Expression of 100,000- M_r Simian Virus 40 (SV40) Tumor Antigen in Mouse Fibroblasts Transfected with Replication-Defective SV40 Genomes

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Simian virus 40 early region mutants which are partially or completely replication defective were tested for their ability to transform postcrisis mouse fibroblasts. All mutants tested were capable of generating anchorage-independent transformants. We have previously reported the presence of a variant tumor antigen of 100,000 M_r (100K protein) generated upon transformation by wild-type simian virus 40 virions which correlates with anchorage-independent growth (Chen et al., *Mol. Cell. Biol.* 1:994-1006, 1981). In this study, none of the mutants tested produced the 100K variant protein at early (before the fifth) passage. Long-term passage (>20 weeks) permitted the expression of this 100K variant in half of the transformants. Thus the phenotype of these mutants is different from both wild-type simian virus 40 (frequently production of 100K by the third passage, and always by the tenth passage) and the origin-minus class of mutants (no production of 100K at any passage).

Simian virus 40 (SV40) is a small DNA virus capable of generating tumors in rodents (16) and of transforming a variety of cells in culture (1, 3, 4, 24, 30, 35, 40). In a nonpermissive interaction at least two early proteins, termed tumor antigens, are generated which correspond to the early proteins of the lytic growth cycle. The small tumor antigen, first reported by Prives and co-workers (33) (M_r , 17,000 [17K protein]), has an unknown function and seems to be dispensable for transformation in at least some species of cells (46). The large tumor antigen (T-ag), first described by Tegtmeyer et al. (44) (94K), is coded for by the same cistron but translated from a message produced by an alternate splicing of the primary RNA transcript (2). This protein has a multitude of functions including a weak poly(dT)-stimulated ATPase activity (15), specific (22, 36) and non-specific (8, 38) DNA binding capability, and the ability to regulate its own synthesis (37, 44), to initiate viral DNA replication (43), to induce cellular DNA synthesis in the lytic cycle (14, 20, 21), and to induce late transcription independent of viral replication (7, 23). The protein is bound to (26) and stabilizes (28) a host embryonic protein (9) (54K in mouse cells and forms oligomers (6), and a small fraction of the protein is ADP ribosylated (19).

At present it is not clear which, if any, of these functions is necessary for the oncogenic potential of SV40. For instance, it has recently been demonstrated that the ability of T-ag to bind specifically to the SV40 origin of replication and the ability to induce viral replication are not required for T-ag to remain transformation competent (17, 42).

Under certain conditions, SV40-cell interactions can generate unusual proteins related to T-ag of 100K, 115K, or 130K (10, 25, 47). Of these, the 100K version is most commonly formed, although its presence seems to be restricted to mouse fibroblasts.

In a set of related mouse cell lines generated from a single line transformed by wild-type SV40 virus (SV101), the

amount of 100K protein correlated extremely well with the ability of the cells to grow in soft agarose when analyzed by rank correlation analysis (12). In these same related cell lines, the presence or absence of a specific set of defective early region DNA sequences was also shown to be related to the presence or absence of the protein that was larger than the wild-type protein (5).

Furthermore, a tandem repeat of the 5' portion of wild-type SV40 early region linked to a complete early region constructed *in vitro* is capable of expressing both 94K and 100K T-ag in a transient monkey cell assay (27).

SV40 mutants which fail to replicate their viral DNA fall into at least two classes: (i) those with defective origins of replication, some of which are unable to bind T-ag (such as the ORI-negative mutants), and (ii) those with mutations in the T-ag-coding region which render the T-ag unable to initiate replication. Here, too, some mutant T-ag (such as the T-ag from the SV40 mutant C2 [32]) can and some (for example the T-ag from the SV40 mutant C6 [39]) cannot bind to the SV40 origin of replication.

In past attempts to understand the genesis of the 100K protein, an origin-defective (and therefore replication-defective) mutant of SV40 was tested for its ability to generate 100K. Under no circumstances could this protein be detected in such ORI-negative transformed cells (11). This suggested that either viral DNA replication or T-ag binding to the viral origin of replication, or both, might be necessary events in the creation of 100K.

