Ori-3 now showed a plating efficiency of $15.0 \pm 1.2/10^4$ cells plated, which is about 10-fold higher than earlier passage (Table 2). The amount of 94K protein remained about the same in Ori-1, whereas it increased by 25-fold in Ori-3 (Table 2). A 25-fold increase in T antigen therefore appears to have resulted in a 10-fold increase in plating efficiency in agarose. Ori-6 also showed an 11-fold increase in 94K T and a 2.5-fold enhancement in agarose growth.

Clones isolated from pSVR1 transfection did not show much change in total 94K and 100K T antigen expression with passage (Table 2). In one clone, the amounts of 94K and 100K T antigen seemed to be regulated inversely, that is, an increase in 100K T resulted in a decrease in 94K T. Growth in agarose also remained essentially the same from the 3rd to the 15th passage (Table 2). Small t antigen did not consistently vary from clone to clone (data not shown).

In wild-type pSVR1 clones, anchorage-independent growth appears to be regulated by both 94K and 100K T antigens. By the time the agarose colonies were scored (3 weeks after plating), most, if not all, of the clones probably expressed 100K T. In the R1 clones both anchorage-independent growth and the sum of 94K and 100K T antigens were approximately the same from clone to clone (Table 2).

Twenty-five subclones were isolated from four primary pSVR1-origin-minus clones, 10 of which were subclones picked from agarose. None of the clones examined by immunoprecipitation showed even trace amounts of the 100K variant T antigen (data not shown). Ori-3 is one of the clones that showed only a small amount of 94K T antigen. We isolated subclones of Ori-3 with higher level of 94K T protein (data not shown). Similarly, from Ori-1, a clone with a higher amount of 94K T, subclones could be isolated (two of six) with lesser amounts of 94K T antigen. The capacity of these subclones to grow in agarose has not been tested. Of the 15 subclones, 8 showed higher amounts of 94K T; 8 of the 10 agarose subclones also showed more 94K protein. Apparently, clonal variation in expression of 94K T continues to occur even after many passages of the cells.

In summary, transformants from wild-type pSVR1 DNA gave rise to primary clones with or without 100K variant T antigen, but with passage and subcloning all clones express this variant T. In contrast, pSVR1-origin-minus transformants never express the 100K variant T antigen. Most pSVR1-origin-minus clones ultimately produce levels of 94K T similar to that of the total amounts of 94K and 100K T in wild-type pSVR1 transformants, and these clones behave like wild-type SV40-transformed cells in the anchorage assay.

Structure of SV40 DNA in mouse cells transformed by pSVR1 or pSVR1-origin-minus plasmids. We have begun to study the integrated viral DNA in the pSVR1 and pSVR1-origin-minus transformants from the third passage as described above. A combination *Bgl*I and *Bam*HI digestion liberates a 2.7-kb fragment of DNA from normal SV40 DNA (Fig. 7A, lane R). This fragment is generated by the same set of enzymes in three pSVR1 clones, R1-1, R1-4, and R1-11 (Fig. 7A, lanes A, B, and C, respectively). In the case of pSVR1-origin-minus transformants, the same size fragment should be liberated by *Bam*HI alone, since a *Bam*HI site has replaced the *Bgl*I site at the SV40 origin of replication. This result is shown for three clones, Ori-1, Ori-3, and Ori-6 (Fig. 7A, lanes D,