To complement our previous studies we have chosen to study the capacity of the C6 SV40 mutant to generate 100K T-ag. The C6 DNA used in this study was recovered by recombinant DNA technology from a monkey cell line designated C6 which was isolated after infection of CV-1 cells with SV40 virus which had been irradiated with UV light (18). Only replication-defective virus would be capable of transforming these cells, since replication-competent virus would have killed the cells. The SV40 early region recovered from these cells was shown to contain 3 base pair (bp) substitutions in the coding region of T-ag. The parent mutant, as well as two different subclones containing respec-

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tively one or two of the three mutations, were tested for transforming ability in rat cells. All three mutants were found to have a transforming efficiency equal to wild-type SV40 in spite of the fact that the T-ag of C6 is incapable of initiating viral replication (17).

We have tested the C6 mutant and its subclones in mouse cells for the ability to generate the 100K protein.

MATERIALS AND METHODS

Plasmids. Plasmids of the C6 lineage included pK1 (containing wild-type SV40), pC6 (the initial mutant isolated from SV40-transformed monkey cells containing 3 mutations at 5,074, 5,011, and 4,360 bp in the SV40 [46] numbering system), [pC6-1] (containing the mutations at 5,074 and 5,011 bp), and [pC6-2] (containing only the mutation at 4,360 bp). The mutation at 5,074 bp changes a guanine to a thymine, resulting in a change of amino acid number 30 from a methionine to an isoleucine. The mutation at 5,011 bp changes an adenine to a thymine and effects amino acid number 51, changing it from a lysine to an asparagine. The change at 4,360 bp switches an adenine to a cytosine and amino acid number 153 from an asparagine to a threonine. The mutation in [pC6-2] maps to the region which has been identified as critical for the specific binding of wild-type T-ag to the SV40 origin of replication (31). Plasmids [pC6] and [pC6-2] are completely replication defective, and [pC6-1] replicates with about 1/100th the efficiency of wild-type SV40.

The plasmid [pK10] was constructed from a virus which grew out of a [pC6-1] infection of monkey cells. It is a second-site revertant with a third mutation at 4,977 bp (changing a guanine to an adenine and amino acid number 63 from a glutamine to a lysine). All pC6-related plasmids are described in detail elsewhere (17).

The plasmid containing the origin defective mutant of SV40 (pSVR1-ORI⁻) (11) was supplied by David Grass (Columbia University). It is referred to in this paper as pORI⁻.

All plasmids were prepared according to standard laboratory protocols.

Transformations. NIH 3T3 cells (provided by M. Wigler) were grown in Dulbecco modified Eagle medium (GIBCO Laboratories; 430-2100) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% (vol/vol) fetal calf serum (GIBCO; lot no. 31P6223). Cells were prepared and transfected exactly as described previously (49). The day after the transfection, cells were split directly into 60-mm dishes (Nunc) at 10⁵ cells per dish or into growth medium containing 0.33% agarose (Becton Dickinson and Co.) and 10% fetal calf serum, which was overlaid onto a base of 0.5% agarose in growth medium containing 10% fetal calf serum. Cells plated directly on plastic were switched to 1% fetal calf serum the second day after transfection, and all cells were subsequently fed twice weekly. Transformants were picked 2 to 3 weeks later as either dense foci in 1% serum or as colonies in agarose. Cells were recloned to pure populations (monitored by T-ag nuclear fluorescent staining performed as described previously [48]) when necessary by plating 10² cells onto a 60-mm dish and picking a well-isolated colony approximately 2 weeks later. Cells were then routinely carried by plating 10³ cells per 60-mm dish and transferring them to a new dish at the same density on the average 11.5 days later.

The part of the designation for each cell line which is in square brackets indicates the plasmid which was used to transform it, and the letter following it indicates which clone

it was. For example, [pC6]-K was generated by the parental plasmid [pC6], and [pC6-1]-E1 was transformed by the plasmid [pC6-1]. A lowercase a indicates a selection through the agarose assay; otherwise clones were picked as dense foci in 1% fetal calf serum. Thus [pC6]a-R was a transformant originally picked from an agarose dish, and [pC6-1]-E2a1 is an agarose subclone of the [pC6-1]-E2 cell line.

Radiolabeling and immunoprecipitation of tumor antigens.

Cells were plated at 5 × 10⁵ cells onto a 60-mm dish. The following day they were labeled with 100 µCi of [³⁵S]methionine (Amersham Corp.; >700 µCi/mmol) per ml in Dulbecco modified Eagle medium minus methionine (GIBCO; lot no. 82-5040) plus 2% dialyzed fetal calf serum. After 4 h with intermittent rocking the dishes were rinsed thrice with phosphate-buffered saline (4°C) and extracted with 1 ml per dish of buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 8.0), 0.14 M NaCl, 1 mM MgCl₂, 1% Nonidet P-40 (Bethesda Research Laboratories), and 300 µg of freshly dissolved phenylmethylsulfonyl fluoride per ml. After 20-min extractions the cells were scraped into a tube and spun at 2,000 × *g* for 5 min, and the supernatant was collected. Trichloroacetic acid-precipitable counts were determined at this point. The supernatant was then spun for at least 45 min at 48,000 × *g* to remove particulate matter. Equal trichloroacetic acid-precipitable counts were brought to 1-ml volumes with the extraction buffer, and one-half of each sample was incubated with 15 ml of either normal hamster serum or serum from a hamster bearing an SV40 tumor. After at least 2 h on ice the immune complexes were collected for 20 min with 50 µl of a 10% (vol/vol) solution of IgSorb (The Enzyme Center) which had been previously washed in 1 M NaCl buffer (1 M NaCl, 5 mM EDTA, 10 mM Tris, pH 8.0). The precipitates were pelleted at 2,000 × *g* for 5 min and washed once in 140 mM NaCl–5 mM EDTA–10 mM Tris (pH 8.0) and twice in the 1 M NaCl buffer. Proteins were eluted at 100°C as described previously (33). Proteins were separated on polyacrylamide gels prepared as described previously (33), but with an acrylamide/bisacrylamide ratio of 30:0.375. The gels were treated with En³Hance (New England Nuclear Corp.) and autoradiographed at –70°C on Kodak SB-5 X-ray film.

Assays. Agarose assays were performed as described above for the transformation assay and scored 3 weeks later.

RESULTS

Early-passage pC6 transformants. Nineteen clonal cell lines from four different transfection experiments were subjected to analysis by immunoprecipitation and agarose assay. The cell lines included 12 lines from [pC6], 2 from [pC6-1], 3 from [pC6-2], 1 from [pK10], and one from the wild type [pK1].

Growth in agarose is well correlated with the ability to produce 100K in wild-type systems (12). The absolute plating efficiency in agarose of each of these cell lines generated by the pC6 plasmids was therefore assayed (Table 1). All but two of the cell lines had a significant plating efficiency in agarose (i.e., greater than 100-fold above background). Note, however, that all the transformed clones generated by C6 mutants are from 2- to 100-fold less efficient at growth in agarose than SV101, a standard full transformant (45) which is used in this laboratory as a reference. The difference in the ability of mutant-transformed cells to grow in soft agarose compared with wild type-transformed clones is even more pronounced when measured against [pK1]-L1, the wild-type transformant generated in this series of experiments.

TABLE 1. Anchorage independence of pC-related mouse transformants

Cell line	Transformed by:	Passage no.	APE (%) ^a
[pK1]-L1	pK1 (wild type)	12	9.1
[pC6]-G1	pC6	9	0.2
[pC6]-J	pC6	9	2.2
[pC6]-K	pC6	6	0.4
[pC6]-L1	pC6		ND ^b
[pC6]-L2	pC6	8	0.75
[pC6]-L3	pC6	9	0.15
[pC6]-L4	pC6	11	<0.05
[pC6]-a-R	pC6	4	3.1
[pC6]-S	pC6	4	1.8
[pC6]-T	pC6	4	0.05
[pC6]-a-U	pC6	4	1.0 ^c
[pC6]-a-W	pC6	4	0.95
[pC6-1]-E1	pC6-1	15	0.07 ^c
[pC6-1]-E2	pC6-1	14	0.30
[pC6-2]-B	pC6-2	14	0.02 ^c
[pC6-2]-G1	pC6-2	14	<0.05
[pC6-2]-K	pC6-2	10	0.13 ^c
[pK10]	pK10	11	0.10 ^c
SV101	SV40		4.9
NIH3T3			<0.0005 ^d

^a Plating efficiency in 0.33% agarose scored as visible colonies 3 weeks after plating 10³ cells per dish (except where indicated). Average of two dishes.