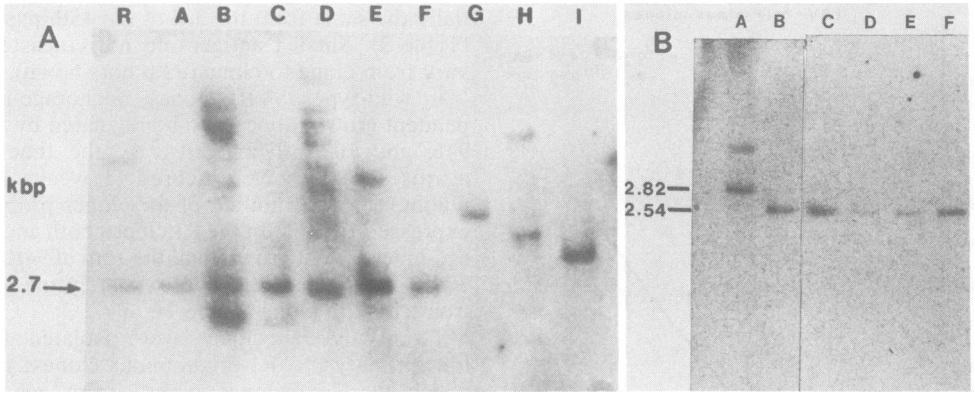

FIG. 7. (A) Genomic DNA digestion and Southern blotting were carried as described previously (35; Blanck et al., in press). Lane R contains SV40 DNA digested with *Bgl*I and *Bam*HI; the 2.7-kb fragment was detected as expected. Lanes A through C contain R1-1, R1-4, and R1-11, respectively, digested with *Bgl*I and *Bam*HI; again, a 2.7-kb fragment was observed in each clone. Lanes D through F contain Ori-1, Ori-3, and Ori-6, respectively; the 2.7-kb fragment was generated by digestion with *Bam*HI alone. Identical digestion with R1 clones did not give rise to the 2.7-kb fragment (lanes G through I). (B) Lanes A and B contain SV40 DNA digested with *Msp*-*Bcl*I and *Bgl*I-*Bcl*I, respectively. Lanes C and E contain DNA from Ori-6 digested with *Msp*-*Bcl*I at early and late passage, respectively. Lanes D and F contain DNA from Ori-6 digested with *Hpa*II-*Bcl*I at early and late passage, respectively.

E, and F, respectively). As expected, *Bam*HI digestion alone did not generate a 2.7-kb fragment from the pSVR1 transformants (Fig. 7A, lanes G, H, and I).

We considered the possibility that the difference in T-antigen expression between pSVR1-origin-minus clones at early and late passage was due to methylation of the SV40 DNA. Since the addition of *Bam* linkers at the SV40 origin in pSVR1-origin-minus plasmid created two *Hpa*II-*Msp* sites on either side of the *Bam*HI site, it was possible to examine the state of methylation near the start of transcription of the early region.

DNA from Ori-1, Ori-3, and Ori-6 at early and late passage was digested first with either *Hpa*II or *Msp* and then with *Bcl*I. The digests were blotted as described previously (1a, 35). *Msp* cuts independently of methylation at cytosine, whereas *Hpa*II will only cut at demethylated cytosine. Results from all three clones indicate no detectable methylation of the *Hpa*II-*Msp*-*Bcl*I fragment. Also the intensity of the integrated SV40 DNA is about the same for all cell lines at either early or late passage. These results suggest that the differences in 94K T antigen expression between Ori-1, Ori-3, or Ori-6 appear not to be due to either amplification or methylation of the integrated SV40 DNA. *Hpa*II-*Msp*-*Bcl*I digestions of Ori-6 are shown in Fig. 7B.

DISCUSSION

Mouse NIH3T3 cells can be transformed equally well with recombinant plasmid DNAs lacking or containing the origin of SV40. Transformants that resulted from transfection with wild-type pSVR1 showed viral specific proteins of 17K, 94K, and 100K. A functional SV40 origin of replication is required not only for the lytic growth of the virus in permissive monkey cells, but also for the generation of the 100K super T antigen. Thus, mutation in the origin of replication region of SV40 DNA totally prevents the expression of the 100K super T antigen, but does not necessarily affect synthesis of the normal 94K T antigen. pSVR1-origin-minus transformants grow in agarose more efficiently when they produce relatively large amounts of 94K T antigen. Early passage pSVR1-origin-minus transformants make abnormally low amounts of 94K T antigen. This reduction is apparently not due to differences in either copy number or methylation of the integrated SV40 DNA.