^b ND, Not determined.

^c 10⁴ cells per dish.

^d 10⁵ cells per dish.

Fig. 1 shows a representative immunoprecipitation of several of these cell lines at early passage (earlier than passage 10). The particular clone generated by pK1 produces more 100K than 94K and was used to clearly indicate the position of 100K (Fig. 1, lane g). Note that none of the cell lines in this particular panel, other than SV101 and [pK1]-L1, demonstrates detectable 100K T-ag, whereas all have similar amounts of 94K T-ag.

The positive control SV101 (Fig. 1) has only small amounts of detectable 100K relative to 94K, especially when compared with [pK1]-L. An exposure sixfold lower than the one used to make Fig. 1 was the minimum exposure necessary to reveal 100K in a low-level producer such as SV101. Since a sixfold increase in the minimum exposure necessary to detect 100K did not reveal any additional cell lines producing detectable 100K, we can confidently say that there is no detectable 100K in any of the cell lines represented by this autoradiogram. Four other autoradiograms chosen at random (representing 26 different samples) were overexposed an average of four times the minimum exposure necessary to detect 100K in the positive control lane. None of the cell lines examined in this analysis had detectable 100K, even after the longer exposures. All autoradiograms contained a positive control and were carefully examined for the presence or absence of 100K. On the basis of these experiments we feel confident that we have not overlooked any cell line expressing detectable 100K.

The results of all the immunoprecipitations on primary transformants generated with the C6 set of mutants are summarized in Table 2. There are three facts to note based on these results. First, the wild type-transformed clone had detectable 100K protein as soon as it was examined (passage 3). This is consistent with previous results (11). In contrast, the clones generated by C6 mutant DNAs usually did not express detectable 100K T-ag until several passages later,

and some still have not expressed detectable 100K after several months in culture. At least one member of each class has generated 100K, indicating all the mutants examined here retain that capacity, although the time of appearance varies when compared with wild-type situations. Second, none of the transformants generated by these mutants expresses amounts of 100K equal to the amount of 94K present. This is in contrast to the particular wild-type transformant generated in this study and also in contrast to other 100K-containing cells. Third, none of the cell lines generated with the mutant DNAs grows as well in soft agarose as wild-type transformants. This is consistent with previous results which demonstrated a correlation between growth in agarose and expression of 100K (12).

Agarose subclones of pC6-related transformants. Passage through agarose of cells transformed by wild-type virus can select for cells which now produce 100K (12). Subclones were picked from agarose of each of the following cell lines: two from [pK1]-L, four from [pC6]-J, two from [pC6]-L4, four from [pC6-1]-E2, four from [pC6-2]-G1, four from [pC6-2]-K, and four from [pK10]-H. Figure 2 shows a typical immunoprecipitation of these subclones. The data for all of the subclones examined are summarized in Table 3. In spite of the selection through agarose, by passage 6 none of the subclones from the parental mutant [pC6] produced detectable 100K. Two of the four subclones of [pC6-1]-E2 now produced 100K. None of the total of eight subclones from [pC6-2] transformed cells produced detectable 100K, and none of the four subclones from [pK10]-H produced the larger protein. Although subclones derived from transformants produced by mutant DNAs were not passaged as long as the ones indicated in Table 2, their failure to make 100K after agarose subcloning distinguishes them clearly from wild-type agarose subclones (11).

DISCUSSION

Many mouse cell lines transformed by SV40 contain a variant T-ag which migrates similarly to the protein we call 100K (13, 25, 33, 41). Tryptic peptide maps have shown that

TABLE 2. 100K T-ag versus passage in pC6-related mouse transformants^a

Cell line	T-ag at passage no.:																			
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
[pK1]-L	+			+					+				+	+				+	+	
[pC6]-G1			+																	
[pC6]-J								-	-	-										
[pC6]-K					-		-													
[pC6]-L1			-																	
[pC6]-L2								+												
[pC6]-L3								-		-		-								
[pC6]-L4				+	+						+		+							
[pC6]a-R	-																			
[pC6]-S		+																		
[pC6]-T		-																		
[pC6]a-U					-															
[pC6]a-W					-															
[pC6-1]-E1									-					+	+	+		+		
[pC6-1]-E2								-							+					
[pC6-2]-B							-								-			+		
[pC6-2]-G1							-					-		-	-	-				
[pC6-2]-K			-						-		-		-							
[pK10]-H			-							+		+		+						

^a -, Presence of only wild-type 94K when assayed at the indicated passage; +, presence of both 94K and 100K. All determinations of presence or absence of 100K were made in ignorance of the cell line or passage number.

FIG. 1. Autofluorogram of an immunoprecipitation of pC6-related mouse transformants separated on 15% acrylamide gels. Lanes (passage numbers): a, [pC6-2]-K (5); b, [pC6]-L1 (5); c, [pC6-2]-G1 (9); d, [pC6-1]-E1 (10); e, [pK10]-H (5); f, [pC6-1]-E2 (9); g, [pK1]-L (6); h, [pC6]-L1 (5); M, molecular weight markers. The left lane of each pair contained control serum, and the right lane contained hamster antitumor serum. Note the presence of both wild-type 94K and 100K T-ag (indicated by the star) in [pK1]-L (lane g). None of the pC6-related transformants expressed 100K at early passage. SV101 is a cell line transformed by wild-type SV40 which contains both proteins. The faint band in lane c is not 100K. On the original autoradiogram it clearly migrates slower than the 100K band, and comigrates with a background band seen in other lanes (including normal serum lanes) on longer exposures. Also, the marker lane on the left side is slightly lower due to edge effects during the running of the gel.

there are only minor differences between the 100K protein we detect and that which is detected by other workers (S. Chen, personal communication). Others have shown that there is no detectable difference between the N termini (41), C termini (13), or tryptic peptide ion-exchange chromatography elution profiles (25) of authentic T-ag and this 100K protein. However, since a similar T-ag that is larger than the wild-type T-ag can be detected in a cell-free system primed with mRNA from the SV40-transformed mouse cell line SV3T3 (34), and since 100K production requires the presence of a partial tandem repeat of the SV40 early region (27), it is very likely that 94K and 100K differ in their primary amino acid sequence.

Previous work had suggested that SV40 DNA replication might be a prerequisite to the production of this protein. Of 10 transformants produced by wild-type SV40 plasmids, 7 contained 100K initially (passage 3) and all expressed the variant protein as well as 94K after several weeks in culture (11). In contrast, viral mutants lacking an origin of replication (and therefore completely replication defective) make no detectable 100K at any passage (11).

With these facts in mind we tested the ability of the C6-related plasmids, which synthesize T-ag that are completely (pC6, pC6-2) or partly (pC6-1) defective for the initiation of SV40 DNA synthesis, for their ability to generate transformed cell lines which have a 100K T-ag. Only 4 of 18 clones transformed by replication-defective T-ag had 100K at first examination. After several weeks in culture (some cell lines for as long as 6 months) 9 of 18 clones still

did not express 100K. Thus we conclude that C6 mutants are partially defective in their ability to generate 100K T-ag.

When agarose subclones of transformed cell lines generated with wild-type SV40 plasmids are examined, 8 out of 8 of them contain 100K T-ag, including those from clones that were not expressing 100K before passage in agarose (11). This is in contrast to the results seen here with the C6 mutants. Only 2 of 24 subclones of various C6-derived transformants expressed detectable 100K protein immediately after agarose subcloning.