We have reported a correlation between the amounts of the 100K T antigen and the ability to grow in agarose in mouse cells transformed by wild-type SV40 virus (2). Since that study was carried out with cell lines transformed by virions, the possibility remained that 100K T resulted from integration of a preexisting variant or a defective virus in the virus stock. Therefore, we have used a plasmid containing wild-type SV40 sequences for the present study. From the experiments reported here, it is clear that variant 100K T protein can be generated in cells transformed by molecularly cloned wild-type SV40 DNA.

The mutant plasmid pSVR1-origin-minus gave rise to transformants that showed an unexpected

disparity in the amount of SV40 T antigen. All clones and subclones isolated from this plasmid showed both 17K t and 94K T antigens, but none showed the variant 100K T antigen. Instead, some of the clones initially showed 40- to 50-fold lower amounts of the 94K T antigen. This reduced 94K T-antigen expression was reflected in the reduced ability of these clones to form colonies in agarose.

One explanation for our results is that sequences encoding the 100K variant T antigen come about from replication of the input DNA during integration. Extensive rearrangements involving both the input DNA and the flanking host sequences after SV40 infection have been shown before (1, 1a, 3, 5, 15, 16, 24). Lacking a functional SV40 origin, the input DNA cannot replicate and therefore probably cannot rearrange. This may be why the 100K super T cannot be generated in cells isolated from pSVR1-origin-minus plasmid transfection. However, it is important to note that some wild-type pSVR1-transformed clones apparently contain only a wild-type-sized *Bgl*I-*Bam*HI early region (2.7 kb), and nevertheless synthesize the 100K T antigen (Fig. 4 and 7).

pSVR1-origin-minus plasmid is unable to replicate in permissive monkey cells. However, this plasmid remains able to transform mouse cells. The separation of replication and transformation in SV40 has been shown previously by Gluzman et al. (8–10). Although a functional origin of replication and T are both required for the replication of the virus, the former is not needed in transformation. Recently Clayton et al. (4) reported transformation of rat cells with a plasmid encoding a 145K super T, but not wild-type 94K T antigen. Despite retention of a functional origin, this plasmid cannot replicate in permissive monkey cells, probably due to a defect in the 145K super T antigen.

Blanck et al. (1a) used the SV40-transformed Swiss-3T3 SV101 cell line and its subclones and revertants to show that anchorage-independent subclones of SV101 retain more defective SV40 DNA than do anchorage-dependent subclones. With a set of restriction enzymes, defective SV40 DNA fragments of 4.3 or 3.7 kb were seen in SV101 and all anchorage-independent subclones of SV101. The 100K variant T antigen was shown to correlate with anchorage-independent growth (2). Therefore, it seemed likely that in SV101 the 100K protein is encoded by either one or both of the defective DNAs. To compare 100K-encoding sequences, we are now examining in detail the SV40 DNA patterns in cells isolated from both wild-type pSVR1 and mutant pSVR1-origin-minus transfection. In addition, SV40-containing sequences from SV101 DNA have recently been obtained by cloning into bacteriophage lambda (A. Levitt, manuscript in preparation). Whether the same 100K T antigen is encoded by one of these DNA fragments, and what role the SV40 origin plays in the generation of these fragments, will be determined.

Some of the clones isolated from pSVR1-origin-minus transfections originally showed a reduced level of the 94K T antigen relative to other clones. The conversion to higher level producers of 94K T observed at later passage in these clones probably is not due to amplification of the integrated SV40 DNA. Also, methylation at both new *Hpa*II sites near the origin of replication is apparently not responsible for the differences observed in 94K T-antigen expression. We are in the process of determining whether the dramatic increase in T-antigen expression results from a higher rate of transcription or a posttranscriptional modification that renders the protein or RNA more stable or both.

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