Previous work from this laboratory indicated a correlation between the ability to grow in soft agarose and the presence of 100K when the original transformants were generated by SV40 virions (12). The results presented here are consistent with that observation to a limited extent. None of the transformants generated by the C6 mutants exhibited 100K in amounts comparable to SV101 or to [pK1]-L1. This may indicate low but undetectable levels of 100K correlating with the lower ability of these cells to grow in agarose when compared with wild type-generated clones. However some mutant-derived transformants (e.g., [pC6]-J) did not express detectable 100K yet grew fairly well in agarose (2.2% plating efficiency). This may indicate that when 100K generation is difficult or impossible other pathways are followed which result in anchorage-independent growth. This phenomenon has already been observed in origin-negative transformants (11). Also, spontaneous (non-SV40-dependent) anchorage-independent cells clearly arise in the absence of any SV40 T-ag.

FIG. 2 Autofluorogram of immunoprecipitations of subclones derived from pC6-related transformants. Lanes: a, [pC6]-L4-a2; b, [pC6-1]-E1-a3; c, [pC6-1]-E1-a4; d, [pC6-1]-E2-a4; e, [pC6-2]-G1-a3; f, [pC6-2]-G1-a4; g, [pC6-2]-K-a2; h, [pC6-2]-K-a3; i, [pK10]-H-a2; M, molecular weight markers. All cells were assayed at passage 4 after subcloning. A summary of the results of all the immunoprecipitations done with subclones appears in Table 3. On the original autoradiogram lane c had a weak 100K band. Otherwise, all clones assayed in this experiment had no detectable 100K.

The genesis of 100K is still an open question. Given the apparent ubiquitous presence of this variant T-ag in mouse cell transformants generated in several laboratories with several strains and stocks of wild-type SV40, it seems reasonable to assume that, during the acute phase of SV40

infection of mouse cells some specific mechanism is at work which consistently generates this T-ag-related polypeptide.

In two cases the DNA sequences which encode the information for the production of 100K T-ag have been cloned. Analysis of the sequence indicates that a partial repeat of the SV40 early region is sufficient to generate 100K (27). One speculation is that this particular type of duplication arises during the few rounds of viral DNA replication which follow infection or transfection of mouse cells. This would be consistent with the hypothesis that replication is required for the generation of 100K T-ag.

Although we do not completely understand the generation of this 100K protein in cells transformed by the mutants which are completely replication defective (pC6 and pC6-2), it should be pointed out that the viral origin of replication is still intact and presumably still capable of being activated by cellular factors in lieu of functional T-ag. T-ag can induce cellular replication (14, 20, 21). Perhaps the host molecules which normally act on cellular origins of replication can induce SV40 replication from the viral origin of replication in a complementary fashion, albeit very inefficiently.

We conclude from these studies that unlike wild-type SV40, which generates sequences encoding 100K at high frequency in transformation and after passage through agarose, C6-type mutants can only produce 100K after extended time in culture and then only occasionally, perhaps only through unknown host factors acting on the integrated DNA.

While this manuscript was in preparation, Manos and Gluzman (29) reported that the replicative function of SV40 T-ag is important in generating 100K T-ag. They based this conclusion on the inability of other SV40 mutants in the C series to produce 100K when used to transform mouse cells.

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TABLE 3. 100K T-ag versus passage in subclones of pC6-related mouse transformants^a

Cell line	T-ag at passage no.:					
	1	2	3	4	5	6
[pK1]-L-a1					+	+
[pK1]-L-a2				+		
[pC6]-J-a1				-		-
[pC6]-J-a2				-	-	-
[pC6]-J-a3						-
[pC6]-J-a4					-	-
[pC6]-L4-a1						-
[pC6]-L4-a2				-	-	
[pC6-1]-E1-a3				-		
[pC6-1]-E1-a4				+		
[pC6-1]-E2-a1					+	
[pC6-1]-E2-a2					-	
[pC6-1]-E2-a3					+	
[pC6-1]-E2-a4				-		
[pC6-2]-G1-a1				-		-
[pC6-2]-G1-a2						-
[pC6-2]-G1-a3				-	-	
[pC6-2]-G1-a4				-	-	
[pC6-2]-K-a1					-	-
[pC6-2]-K-a2					-	
[pC6-2]-K-a3				-	-	
[pC6-2]-K-a4				-		
[pK10]-H-a1				-	-	-
[pK10]-H-a2					-	
[pK10]-H-a3				-		
[pK10]-H-a4				-		

^a See footnote a of Table 2.

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